University of Alberta

The role of EmhABC efflux pump in *Pseudomonas fluorescens* LP6a

by

Abigail Adewunmi Adebusuyi

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Dedication

This work is dedicated “to God who is able to do exceedingly abundantly above all that we ask or think….” (Eph 3^{20})

Abstract

Efflux pumps belonging to the resistance-nodulation-division (RND) superfamily in bacteria are involved in antibiotic resistance and solvent tolerance but have an unknown physiological role. EmhABC, a RND-type efflux pump in the hydrocarbon-degrading bacterium *Pseudomonas fluorescens* LP6a, extrudes hydrophobic antibiotics, dyes and polycyclic aromatic hydrocarbons (PAHs) including phenanthrene and anthracene but not naphthalene. Because PAH substrates of EmhABC are also carbon sources for LP6a, the authentic physiological role of this pump in LP6a was determined. The effects of physico-chemical factors such as temperature or antibiotics on the activity and expression of EmhABC were examined in order to deduce its authentic role(s) in LP6a. Based on expression studies, efflux assays and membrane fatty acid analysis, induction of EmhABC expression by physico-chemical factors is linked to modulation of membrane fatty acids. Physico-chemical factors such as variation in incubation temperature, pH and increased Mg^{2+} concentration induced the expression of EmhABC, whereas pump substrates such as phenanthrene and chloramphenicol did not. The active efflux of phenanthrene decreased the efficiency of phenanthrene degradation by LP6a but the presence of EmhABC was important for efficient degradation of naphthalene. This suggests that the activity of EmhABC in LP6a has implications for bioremediation and biocatalytic transformation of PAHs and heterocycles. The deleterious effect of an antibiotic or other

compound on cell membrane integrity and fatty acid composition may be the signal that initiates the induction of the EmhABC efflux pump, and inducers of bacterial efflux pumps may include environmental factors rather than the substrates per se. For effective treatment of bacterial infections, the factors affecting a bacterial pathogen in its environment and the effect of the antibiotic on the membrane should be considered. These observations suggest that the EmhABC efflux pump may be involved in the management of membrane stress. Efflux of fatty acids replaced as a result of membrane damage or phospholipid turnover may be the authentic physiological role of the EmhABC efflux pump in *P. fluorescens* LP6a.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

BAME – Bacterial acid methyl ester

CTAB - Cetyltrimethylammonium bromide

DBT – Dibenzothiophene

DPM – Disintegration per minute

EPIs – Effux pump inhibitors

FA – Fatty acid

FAME – Fatty acid methyl ester

GC-MS – Gas chromatography-mass spectrometry

HAE-1 – Hydrophobe/amphiphile efflux-1

HTOB - *Trans*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid

PAHs – polycyclic aromatic hydrocarbons

PCR – Polymerase chain reaction

PI – Propidium iodide

RND – Resistance-nodulation-division

TMS – Trans membrane segments

TSB – Trypticase soy broth

1. Introduction

Efflux pumps of the resistance-nodulation-division (RND) superfamily are major determinants of antibiotic resistance and virulence (Kumar and Schweizer 2005; Martinez et al. 2009) but may have other unidentified functions that may be associated with the environment. Identification and the characterization of the RND-type efflux pump EmhABC in *Pseudomonas fluorescens* LP6a revealed that polycyclic aromatic hydrocarbons (PAHs) are substrates of the pump as well as hydrophobic antimicrobials (Bugg et al. 2000; Hearn et al. 2003). Unlike other substrates of RND efflux pumps, PAHs have no known deleterious effects on *P. fluorescens* (Bugg et al. 2000; Hearn et al. 2003), so efflux of PAH compounds that can be utilized as sole source of carbon and energy was unexpected (Bugg et al. 2000; Foght and Westlake 1996). The atypical trend of effluxing a usable carbon substrate discovered in *P. fluorescens* LP6a (Bugg et al. 2000; Hearn et al. 2003) raised my interest to do a follow up study to determine the "authentic" physiological role of the EmhABC efflux pump in this strain. Here "authentic substrate(s)" is defined as naturally occurring compounds present in the natural environment, or compounds produced by or components of bacterial species expressing RND efflux pumps, in contrast to anthropogenic substrates such as semisynthetic or synthetic antimicrobials.

1.1. History of bacterial efflux pumps

In 1976, the first example of an efflux pump was discovered (Juliano and Ling 1976; Kartner et al. 1983). The P-glycoprotein, discovered in mammalian tumour cells, is responsible for the efflux and resistance to anti-tumour drugs (Juliano and Ling 1976; Kartner et al. 1983). Shortly after the discovery of the P-glycoprotein, the tetracycline efflux pump was found in *Escherichia coli* in the late 1970's (Ball et al. 1980; Levy and McMurry 1978; McMurry et al. 1980). The tetracycline efflux pump is plasmid-encoded and was assumed to be acquired from tetracycline-producing species by horizontal gene transfer (Davies 1994; Pang et al. 1994). This assumption was disproven by evidence gathered from subsequent study of efflux pumps in bacteria and archaea (George and Levy 1983a; Paulsen et al. 2001). Efflux pumps genes are not exclusively plasmid-encoded, nor specific for a single antibiotic nor are they present only in antibiotic-producers (George and Levy 1983a, b). Indeed, genes encoding most efflux pumps described to date are located on the chromosome (Hearn et al. 2003; Paulsen et al. 2001). Screening of available bacterial genome sequences revealed that efflux pumps comprise 6-18% of all transporters found in bacteria (Kumar and Schweizer 2005; Martinez et al. 2009; Paulsen et al. 1998), which in essence highlights their importance to bacterial cells.

1.1.1. Bacterial efflux pump classes

Bacterial efflux pumps are grouped into five classes based on their energy source, the number of protein components (single or multiple), the number of transmembrane segments (TMS) and the substrates transported (Piddock 2006). The five classes comprise the major facilitator superfamily (MFS), the adenosine triphosphate (ATP) – binding cassette (ABC) superfamily, the small drug resistance (SMR) family, the multidrug and toxic compound extrusion (MATE) family and the resistance-nodulation-division (RND) superfamily (Lubelski et al. 2007; Law et al. 2008; Tseng et al. 1999; Chung and Saier 2001; Moriyama et al. 2008). The characteristics of these efflux pumps are listed in Table 1.1. The RND efflux pumps will be the major focus of the remainder of this review.

Table 1.1 Features of different classes of bacterial efflux pumps

Features	Bacterial efflux pumps*				
	ABC	MATE	MFS	RND	SMR
Energy source	ATP	Na+ gradient	Proton	Proton	Proton
Number of component	1 or 3	1	1 or 3	3	1
Number of TMS	6	12	12 or 14	12	4
Substrates	Drugs, amino acids, sugars	Drugs, cationic dyes	Drugs, cationic dyes	Drugs, cationic dyes, solvents	Drugs, dyes, cations
Example	MacAB-TolC, LmrA	NorM, YdhE	EmrAB-TolC, Nor A	AcrAB-TolC, EmhABC	EmrE, QacC

*, Sources for the data on bacterial efflux pumps can be found in Kumar and Schweizer (2005) and Nikaido (2009)

1.2. What are RND efflux pumps?

RND efflux pumps are integral membrane proteins (Dinh et al. 1994; Paulsen et al. 1997) that play a key role in acquired or intrinsic multidrug resistance in Gram-negative bacteria (Martinez et al. 2009; Nikaido 2009; Nikaido and Takatsuka 2009; Tikhonova et al. 2011). While this family of efflux pumps is common in Gram-negative bacteria (Zgurskaya and Nikaido 2000), representatives have been found in all domains of life (Blair and Piddock 2009; Tseng et al. 1999). So they are "an ancient, ubiquitous and diverse family" (Tseng et al. 1999) of efflux pumps. Most RND efflux pumps are encoded by genes located on the chromosome (Kumar and Schweizer 2005) and are conserved in most bacterial species (Piddock 2006; Morita et al. 2006). However, plasmid-encoded RND efflux pumps such as the TtgGHI in *Pseudomonas putida* DOT-T1E have been reported (Rodriguez-Herva et al. 2007).

The RND efflux pump functions as a three-component system in association with an outer membrane protein (OMP) and a periplasmic adaptor protein (PAP) formerly referred to as the membrane fusion protein (Blair and Piddock 2009; Dinh et al. 1994; Kumar and Schweizer 2005; Paulsen et al. 1997). Similar to MFS and SMR efflux pumps (Table 1.1), RND efflux pumps are powered by the proton motive force (Nikaido 1996). Most RND efflux pumps have 12 TMS (Kumar and Schweizer 2005) or 6 TMS in *Mycobacterium jannaschii* (Tseng et al. 1999). AcrAB-

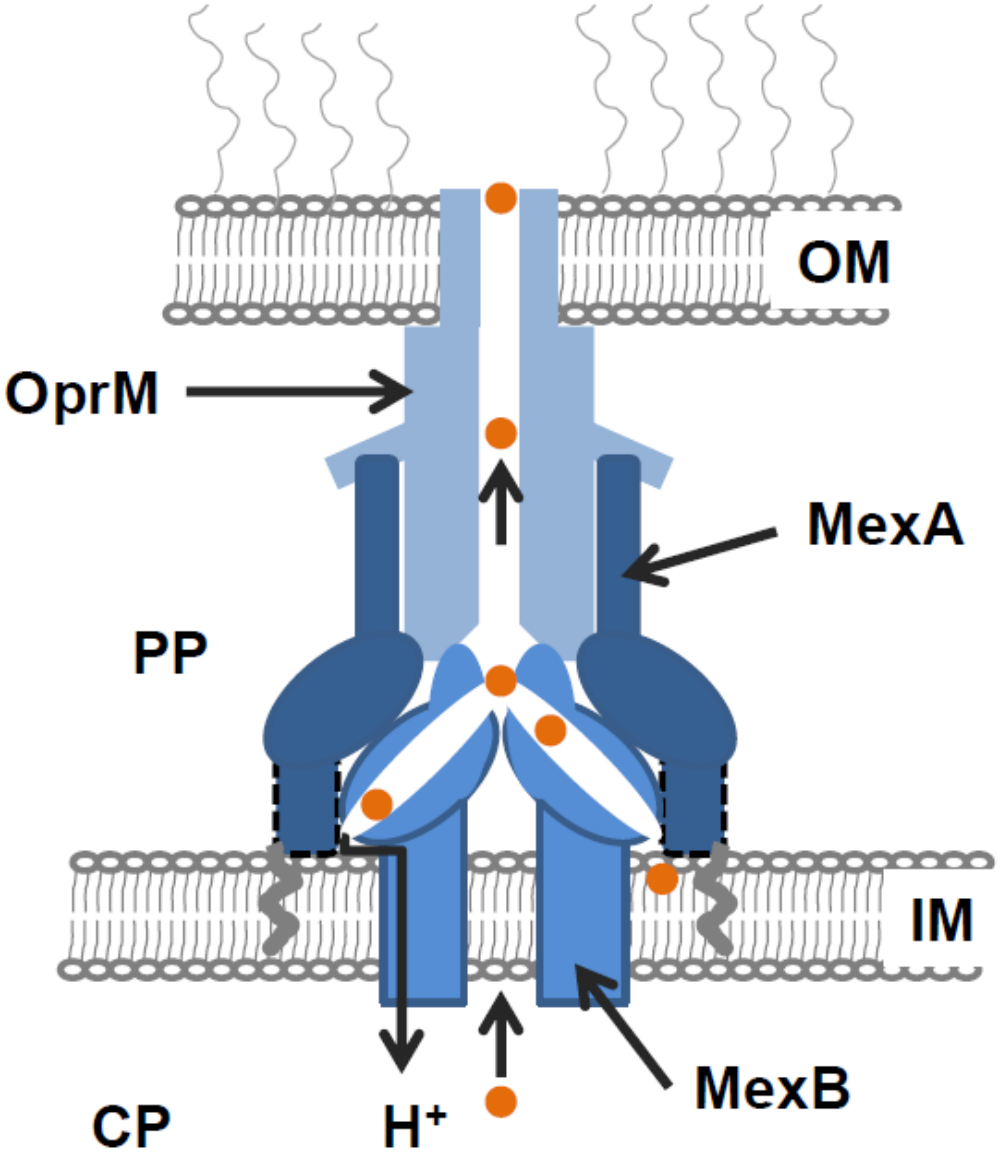

Figure 1.1 Schematic of a RND efflux pump based on the MexAB-OprM/AcrAB-TolC model by Symmons et al. (2009). RND transporter (MexB) is embedded in the inner membrane (IM); MexA, the periplasmic adaptor protein, is connected to the IM by a fatty acid (zigzag line) and the outer membrane protein OprM is the exit channel in the outer membrane (OM). CP, cytoplasm; PP, periplasm; H^+, proton; and Orange circle, efflux pump substrates (e.g. antibiotics).

TolC is an example of a RND efflux pump in *E. coli*, involved in the efflux of cationic dyes and antibiotics (Ma et al. 1993; 1995). The structures of AcrB of *E. coli* and MexB of *P. aeruginosa* have been solved by crystallography (See schematic of RND efflux pumps in Fig. 1.1; Murakami et al. 2002; Seeger et al. 2006; Sennhauser 2009; Symmons et al. 2009) and found to have common features (Blair and Piddock 2009; Sennhauser 2009) discussed below.

1.2.1. Structure and function of RND efflux pumps

RND efflux proteins such as AcrB associate with the PAP (AcrA) and OMP (TolC) to span the entire cell envelope (Symmons et al. 2009). The AcrB is the energy transducer and is responsible for substrate binding (Murakami et al. 2006; Takatsuka and Nikaido 2009). Each protomer of the AcrB asymmetric homotrimer contains 12 trans-membrane (TM) α-helices and two large periplasmic loops, which connect TM1 with TM2 and TM7 with TM8 (Eicher et al. 2009; Murakami et al. 2002; Takatsuka and Nikaido 2009). The periplasmic loops of RND efflux pumps such as AcrB and MexB (Fig. 1.1) extend 7 nm into the periplasm (Eicher et al. 2009; Trepout et al. 2010) and can be divided into TolC docking and porter domains. Contrary to the presumption that the PAP, AcrA, connects AcrB to TolC, new evidence shows that the AcrB docking domain interacts with the TolC periplasmic domain in the periplasm (Symmons et al. 2009; Tikhonova et al. 2011). The direct interaction of AcrB and TolC periplasmic domains was supported by genetic experiments and in vivo cysteine

cross-linking studies (Tamura et al. 2005; Weeks et al. 2010). Because there is limited interaction between AcrB and TolC, AcrA is thought to stabilize the AcrB-TolC assembly (Tikhonova et al. 2011). AcrA crystal structure suggests it interacts with both AcrB and TolC and may be involved in the recruitment of TolC (Mikolosko et al. 2006; Tikhonova et al. 2009; 2011). AcrA interacts with AcrB with its lipoyl-binding, α–β-barrel and membrane proximal domains, whereas interaction with TolC is via its α-helical hairpin (Lobedanz et al. 2007; Symmons et al. 2009). Detailed reviews of the structure and mechanism of RND efflux pumps are available in the literature (Blair and Piddock 2009; Eicher et al. 2009).

1.2.2. Mechanism of transport by RND efflux pumps

Substrate transport in RND efflux pumps is hypothesized to occur by the functionally rotating mechanism based on X-ray crystallography studies of the asymmetric trimer of AcrB (Murakami et al. 2006; Sennhauser et al. 2007; Pos 2009; Takatsuka and Nikaido 2009). Each protomer goes through a cycle of three-step conformational changes (i.e. access [loose], binding [tight] and extrusion [open]) during substrate export (Takatsuka and Nikaido 2009). Substrate transport through these conformational changes is initiated by the protonation of one of the key residues (Asp407, Asp408, Lys 940 or Thr978) in the AcrB transmembrane domains (Li and Nikaido 2009). Biochemical studies by Takatsuka and Nikaido (2009) and Seeger et al. (2008) with disulfide cross-linking, in addition to the behaviour of covalently linked AcrB

protomers, have confirmed the functional rotating mechanism of substrate transport. This mechanism supports substrate capture in the periplasm but is unclear on how and where hydrophobic substrates such as PAHs that partition into the inner membrane (Pos 2009) are captured. A full review on the functional rotating mechanism can be found in Eicher et al. (2009).

Substrate capture and binding by the periplasmic domains is well characterized using chimeric constructs of RND efflux pumps (Murakami et al. 2002; Ohene-Agyei et al. 2012). Ohene-Agyei et al. (2012) also recently reported substrate capture from the cytoplasm by mutating conserved phenylalanine residues on the cytoplasmic side of the central cavity of the MexB efflux pump in *P. aeruginosa* to alanine residues. Hearn et al. (2006) reported that mutations in some conserved central cavity residues of the EmhABC efflux pump transmembrane domain affected the transport of PAHs that likely partitioned into the inner membrane. The evidence that the transmembrane domains of RND efflux pumps is involved in substrate binding is contested by Nikaido and Takatsuka (2009) who hypothesize that the transmembrane domain of these pumps is solely for proton translocation. Thus the exact mechanism has yet to be fully resolved.

1.3. RND efflux pump divisions

Phylogenetic analysis of RND efflux pumps by Tseng et al. (1999) defined seven distinct families within the superfamily along with putative

proteins that do not fall into any of the seven families (Meguro et al. 2005). These seven families are:(1) the hydrophobe/amphiphile efflux-1 (HAE1) family, (2) the heavy metal efflux (HME) family, (3) the nodulation factor exporter (NFE) family, (4) the SecDF protein-secretion accessory protein family, (5) the hydrophobe/amphiphile efflux-2 (HAE2) family, (6) the eukaryotic sterol homeostasis (ESH) family, and (7) the hydrophobe/amphiphile efflux-3 (HAE3) family (Meguro et al. 2005; Tseng et al. 1999). Proteins in the seven families have similar sizes and topological patterns (Tseng et al. 1999).

The HAE1, HME and NFE families are found in Gram-negative bacteria (Tseng et al. 1999). HAE1 efflux pumps such as AcrB in *E. coli* are involved in the transport of drugs and other hydrophobic compounds (Nikaido 1996), whereas the HME and NFE families respectively export heavy metals (Rensing et al. 1997) and lipooligosaccharide signaling molecules required for the nodulation of legumes by rhizobia (Baev et al. 1991; Göttfert 1993). The HAE2 family of efflux pumps is found in Gram-positive bacteria; an example is the actinorhodin transport-associated protein ActII3 in *Streptomyces coelicolor* (Férnandez-Moreno et al. 1991). The ESH family pumps are found in eukaryotes, for example the human Niemann-Pick C disease protein that is involved in cholesterol homeostasis (Loftus et al. 1997). The seventh family, i.e. HAE3, is found in archaea and spirochetes and is functionally uncharacterized but assumed to be involved in drug efflux because of their similarity to the

HAE2 family (Tseng et al. 1999). The following sections are focused on the HAE1 family of RND efflux pumps.

1.4. Substrates of RND efflux pumps

The association of a HAE1 RND efflux pump with the OMP and PAP allows for effective efflux of substrates across the inner and outer membrane of the Gram-negative cell to the extracellular medium (Lomovskaya and Totrov 2005; Nikaido 1996). The HAE1 family of RND pumps effluxes structurally diverse compounds, but mostly drugs and hydrophobic compounds (Nikaido 1996). Examples (Table 1.2) include hydrophobic antibiotics such as tetracycline and chloramphenicol (Hearn et al. 2003; Nikaido 1996), solvents such as toluene (Kieboom et al. 1998; Ramos et al. 1998), cationic dyes such as ethidium bromide, crystal violet and rhodamine 6G (Hearn et al. 2003; Nikaido 1996) and detergents (Zgurskaya and Nikaido 2000). Other substrates of the HAE1 efflux pumps are bile salts (Nikaido 1996), fatty acids (Ma et al. 1995), polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene, anthracene and fluoranthene (Bugg et al. 2000; Hearn et al. 2003; 2006), and quorum sensing molecules (Evans et al. 1998; Kōhler et al. 2001; Minagawa et al. 2012). Structures of some HAE1 efflux pump substrates are shown in Fig. 1.2.

A HAE1 efflux pump may have preference for one or more of the different categories of substrates listed above. The well-studied AcrAB-

Table 1.2 Substrates, regulators and inducers of some RND efflux pumps[a]

Organism	RND system	Substrates[b]	Regulator	Inducers[b]
E. coli	AcrAB-TolC	AC,BS,CM,CP,CV,EB,FA,FQ,ML,NO,OS, PN, SDS, TC,TM,TR,TX	AcrR, MarA, SoxS, Rob, SdiA	BS, ET, FA, NaCl, SAL
P. aeruginosa	MexAB-OprM	AC, AG, CM, CP, CV,EB, ML, NO, OS, PN, SDS, SF, TC, TM, TR	MexR, NalC (PA3721), NalD	PCP
P. aeruginosa	MexXY-OprM	AG,FQ, ML,TC	MexZ	AG, ML, TC
P. putida	TtgABC	CM, FL, OS, PN, TC	TtgR	CM, FL, TC
P. fluorescens	EmhABC	CM, FQ, PAHs, R6G, TC	EmhR	TC

[a], Data on RND efflux pumps included in this table can be found in Alguel et al. (2007); Hearn et al. (2003); Jeannot et al. (2005); Ma et al. (1995); Muller et al. (2007); Nikaido (2009).

[b], Abbreviations: AC, acriflavine; AG, aminoglycosides; BS, bile salts; CM, chloramphenicol; CP, cephalosporins; CV, crystal violet; EB, ethidium bromide; ET, ethanol; FA, fatty acids; FL, flavonoids; FQ, fluoroquinolones; ML, macrolides; NO, novobiocin; OS, organic solvents; PAHs, polycyclic aromatic hydrocarbons; PCP, pentachlorophenol; PN, penicillins; R6G, rhodamine-6-G; SAL, salicylate; SDS, sodium dodecylsulfate; SF, sulfonamides; TC, tetracyclines; TM, trimethoprim; TR, triclosan; TX, Triton X-100.

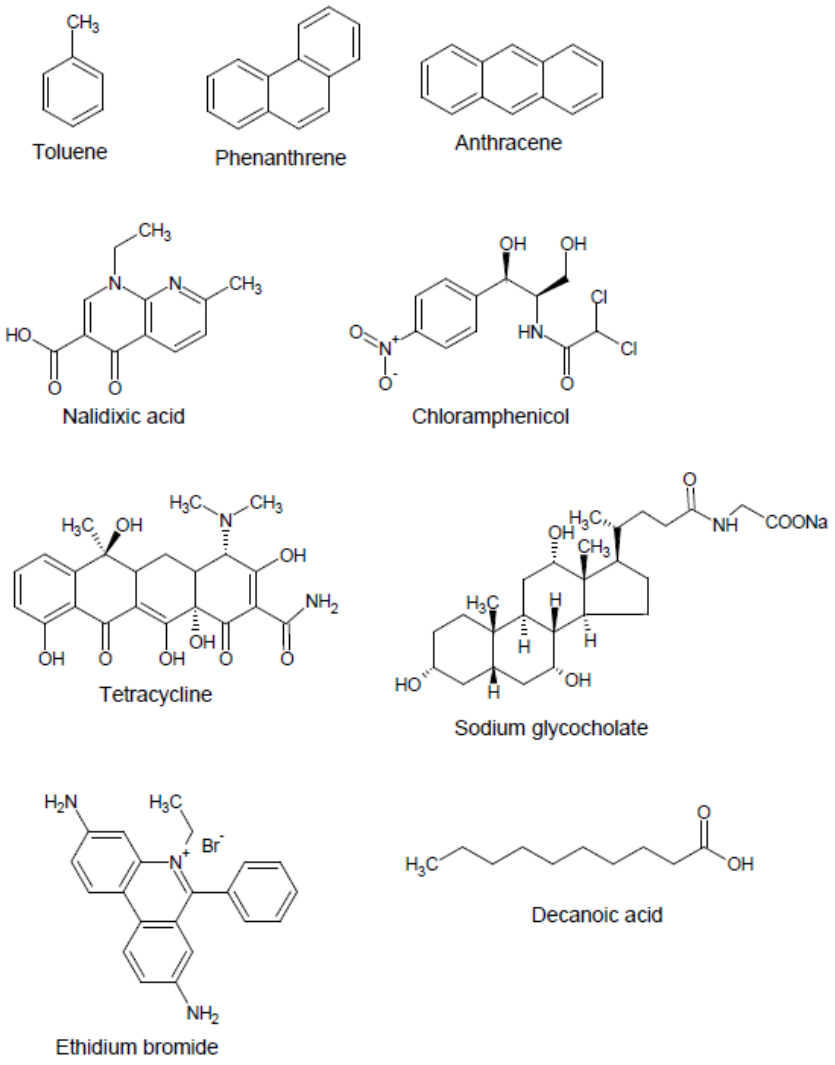

Figure 1.2 Some substrates of RND efflux pumps in the HAE1 family.

TolC efflux pumps in *E. coli* and MexAB-OprM in *P. aeruginosa* have broad substrate specificity for different antibiotics, fatty acids and cationic dyes (Nikaido 2009). Others like the *P. putida* Ttg and Srp efflux pumps have high affinity for organic solvents although they still efflux antimicrobials (Kieboom et al. 1998; Ramos et al. 1998). Gram-negative

bacteria that have non-functional efflux pumps are susceptible to various antimicrobial substrates, whereas over-expression of efflux pumps such as AcrB confers multi-drug resistance on *E. coli* (Blair and Piddock 2009).

1.5. Prevalence of the HAE1 family of RND efflux pumps

Homologues of HAE1 efflux pumps in *E. coli* and *P. aeruginosa* are present in other aerobic and anaerobic Gram-negative species (Ikeda and Yoshimura 2002; Kumar and Schweizer 2005). Examples of clinically relevant Gram-negative bacteria include the CeoAB-OpcM of *Burkholderia cepacia,* responsible for the efflux of chloramphenicol and fluoroquinolones (Zhang et al. 2001) and the MtrCDE RND pump of *Neisseria gonorrhoeae*, which is involved in the efflux of antibiotics, dyes and detergents (Hagman et al. 1995). In Gram-negatives that may be of biotechnological relevance, TtgABC, TtGDEF and TtgGHI in *P. putida* DOT-T1E have been studied extensively for their role in toluene tolerance (Ramos et al. 1998; Mosqueda and Ramos 2000; Rojas et al. 2001; Rodriguez-Herva et al. 2007). The EmhABC efflux pump in *P. fluorescens* LP6a is responsible for the efflux of PAHs such as phenanthrene and anthracene in addition to hydrophobic antibiotics and dyes (Bugg et al. 2000; Hearn et al. 2003). The discovery and function of the EmhABC efflux pump will be discussed in detail section 1.11.

RND-type efflux pump genes have been identified in anaerobic bacteria such as *Desulfatibacillium alkenivorans* and *Geobacter* species that can anaerobically degrade hydrocarbons (Aklujkar et al. 2009;

Callaghan et al. 2012) based on bioinformatics annotation of their sequenced genomes. The function of the putative HAE1 efflux pumps in these anaerobic bacteria has not been demonstrated as most research on RND efflux pumps has been performed using aerobic bacteria. A report on a functional AcrAB-TolC homolog, XepCAB in the anaerobe *Porphyromonas gingivalis,* showed that it is responsible for the extrusion of rifampin, puromycin and ethidium bromide (Ikeda and Yoshimura 2002).

1.6. Regulation of expression of RND efflux pumps

Some RND efflux pumps are produced constitutively, whereas others are inducible. The TtgABC efflux pump of *P. putida* is produced constitutively and its expression is not affected by the presence of solvent substrates (Duque et al. 2001; Terán et al. 2006). Conversely, the TtgDEF and TtgGHI efflux pumps in the same strain are induced by sub-lethal concentrations of toluene (Mosqueda et al. 1999; Rojas et al. 2003). Also, more than one RND efflux pump can be functional concurrently in a bacterial cell (Poole 2008). For example, 12 RND efflux pumps have been identified in the *P. aeruginosa* genome (Kumar and Schweizer 2005; Poole 2008), of which 11 have been characterized and some are known to be expressed simultaneously (Kumar and Schweizer; 2005, 2011). MexAB and MexXY are simultaneously expressed in *P. aeruginosa*, thus boosting the resistance of this microbe to a wide range of antimicrobial compounds (Llanes et al. 2004). Because RND efflux pumps are energy-driven,

induction of genes that encode constitutively expressed or inducible efflux pumps is complex and tightly coordinated (Poole 2008, Table 1.2).

1.6.1. Local regulation

Local regulators are involved in the regulation of RND efflux pump gene transcription (Kumar and Schweizer 2005). Most operons encoding RND efflux pumps have a linked regulatory gene that is typically divergently transcribed from the efflux pump genes (Kumar and Schweizer 2005), however the effector molecules that bind to these regulators are unknown (Nikaido 2009). The best-characterized local regulators of RND efflux pumps are in the TetR repressor family (Li et al. 1995; Orth et al. 2000). AcrR in *E. coli* is a TetR-like repressor able to repress *acrAB* and *acrR* expression (Kumar and Schweizer 2005). Mutations in *acrR* resulted in over-expression of the *acrAB* genes and increased resistance to fluoroquinolones (Wang et al. 2001). Other local regulators of RND efflux pumps of the TetR family include MexZ in *P. aeruginosa,* involved in repression of *mexY* (Westbrock-Wadman et al. 1999), and SmeT in *Stenotrophomonas maltophilia* that represses the expression of genes encoding the SmeDEF efflux pump (Zhang et al. 2001). In *P. aeruginosa*, the linked regulator of RND efflux pump genes is either a repressor or an activator (Kumar and Schweizer 2005). For example, MexR is a repressor of the MarR family (Li et al. 1995) that represses the transcription of the *mexAB genes* (Evans et al. 2001). Over-expression of the main efflux pump of *P. aeruginosa*, the MexAB-OprM efflux pump that has a broad

substrate range, is usually caused by mutations in the adjacent repressor gene *mexR* (Higgins et al. 2003; Saito et al. 1999). However, over-expression of MexAB-OprM is not always dependent on mutations in *mexR* (Beinlich et al. 2001; Ziha Zafiri et al. 1999). MexT a member of the LysR family of regulators activate the transcription of the *mexEF* genes in *P. aeruginosa* (Kohler et al. 1999) but mutations in *mexT* do not cause over-expression of *mexEF* (Masuda et al. 2001; Sobel et al. 2005). Over-expression of *mexEF-oprN* in *P. aeruginosa* is caused by mutations in a gene that encodes the synthesis of an uncharacterized protein, PA2491 (Sobel et al. 2005). Another RND efflux pump in *P. aeruginosa*, MexCD-OprJ, which confers resistance to different antimicrobials, cationic dyes and organic solvents, is only expressed in strains with mutations in the *nfxB* gene (Poole et al. 1996). In *P. putida* S12, Sun et al. (2011) found that binding of the local repressor SrpS of the SrpABC efflux pump to organic solvent substrates or to its antirepressor SrpR induces the expression of the SrpABC efflux pump. The clinical implication of local regulators is that most pathogenic bacteria that over-express RND efflux pumps carry mutations in the local regulatory gene (Nikaido 2009).

1.6.2. Global regulation

Global regulators also regulate expression of RND efflux pumps. The best-studied example is the *acrAB* operon in *E. coli*, which is positively regulated by global activators of the AraC family: MarA, SoxS and Rob (Grkovic et al. 2002; Li and Nikaido 2004; Nikaido 2009). The

level of AcrAB regulation by MarA and SoxS depends on their concentration in the cell (Nikaido 2009). MarA is regulated by MarR, a repressor protein that is inactivated by salicylate (Li and Nikaido 2004; Nikaido 2009). The repressor SoxR, which is inactivated by superoxide (Nikaido 2009), determines SoxS concentration in the cell. Rob is inactivated when it is bound to molecules such as fatty acids (Rosner et al. 2002) and bile salts (Rosenberg et al. 2003). In *P. aeruginosa*, the global regulator MvaT has been shown to repress the expression of MexEF-OprN efflux pump (Westfall et al. 2006). This indicates that substrates in the natural environment of a bacterium may be the inducers of its RND efflux pumps.

1.6.3. Regulation by two-component systems

Some RND efflux pumps are regulated by two-component systems, which play a role in bacterial adaptation to their environment (Kumar and Schweizer 2005). The two-component systems involved in the regulation of RND efflux pump genes are often located nearby, so the response regulators act as an activator. For example in *E. coli*, the BaeSR and EvgAS two-component systems regulate expression of the MdtABC and the AcrAB-TolC pumps respectively (Baranova and Nikaido 2002; Nagakubo et al. 2002; Eguchi et al. 2003). The PhoP-PhoQ two-component system, which responds to the concentrations of Ca^{2+} and Mg^{2+} in the environment, has also been linked to the activation of RND efflux pumps (Eguchi et al. 2003).

1.7. New opinions concerning the role(s) of RND efflux pumps in bacteria

Generally, RND efflux pumps are regarded as a mechanism of detoxification based on their involvement in the efflux of antibiotics and solvents that are toxic to the bacterial cell. Over the years, other functions of RND efflux pumps in Gram-negative bacteria have been uncovered such as cell-cell communication (Minagawa et al. 2012), membrane stress response, oxidative and nitrosative stress responses (Evans et al. 1998; Fraud et al. 2008; Jeannot et al. 2005), and removal of toxins and toxic metabolites (Helling et al. 2002; Taylor et al. 2012; Tian et al. 2010). If the authentic substrates of RND pumps are not antibiotics or solvents, the question arises, 'what are the authentic substrates of these pumps in bacterial cells or what promotes the assembly of these pumps'? Although there is controversy about the authentic physiological role(s) of RND efflux pumps in bacteria, evidence has been reported in the literature that they have physiological functions beyond antibiotic efflux. Recently, Kumar and Schweizer (2011) reported the over-expression of MexEF-OprN in *P. aeruginosa* to be dependent on metabolic stress but independent of antibiotic exposure. *P. aeruginosa* mutants expressing the Mex-RND efflux pumps have been isolated in experimentally infected mice in the absence of antibiotics (Poole et al. 2008). In addition, RND efflux pumps transport structurally diverse compounds but are not induced by some of the substrates transported (Table 1.2). The *P. fluorescens* LP6a EmhABC

efflux pump is not induced by phenanthrene although this PAH is a substrate (Bugg et al. 2000; Hearn et al. 2003). Bile salts are substrates and inducers of the AcrB efflux pump of *E. coli* (Nikaido 2009; Thanassi et al. 1997), but there is also a physiological aspect in that bile salts are found in the environment where *E. coli* thrives (Thanassi et al. 1997).

Expression of MexXY in *P. aeruginosa* is induced by antibiotics that interact with the ribosome, such as tetracycline, chloramphenicol, macrolides and aminoglycosides, in a concentration-dependent manner (Jeannot et al. 2005; Masuda et al. 2000). On the other hand, antibiotics that target the cell wall such as β-lactams, or fluoroquinolones that interfere with DNA replication, do not affect the expression of *mexXY* (Jeannot et al. 2005) emphasizing that the MexXY may have additional functions in *P. aeruginosa*. Expression studies by Jeannot et al. (2005) showed a physiological relationship between the MexXY efflux pump and ribosome disruption, as follows: ribosome disruption by antibiotics leads to mistranslation of proteins, production of abnormal polypeptides and the induction of a protein (PA5471) with unspecified function (Morita et al. 2006; Poole et al. 2008). In turn, the PA5471 induced by the presence of abnormal polypeptides directly or indirectly induces *mexXY* expression in *P. aeruginosa* (Morita et al. 2006). Other reports of substrate induction of RND efflux pumps include induction of TtgDEF and TtgGHI in *P. putida* DOT-T1E by toluene (Mosqueda et al. 1999; Rojas et al. 2001). Conversely, TtgABC is expressed at high levels in the presence or

absence of toluene (Duque et al. 2001). This indicates that toluene may not be the primary inducer of Ttg efflux pumps but that the effect of toluene on the cell membrane may be the trigger that induces their expression.

The association of the cell membrane with efflux pump expression was established by Fraud et al. (2008) and Muller et al. (2007) who attributed the up-regulation of RND efflux pumps to membrane-damaging agents. Fraud et al. (2008) tested the effect of several membrane-active agents such as detergent, solvents and cationic antimicrobials on the expression of *mexCD-oprJ* in *P. aeruginosa* and found that they all induced the expression of the efflux genes in an AlgU-dependent manner. AlgU is a homologue of the RpoE sigma factor of *E. coli*, which regulates the expression of several genes involved in envelope stress response (Fraud et al. 2008; Poole 2008). This suggests an apparent connection between membrane damage and the recruitment of RND efflux pumps. Fraud et al. (2008) concluded that MexCD-OprJ may be responsible for the efflux of membrane fatty acids damaged or replaced due to the action of or adaptation to the membrane-active agent. In addition, MexCD-OprJ may be involved in normal membrane turnover to repair membrane stress because of bacterial growth (Fraud et al. 2008; Poole 2008). To this effect, Poole (2008) has proposed two models for the induction of Mex efflux pumps in *P. aeruginosa*. Induction of RND efflux pumps may be the consequence of the action of the efflux pump substrates (Poole 2008).

1.8. Function of RND efflux pumps in the environment

The role of RND efflux pumps in the survival of bacterial species in their environment have been documented over the years. RND efflux pumps in Gram-negative bacteria found in the soil, such as *Pseudomonas* species, are mainly involved in the efflux of foreign compounds found in that environment (Hearn et al. 2003; Huertas et al. 2000; Nikaido 2009). *P. fluorescens* LP6a isolated from petroleum-contaminated soil (Foght and Westlake 1991) efflux PAHs such as phenanthrene (Bugg et al. 2000; Hearn et al. 2003), a component of petroleum (See section 1.10 for more details), in addition to hydrophobic antibiotics. Buckley et al. (2006) showed that prolonged colonization of chickens by *Salmonella* species is dependent on the AcrAB-TolC efflux pump. Although this finding was countered with the explanation that AcrAB-TolC is required for survival in the presence bile salts found in the chicken intestine (Nikaido 2009; Webber et al. 2009), this does not explain the involvement of the efflux pump in the *in vitro* adhesion to and invasion of epithelial cells and macrophages by *Salmonella* species (Webber et al. 2009). *Salmonella* species lacking the AcrAB-TolC efflux pump have also been shown to have reduced ability to kill infected mice (Nishino et al. 2006). In the plant pathogen *P. syringae*, loss of the PseABC RND efflux pumps caused decreased secretion of lipopeptide phytotoxins, thereby reducing its ability to invade the plant host (Kang and Gross 2005). While there may be

explanations for the function of RND efflux pumps in the environment, loss of function of some RND efflux pumps of *P. aeruginosa* resulted in loss of invasiveness in epithelial cell cultures by unknown mechanisms (Hirakata et al. 2002).

1.9. What is the connection? Environmental factors, bacterial cell membranes and RND efflux pumps

Because the bacterial membrane delimits the cell from the environment, it is “fashioned” strategically to be modulated in response to environmental perturbations (Denich et al. 2003; Zhang and Rock 2008). Slight changes in membrane fatty acids (FA) or phospholipid head group composition can change the lipid packing within the membrane, thereby affecting its stability, fluidity and lipid-protein interaction (Denich et al. 2003; Phillips et al. 2009; Zhang and Rock 2008). Logically therefore, perturbation of the cell membrane will affect stability and function of RND efflux pumps that are embedded in the cytoplasmic membrane (Denich et al. 2003; Lomovskaya and Totrov 2005). Although phospholipid head groups are modified in response to environmental changes, the modification of the FA chains is more pronounced (Denich et al. 2003).

A common mechanism for modifying bacterial membrane FAs is to change the ratio of saturated to unsaturated FAs (Russell 1984). Saturated FAs are packed closely in the membrane giving more rigidity and stability to the membrane (Hazel and Williams 1990), whereas

unsaturated FAs have a kink in their structure due to the *cis*-double bond (Fig.1.3) and cannot pack as closely as saturated FA, causing the membrane to be de-stabilized (Gruner et al. 1985; Hazel and Williams 1990). Some bacterial species also have an alternative mechanism to convert existing *cis*-unsaturated FAs to their *trans*-isomers (Diefenbach et al. 1992). *Trans*-unsaturated FAs do not pack as closely as saturated FAs because they retain a slight kink in their conformation (Fig. 1.3) but behave like saturated FAs in the membrane (Diefenbach et al. 1992; Zhang and Rock 2008). Changes in the ratio of *cis:trans* unsaturated FA are observed in the absence of growth in *P. putida* strains exposed to phenol (Diefenbach et al. 1992) or toluene (Junker and Ramos 1999). Both changes in the ratio of saturated:unsaturated FAs and *cis:trans* unsaturated FAs are short-term responses to modify the cell membrane (Russell 1984). Long-term changes in the cell membrane composition include changes in the FA chain length and branching (Russell 1984). Changes in the proportion of cyclopropane FAs, which increase the fluidity of the cytoplasmic membrane, are also common in Gram-negative organisms (Eze and McElhaney 1981; Russell 1984).

Deviation from optimum environmental factors such as temperature, pH, pressure, divalent cations and chemicals, nutrients, water activity and the age of culture affects the cell membrane composition (Denich et al. 2003) and probably the function of proteins embedded in the membrane such as RND efflux pumps. For example,

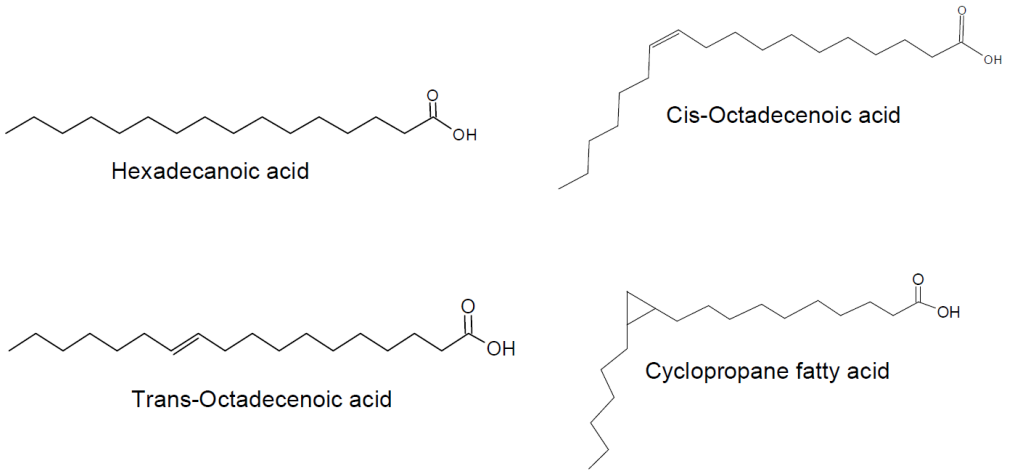

Figure 1.3 Various conformations of membrane fatty acids found in the Gram-negative bacterial membrane.

the proportion of long chain and saturated FAs increases with increased temperature (Keweloh et al. 1991), whereas unsaturated and short chain FAs are preferred at lower temperatures (Kim et al. 2001). While there is extensive literature on the effect of environmental factors on the membrane fatty acid composition of different bacteria, there is no information on how these changes in membrane composition affect the stability and function of RND efflux pumps.

Accumulation of FA degradation products due to membrane modification in response to environmental factors or membrane turnover would cause the disruption of the cell membrane (Zhang and Rock 2008). These FA degradation products must be recycled or eliminated from the

bacterial cells to maintain cell membrane integrity (Zhang and Rock 2008). Recycling is the preferred method of removing these degradation products because it is more energy efficient (Zhang and Rock 2008) but some are eliminated from the cells through unknown mechanism(s). There is a possibility that RND efflux pumps are involved in the transport of such degradation products of FAs (Fraud et al. 2008; Poole 2008). In addition to the association of the Mex efflux pump with efflux of fatty acids (Fraud et al. 2008), the MFS transporter LpIT is also involved in the transport of membrane lipids (Harvat et al. 2005). LpIT is an integral membrane protein of the major facilitator superfamily of membrane transporters involved in the transportation of 2-acylglycerol-phosphoethanolamine, a membrane-disruptive product from the turnover of phosphatidylethanolamine (Harvat et al. 2005). Recently, Doughty et al. (2011) showed that hopanoids found in some bacterial membranes are substrates of the HpnN efflux pump in *Rhodopseudomonas palustris*. Hopanoids are bacterial lipids that play important roles in cell membrane adaptation to temperature, desiccation, pH and cellular differentiation (Jiao and Newman 2007). FA degradation products may be authentic substrates of RND efflux pump since most substrates of these efflux pumps identified to date affect the cell membrane composition and/or integrity.

1.10. *Pseudomonas fluorescens* LP6a, a model to study the natural function of RND efflux pumps

P. fluorescens LP6a was isolated from petroleum condensate-contaminated soil by Foght and Westlake (1991) for its ability to use PAHs such as naphthalene, phenanthrene, anthracene and 2-methylnaphthalene as sole carbon and energy sources (Foght and Westlake 1996). The wild-type LP6a strain can also co-metabolize a variety of hydrocarbon compounds including fluorene, acenaphthene and the heterocyclic aromatic compound dibenzothiophene because its aromatic-degrading enzymes have broad substrate specificity (Foght and Westlake 1996). *P. fluorescens* belongs to the Pseudomonadaceae family, which are Gram-negative rods phylogenetically classified as Gamma-proteobacteria (Woese et al. 1985). *Pseudomonas* species are aerobes and facultative aerobes, non-fermentative chemoorganotrophs capable of utilizing a wide variety of organic substrates (Woese et al. 1985). The PAH catabolic genes in strain LP6a are located on a 63 kb plasmid (pLP6a), separated into the upper- (NahA-F) and lower- (NahG & H) pathways (Foght and Westlake 1996). The arrangement of the PAH catabolic genes into two separate clusters is analogous to archetypal NAH plasmids such as the NAH7 and pWW60 of *P. putida* 1064 and *P. putida* NCIB 98163 but the restriction pattern of plasmid pLP6a is unique (Foght and Westlake 1996). PAHs such as phenanthrene and anthracene used as carbon substrates by *P. fluorescens* LP6a readily partition into the cell membrane

because of their hydrophobic nature. Efflux of PAHs by *P. fluorescens* LP6a was first reported by Bugg et al. (2000). Subsequently Hearn et al. (2003) identified an efflux pump of the RND superfamily to be responsible for PAH efflux in *P. fluorescens* LP6a (See section 1.11). Although efflux of hydrocarbons such as toluene had been reported previously in *P. putida* strains (Kieboom et al. 1998), efflux of PAHs by *P. fluorescens* LP6a seems counterproductive because it expends energy to extrude PAHs that are substrates of cytoplasmic catabolic enzymes. PAH efflux in *P. fluorescens* LP6a is independent of biodegradation because the efflux genes (*emhABC*) are located on the chromosome (Hearn et al. 2003; 2006). Mutants of *P. fluorescens* have been generated such as a cured strain lacking the pLP6a catabolic plasmid (Foght and Westlake, 1996) and strain cLP6a-1, which is the *emhB* disruptant mutant of the cured strain (Hearn et al. 2003). The availability of a variety of mutants and the presence of apparently conflicting mechanisms of efflux and catabolism makes study of efflux and biodegradation of PAH compounds possible using *P. fluorescens* LP6a as a model organism.

1.11. The EmhABC efflux pump

Experiments intended to study PAH uptake (Bugg et al. 2000) led to the discovery of the EmhABC efflux pump in *P. fluorescens* LP6a (Hearn et al. 2003). EmhABC is a HAE1 RND-type efflux pump identified by and characterized for its role in the efflux of PAHs, hence the name Emh

(efflux of multicyclic hydrocarbons) (Hearn et al. 2003; 2006). The RND protein EmhB presumably associates with EmhA (the PAP) and EmhC (the OMP). Based on protein homology, the RND protein EmhB is 85% identical to TtgB, which plays a role in solvent tolerance of *P. putida* DOT-T1E, and to the antibiotic exporting pump ArpB in *P. putida* S12 (Hearn et al. 2003). The EmhABC efflux pump also shares 80% identity with MexB in *P. aeruginosa* PA01 and 67% identity with AcrB in *E. coli* (Hearn et al. 2003). A putative TetR regulatory protein, EmhR, is located upstream of the *emhABC* genes but transcribed divergently from the efflux operon (Hearn et al. 2003). EmhR is assumed to be a local transcriptional regulator of the *emhABC* genes (Hearn et al. 2003), an assumption confirmed by the work of another group (Tian et al. 2010) who determined that EmhR is a repressor of an EmhABC homolog in *P. fluorescens* 2P24. Identified substrates of the EmhABC efflux pump (Table 1.2) include the PAHs phenanthrene, anthracene and fluoranthene but not naphthalene; the hydrophobic antibiotics chloramphenicol, tetracycline, ciprofloxacin and nalidixic acid; the quaternary ammonium cation dequalinium; and the cationic dye rhodamine 6G (Bugg et al. 2000; Hearn et al. 2003; 2006). The efflux of phenanthrene and anthracene was a surprise as these compounds are non-, or slightly toxic to *P. fluorescens* LP6a cells carrying the EmhABC efflux pump (Bugg et al. 2000; Hearn et al. 2003) or other hydrocarbon-degrading species (Sikkema et al. 1995). Efflux of

phenanthrene is not unique to EmhABC efflux pump; it is a substrate of other efflux pumps such as SrpABC and MexAB-OprM (Hearn et al. 2003).

1.12. Significance of RND efflux pumps in bacteria

Regardless of the proposal that antibiotics, solvents and PAHs are incidental substrates of RND efflux pumps, their efflux has clinical (Poole 2008) and biotechnological significance. The interest that has been generated to determine the authentic substrates of the HAE1 family of RND efflux pumps in particular is to understand why this efflux pump transports antimicrobials and hydrocarbon compounds. Identifying the authentic substrates and function of RND efflux in Gram-negative bacteria will be useful to effectively combat antibiotic resistance and improve biotechnological processes involving the use of hydrocarbon solvents or PAHs.

1.12.1. Clinical significance

Bacterial infections can be difficult to treat because of multiple antibiotic resistance due to membrane adaptations and RND efflux pumps. The sensitivity of Gram-negative bacteria to a particular antibiotic decrease when they possess functional RND efflux pumps that transport the antibiotic. For instance, *P. fluorescens* strain cLP6a-1 with a disrupted EmhB efflux pump was more sensitive than the wild type to hydrophobic antibiotics transported by this pump (Hearn et al. 2003; 2006). Temperature and pH affect the efflux of antibiotics by *Serratia marcescens*

(Begic and Worobec 2007), supporting the idea that environmental factors affecting the cell membrane may induce the expression of RND efflux pumps. Over-expression of Mex efflux pumps of *P. aeruginosa*, a soil dwelling bacterium, in cystic fibrosis patients (Poole et al. 2008) may occur because of the change in environmental temperature. Knowledge of the natural substrate(s) and inducers of RND efflux pumps, how RND efflux pumps are regulated and what environmental factors promote their expression will help in selecting effective antibiotic therapies (Poole 2008). If the perturbation of the cell membrane is the trigger for the recruitment of RND efflux pumps, then development of an antibiotic that will not perturb the membrane may be a useful option.

1.12.2. Biotechnological significance

Degradation of hydrocarbon pollutants by bacterial species such as *Pseudomonas* is a safe and cheap method of removing these toxic compounds from a contaminated environment (Daugulis 2001; Mohn et al. 2001) compared to physicochemical methods such as incineration (Mallick et al. 2011). The involvement of RND efflux pumps in hydrocarbon efflux may or may not be beneficial for biotechnology. The presence of RND efflux pumps in bacterial species used for remediation of environments contaminated with solvents such as toluene may be beneficial to reduce the concentration of toluene that accumulates in the cell membrane to manage the effect of toluene on the membrane. Bacterial species such as *P. putida* that can survive high concentrations of toluene or other organic

solvents because of RND efflux pumps may be useful tools in bioremediation or biotransformation involving organic solvents. Conversely, efflux of PAHs such as phenanthrene in *P. fluorescens* LP6a may reduce the concentration available for biodegradation since catabolism of these compounds occurs in the cytoplasm (Bugg et al. 2000).

1.13. Thesis overview and research objectives

The main objective of my research was to identify the authentic role(s) and substrates of the EmhABC efflux pump of *P. fluorescens* LP6a strains. Remarkably, environmental factors such as temperature were inducers of EmhABC efflux pump (Chapter 2, Adebusuyi and Foght 2011). The other objective was to understand how the presence or up-regulation of the EmhABC efflux pump affected the biodegradation of phenanthrene, an EmhABC substrate (Chapter 3, Adebusuyi et al. 2012), of naphthalene, a non-substrate (Chapter 4) and the transport of antibiotic substrates such as chloramphenicol (Chapter 5). From this study, up-regulation of EmhABC efflux pump was found to be dependent on membrane FA modulation. Suggestions are made for future studies (Chapter 6) on how to apply the knowledge of bacterial RND efflux pumps for biotechnological purposes and to study the function of these pumps in anaerobic hydrocarbon-degrading hydrocarbons. This research describes the link between environmental factors, the bacterial membrane, and RND efflux

pumps, which are membrane integral proteins. Besides the identification of novel inducers of the EmhABC efflux pump, alternative substrates of this efflux pump were also discovered.

1.14. Literature cited

Adebusuyi AA, Foght JM (2011) An alternative physiological role for the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a. BMC Microbiol 11:252

Adebusuyi AA, Smith AY, Gray MR, Foght JM (2012) The EmhABC efflux pump decreases the efficiency of phenanthrene biodegradation by *Pseudomonas fluorescens* strain LP6a. Appl Microbiol Biotechnol DOI 10.1007/s00253-012-3932-4

Aklujkar M, Krushkal J, DiBartolo G, Lapidus A, Land ML, Lovley DR (2009) The genome sequence of *Geobacter metallireducens:* features of metabolism, physiology and regulation common and dissimilar to *Geobacter sulfurreducens*. BMC Microbiol 9:109

Baev N, Endre G, Petrovics G, Banfalvi Z, Kondorosi A (1991) Six nodulation genes of *nod* box locus 4 in *Rhizobium meliloti* are involved in nodulation signal production: nodM codes for D-glucosamine synthetase. Mol Gen Genet 228:113-124

Ball PR, Shales SW, Chopra I (1980) Plasmid-mediated tetracycline resistance in *Escherichia coli* involves increased efflux of the antibiotic. Biochem Biophys Res Commun 93:74-81

Baranova N, Nikaido H (2002) The BaeSR two-component regulatory system activates transcription of the *yegMNOB* (*mdtABCD*) transporter gene cluster in *Escherichia coli* and increases its resistance to novobiocin and deoxycholate. J Bacteriol 184:4168-4176

Begic S, Worobec EA (2007) Fluoroquinolone resistance of *Serratia marcescens*: sucrose, salicylate, temperature and pH induction of phenotypic resistance. Can J Microbiol 53:1239-1245

Beinlich KL, Chuanchuen R, Schweizer HP (2001) Contribution of multidrug efflux pumps to multiple antibiotic resistance in veterinary

clinical isolates of *Pseudomonas aeruginosa*. FEMS Microbiol Lett 198:129-134

Blair JMA, Piddock LJV (2009) Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update**.** Curr Opin Microbiol 12:512-519

Buckley AM, Webber MA, Cooles S, Randall LP, La Ragione RM, Woosward MJ, Piddock LJV (2006) The AcrAB-TolC efflux system of *Salmonella enteric* serovar Typhimurium plays a role in pathogenesis. Cell Microbiol 8:847-856

Bugg T, Foght JM, Pickard MA, Gray MR (2000) Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol 66**:**5387-5392

Callaghan AV, Morris BEL, Pereira IAC, McInerney MJ, Austin RN, Groves JT, Kukor JJ, Suflita JM, Young LY, Zylstra GJ, Wawrik B (2012) The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. Environ Microbiol 14:101-113

Chung YJ, Saier Jr. MH (2001) SMR-type multidrug resistance pumps. Curr Opin Drug Disc 4:237-245

Daugulis AJ (2001) Two-phase partitioning bioreactors: a new technology platform for destroying xenobiotics. Trends Biotechnol 19:457-462

Davies J (1994) Inactivation of antibiotics and the dissemination of resistance genes. Science 264:375-382

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Methods 52**:**149-182

Diefenbach R, Heipieper HJ, Keweloh H (1992) The conversion of *cis* into *trans* unsaturated fatty acids in *Pseudomonas putita* P8: evidence for a role in the regulation of membrane fluidity. Appl Microbiol Biotechnol 38:382-387

Dinh T, Paulsen IT, Saier Jr. MH (1994) A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. J Bacteriol 176:3825-3831

Doughty DM, Coleman ML, Hunter RC, Sessions AL, Summons RE, Newman DK (2011) The RND-family transporter, HpnN, is required

for hopanoids localization to the outer membrane of *Rhodopseudomonas palustris* TIE-1. PNAS 108:E1045-E1051

Duque E, Segura A, Mosqueda G, Ramos JL (2001) Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of *Pseudomonas putida*. Mol Microbiol 39:1100-1106

Eguchi Y, Oshima T, Mori H, Aono R, Yamamoto K, Ishihama A, Utsumi R (2003) Transcriptional regulation of drug efflux genes by EvgAS, a two-component system in *Escherichia coli*. Microbiol 149:2819-2828

Eicher T, Brandstatter L, Pos KM (2009) Structural and functional aspects of the multidrug efflux pump AcrB. Biol Chem 390:693-699

Evans K, Adewoye L, Poole K (2001) MexR repressor of the *mexAB-oprM* multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the *mexA-mexR* intergenic region. J Bacteriol 183:807-812

Evans K, Passador L, Srikumar R, Tsang E, Nezezon J, Poole K (1998) Influence of the MexAB-OprM multidrug efflux system on quorum sensing in *Pseudomonas aeruginosa*. J Bacteriol 180:5443-5447

Eze MO, McElhaney RN (1981) The effect of alterations in the fluidity and phase state of the membrane lipids on the passive permeation and facilitated diffusion of glycerol in *Escherichia coli*. J Gen Microbiol 124:299-307

Fernández-Moreno MA, Caballero JL, Hopwood DA, Malpatida F (1991) The act cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell 66:769-780

Foght JM, Westlake DWS (1991) Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. Can J Microbiol 37:924-932

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353-366

Fraud S, Campigotto AJ, Chen Z, Poole K (2008) MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in

chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response factor. Antimicrob Agents Chemother 52:4478-4482

George AM, Levy SB (1983a) Gene in the major cotransduction gap of the *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J Bacteriol 155:541-548

George AM, Levy SB (1983b) Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J bacterial 155:531-540

Göttfert M (1993) Regulation and function of rhizobial nodulation genes. FEMS Microbiol Rev 104:39-64

Grkovic S, Brown MH, Skurray RA (2002) Regulation of bacterial drug export systems. Microbiol Mol Biol Rev 66:671-701

Gruner SM, Cullis PR, Hope MJ, Tilcock CPS (1985) Lipid polymorphism: the molecular basis of nonbilayer phases. Annu Rev Biophys Chem 14:211-238

Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM (1995) Resistance of *Neisseria gonorrhoeae* to antimicrobial hydrophobic agents is modulated by the *mtrRCDE* efflux system. Microbiol 141:611-622

Harvat EM, Zhang YM, Tran CV, Zhang Z, Frank MW, Rock CO, Saier Jr. MH (2005) Lysophospholipid flipping across the *Escherichia coli* inner membrane catalyzed by a transporter (LpIT) belonging to the major facilitator superfamily. J Biol Chem 280:12028-12034

Hazel JR, Williams EE (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. Prog Lipid Res 29:167-227

Hearn EM, Dennis JJ, Gray MR, Foght JM (2003) Identification and characterization of the *emh*ABC efflux system for polycyclic aromatic hydrocarbons in *Pseudomonas fluorescens* cLP6a. J Bacteriol 185:6233-6240

Hearn EM, Gray MR, Foght JM (2006) Mutations in the central cavity and periplasmic domain affect efflux activity of the resistance-nodulation-division pump EmhB from *Pseudomonas fluorescens*

cLP6a. J Bacteriol 188**:**115-123

Helling RB, Janes BK, Kimball H, Tran T, Bundesmann M, Check P, Phelan D, Miller C (2002) Toxic waste disposal in *Escherichia coli*. J Bacteriol 184:3699-3703

Higgins PG, Fluit AC, Milatovic D, Verhoef J, Schmitz FJ (2003) Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. Int J Antimicrob Agents 21:409-413

Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, Kohno S, Hancock RE, Speert DP (2002) Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. J Exp Med 196:109-118

Huertas M-J, Duque E, Molina L, Roselló-Mora R, Mosqueda G, Godoy P, Christensen B, Molin S, Ramos JL (2000) Tolerance to sudden organic solvent shocks by soil bacteria and characterization of *Pseudomonas putida* strains isolated from toluene polluted sites. Environ Sci Technol 34:3395 3400

Ikeda T, Yoshimura F (2002) A resistance-nodulation-cell division family of xenobiotic efflux pump in an obligate anaerobe, *Porphyromonas gingivalis*. Antimicrob Agents Chemother 46:3257-3260

Jeannot K, Sobel ML, Garch FE, Poole K, Plesiat P (2005) Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribose interaction. J Bacteriol 187:5341-5346

Jiao Y, Newman DK (2007) The pio operon is essential for phototrophic Fe(II) oxidation in *Rhodopseudomonas palustris* TIE-1. J bacterial 189:1765-1773

Juliano RL, Ling V (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta 455:152-162

Junker F, Ramos JL (1999) Involvement of the cis/trans isomerise Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181:5693-5700

Kang H, Gross DC (2005) Characterization of a resistance-nodulation-cell division transporter system associated with the *syr-syp* genomic island of *Pseudomonas syringae* pv. Syringae. Appl Environ Microbiol 71:5056-5065

Kartner N, Riordan JR, Ling V (1983) Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science 221:1285-1288

Keweloh H, Diefenbach R, Rehm HJ (1991) Increase of phenol tolerance of *Escherichia coli* by alterations of the fatty acid composition of the membrane lipids. Arch Microbiol 157:49-53

Kieboom J, Dennis JJ, de Bont JAM, Zylstra GJ (1998) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85-91

Kim IS, Lee H, Trevors JT (2001) Effects of 2,2′,5,5′-tetrachlorbiphenyl and biphenyl on cell membranes of *Ralstonia eutropha* H850. FEMS Microbiol Lett 200:17-24

Kohler T, Epp SF, Curty LK, Pechere JC (1999) Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of *Pseudomonas aeruginosa*. J Bacteriol 181:6300-6305

Kōhler T, van Delden C, Curty LK, Hamzehpour MM, Pechere JC (2001) Overexpression of the MexEF-OprN multidrug efflux system affects cell-to-cell signaling in *Pseudomonas aeruginosa*. J bacterial 183:5213-5222

Kumar A, Schweizer HP (2005) Bacterial resistance to antibiotics: Active efflux and reduced uptake. Adv Drug Del Revs 57:1486-1513

Kumar A, Schweizer HP (2011) Evidence of MexT-independent overexpression of MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa* in presence of metabolic stress. PLoS ONE 6(10):e26520 doi:10.1371/journal.pone.0026520

Law CJ, Maloney PC, Wang DN (2008) Ins and outs of major facilitator superfamily antiporters. Annu Rev Microbiol 62:289-305

Levy SB, McMurry L (1978) Plasmid-determined tetracycline resistance involves new transport systems for tetracycline. Nature 276:90-92

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

Li XZ, Nikaido H (2009) Efflux-mediated drug resistance in bacteria: An update. Drugs 69:1555-1623

Li XZ, Nikaido H, Poole K (1995) Role of *mexA-mexB-oprM* in antibiotic

efflux in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 39:1948-1953

Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plesiat P (2004) Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrob Agents Chemother 48:1797-1802

Lobedanz S, Bokma E, Symmons MF, Koronakis E, Hughes C, Koronakis V (2007). A periplasmic coiled-coil interface underlying TolC recruitment and the assembly of bacterial drug efflux pumps. PNAS 104:4612-4617

Loftus SK, Morris JA, Carstea ED, Gu JZ, Cummings C, Brown A, Ellison J, Ohno K, Rosenfeld MA, Tagle DA, Pentchev PG, Pavan WJ (1997) Murine model of Niemann-Pick C disease: Mutation in a cholesterol homeostasis gene. Science 277:232-235

Lomovskaya O, Totrov M (2005) Vacuuming the periplasm. J Bacteriol 187:1879-1883

Lubelski J, Konings WN, Driessen AJ (2007) Distribution and physiology of ABC-type transporters contributing to multidrug resistance in bacteria. Microbiol Mol Biol Rev 71:463-476

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

Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE (1995) Genes *acrA* and *acrB* encode a stress-induced efflux system of *Escherichia coli*. Mol Microbiol 16:45-55

Mallick S, Chakraborty J, Dutta TK (2011) Role of oxygenases in guiding diverse metabolic pathways in the bacterial degradation of low-molecular-weight polycyclic aromatic hydrocarbons: A review. Crit Rev Microbiol 37:64-90

Martinez JL, Sánchez MB, Martinez-Solano L, Hernandez A, Garmendia L, Fajardo A, Alvarez-Ortega C (2009) Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev 33:430-449

Masuda N, Sakagawa E, Ohya S, Gotoh N, Nishino K (2001) Hypersusceptibility of the *Pseudomonas aeruginosa nfxB* mutant to β-lactams due to reduced expression of the AmpC β-lactamase.

Antimicrob Agents Chemother 45:1284-1286

Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T (2000) Contribution of the MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 44:2242-2246

McMurry L, Petrucci Jr. RE, Levy SB (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. PNAS 77:3974-3977

Meguro N, Kodama Y, Gallegos M-T, Watanabe K (2005) Molecular characterization of resistance-nodulation-division transporters from solvent- and drug-resistant bacteria in petroleum-contaminated soil. Appl Environ Microbiol 71:580-589

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

Minagawa S, Inami H, Kato T, Sawada S, Yasuki T, Miyairi S, Horikawa M, Okuda J, Gotoh N (2012) RND type efflux pump system MexAB-OprM of *Pseudomonas aeruginosa* selects bacterial languages, 3-oxo-acyl-homoserine lactones, for cell-to-cell communication. BMC Microbiol 12:70 doi:10.1186/1471-2180-12-70

Mohn WW, Radziminski CZ, Fortin MC, Reimer KJ (2001) On site bioremediation of hydrocarbon-contaminated Arctic tundra soils in inoculated biopiles. Appl Microbiol Biotechnol 57:242-247

Morita Y, Sobel ML, Poole K (2006) Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: Involvement of the antibiotic-inducible PA5471 gene product. J Bacteriol 188:1847-1855

Moriyama Y, Hiasa M, Matsumoto T, Omote H (2008) Multidrug and toxic compound extrusion (MATE)-type proteins as anchor transporters for the excretion of metabolic waste products and xenobiotics. Xenobiotica 38:1107-1118

Mosqueda G, Ramos JL (2000) A set of genes encoding a second toluene efflux system in *Pseudomonas putida* DOT-T1E is linked to the *tod* genes for toluene metabolism. J Bacteriol 182:937-943

Mosqueda G, Ramos-González MI, Ramos JL (1999) Toluene metabolism by the solvent-tolerant *Pseudomonas putida* DOT-T1E strain, and its role in solvent impermeabilization. Gene 232:69-76

Muller JF, Stevens AM, Craig J, Love NG (2007) Transcriptome analysis reveals that multidrug efflux genes are upregulated to protect *Pseudomonas aeruginosa* from pentachlorophenol stress. Appl Environ Microbiol 73:4550-4558

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

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

Nagakubo S, Nishino k, Hirata T, Yamaguchi (2002) The putative response regulator BaeR stimulates multidrug resistance of *Escherichia coli* via a novel multidrug exporter system, MdtABC. J Bacteriol 184:4161-4167

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

Nikaido H (2009) Multidrug resistance in bacteria. Annu Rev Biochem 78:119-146

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

Nishino K, Latifi T, Groisman EA (2006) Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enteric* serovar Typhimurium. Mol Microbiol 59:126-141

Ohene-Agyei T, Lea JD, Venter H (2012) Mutations in MexB that affect the efflux of antibiotics with cytoplasmic targets. FEMS Microbiol Lett doi:10.1111/j.1574-6968.2012.02594.x

Orth P, Schnappinger D, Hillen W, Saenger W, Hinrichs W (2000) Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. Nature Struct Biol 7: 215-219

Pang Y, Brown BA, Steingrube VA, Wallace Jr. RJ, Roberts MC (1994) Tetracycline resistance determinants in *Mycobacterium* and

Streptomyces species. Antimicrob Agents Chemother 38:1408-1412

Paulsen IT, Chen J, Nelson KE, Seier MH Jr. (2001) Comparative genomics of microbial drug efflux systems. J Mol Microbiol Biotechnol 3:145-150

Paulsen IT, Park JH, Choi PS, Saier Jr. MH (1997) A family of gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from gram-negative bacteria. FEMS Microbiol Lett 156:1-8

Paulsen IT, Sliwinski MK, Saier Jr. MH (1998) Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. J Mol Biol 277:573-592

Phillips R, Ursell T, Wiggins P, Sens P (2009) Emerging roles for lipids in shaping membrane-protein function. Nature 459:379-385

Piddock LJV (2006) Multidrug-resistance efflux pumps – not just for resistance. Nature Rev Microbiol 4:629-636

Poole K (2008) Bacterial multidrug efflux pumps serve other functions. Microbe 3:179-185

Poole K, Gotoh N, Tsujimoto H, Zhao Q, Wada A, Yamasaki T, Neshat S, Yamagishi J-I, Li X-Z, Nishino T (1996) Overexpression of the *mexC-mexD-oprJ* efflux operon in *nfxB* multidrug resistant strains of *Pseudomonas aeruginosa*. Mol Microbiol 21:713-724

Pos KM (2009) Drug transport mechanism of the AcrB efflux pump. Biochim Biophys Acta 1794:782-793

Ramos J-L, Duque E, Godoy P, Segura A (1998) Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323-3329

Rensing C, Pribyl T, Nies DH (1997) New functions for the three subunits of the CzcCBA cation-proton antiporter. J Bacteriol 179:6871-6879

Rodriguez-Herva JJ, Garcia V, Hurtado A, Segura A, Ramos JL (2007) The *ttgGHI* solvent efflux pump operon of *Pseudomonas putida* DOT-T1E is located on a large self-transmissible plasmid. Environ Microbiol 9:1550-1561.

Rojas A, Duque E, Mosqueda G, Golden G, Hurtado A, Ramos JL, Segura A (2001) Three efflux pumps are required to provide efficient tolerance to toluene in *Pseudomonas putida* DOT-T1E. J Bacteriol 183:3967-3973

Rojas A, Segura A, Guazzaroni ME, Terán W, Hurtado A, Gallegos MT, Ramos JL (2003) *In vivo* and *in vitro* evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of *Pseudomonas putida*. J Bacteriol 185:4755-4763

Rosenberg EY, Bertenthal D, Nilles ML, Bertrand KP, Nikaido H (2003) Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. Mol Microbiol 48:1609-1619

Rosner JL, Dangi B, Gronenborn AM, Martin RG (2002) Posttranscriptional activation of the transcriptional activator Rob by dipyridyl in *Escherichia coli*. J Bacteriol 184:1407-1416

Russell NJ (1984) Mechanisms of thermal adaptation in bacteria: blueprints for survival. Trends Biochem Sci 9:108-112

Saito K, Yoneyama H, Nakae T (1999) *nalB*-type mutations causing the overexpression of the MexAB-OprM efflux pump are located in the *mexR* gene of the *Pseudomonas aeruginosa* chromosome. FEMS Microbiol Lett 179:67-72

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

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

Sikkema J, De Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222

Sobel ML, Neshat S, Poole K (2005) Mutations in PA2491 (*mexS*) promote MexT-dependent *mexEF-oprN* expression and multidrug resistance in a clinical strain of *Pseudomonas aeruginosa*. J Bacteriol 187:1246-1253

Sun X, Zahir Z, Lynch KH, Dennis JJ (2011) An antirepressor, SrpR, is involved in transcriptional regulation of the SrpABC solvent

tolerance efflux pump of *Pseudomonas putida* S12. J Bacteriol 193:2717-2725

Symmons MF, Bokma E, Koronakis E, Hughes C, Koronakis V (2009) The assembled structure of a complete tripartite bacterial multidrug efflux pump. PNAS 106:7173-7178

Takatsuka Y, Nikaido H (2009) Covalently linked trimer of the AcrB multidrug efflux pump provides support for the functional rotating mechanism. J Bacteriol 191:1729-1737

Tamura N, Murakami S, Oyama Y, Ishiguro M, Yamaguchi A (2005) Direct interaction of multidrug efflux transporter AcrB and outer membrane channel TolC detected via site-directed disulfide cross-linking. Biochem 44:11115-11121

Taylor DL, Bina XR, Bina JE (2012) *Vibrio cholera vexH* encodes a multiple drug efflux pump that contributes to the production of cholera toxin and the toxin co-regulated pilus. PLoS ONE 7(5):e38208 doi:10.1371/journal.pone.0038298

Terán W, Krell T, Ramos JL, Gallegos MT (2006) Effector-repressor interactions, binding of a single effector molecule to the operator-bound TtgR homodimer mediates depression. J Biol Chem 281:7102-7109

Thanassi DG, Cheng LW, Nikaido H (1997) Active efflux of bile salts by *Escherichia coli*. J Bacteriol 179:2512-2518

Tian T, Wu X-G, Duan H-M, Zhang L-Q (2010) The resistance-nodulation-division efflux pump EmhABC influences the production of 2,4-diacetylphloroglucinol in *Pseudomonas fluorescens* 2P24. Microbiol 156:39-48

Tikhonova EB, Dastidar V, Rybenkov VV, Zgurskaya HI (2009) Kinetic control of TolC recruitment by multidrug efflux complexes. PNAS 106:16416-16421

Tikhonova EB, Yamada Y, Zgurskaya HI (2011) Sequential mechanism of assembly of multidrug efflux pump AcrAB-TolC. Chem Biol 18:454-463

Trepout S, Taveau J-C, Benabdelhak H, Granier T, Ducruix A, Frangakis AS, Lambert O (2010) Structure of reconstituted bacterial membrane efflux pump by cryo-electron tomography. Biochim Biophys Acta 1798:1953-1960

Tseng T-T, Gratwick KS, Kollman J, Park D, Nies DH, Goffeau A, Saier Jr. MH (1999) The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins. J Mol Microbiol Biotechnol 1:107-125

Wang H, Dzink-Fox JL, Chen M, Levy SB (2001) Genetic characterization of highly fluoroquinolone-resistant clinical *Escherichia coli* strains from China: role of *acrR* mutations. Antimicrob Agents Chemother 45:1515-1521

Webber MA, Bailey AM, Blair JMA, Morgan E, Stevens MP, Hinton JCD, Ivens A, Wain J, Piddock LJV (2009) The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. J Bacteriol 191**:**4276-4285

Weeks JW, Celaya-Kolb T, Pecora S, Misra R (2010) AcrA suppressor alterations reverse the drug hypersensitivity phenotype of a TolC mutant by inducing TolC aperture opening. Mol Microbiol 75:1468-1483

Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P, Nguyen LY, Shawar RM, Folger KR, Stover CK (1999) Characterization of a *Pseudomonas aeruginosa* efflux pump contributing to aminoglycoside impermeability. Antimicrob Agents Chemother 33:2975-2983

Westfall LW, Carty NL, Layland N, Kuan P, Colmer-Hamood JA, Hamood AN (2006) *mvaT* mutation modifies the expression of the *Pseudomonas aeruginosa* multidrug efflux operon *mexEF-oprN*. FEMS Microbiol Lett 255:247-254

Woese CR, Stackebrandt E, Macke TJ, Fox GE (1985) A phylogenetic definition of the major eubacterial taxa. Syst Appl Microbiol 6:143-151

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

Zhang L, Li X-Z, Poole K (2001) SmeDEF multidrug efflux pump contributes to intrinsic multidrug resistance in *Stenotrophomonas maltophilia*. Antimicrob Agents Chemother 45:3497-3503

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nature Rev Microbiol 6**:**222-233

Ziha Zafiri I, Llanes C, Kohler T, Pechere J-C, Plesiat P (1999) In vivo emergence of multidrug resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob Agents Chemother 43:287-291

2. An alternative physiological role for the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a[1]

2.1. Introduction

The role of resistance-nodulation-division (RND) efflux pumps in efflux of antibiotics, solvents and polycyclic aromatic hydrocarbons (PAHs) by Gram-negative bacteria (Hearn et al. 2003; Kieboom et al. 1998; Nikaido 1996) raises the question of their "authentic" physiological role in bacteria. Knowledge of the authentic substrates and inducers of RND efflux pumps will be useful to effectively combat antibiotic resistance and improve biocatalytic processes such as production of enantio-pure compounds from hydrocarbons or bioremediation of PAH pollutants. Previous studies seeking the inducers of genes encoding RND efflux pumps focussed on known substrates of the pumps (Fraud et al. 2008; Morita et al. 2006). However, such studies showed that substrates are often not inducers, and the pumps are present and active in bacterial cells that have not been exposed to antibiotics or solvents (Morita et al. 2006; Piddock 2006). Furthermore, genes encoding RND efflux pumps can be induced by stress responses such as ribosome disruption or membrane-damaging agents (Fraud et al. 2008; Jeannot et al. 2005; Lin et al. 2005; Poole 2008). These observations suggest a physiological

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function for RND efflux systems beyond the transport of antibiotics or solvents.

The first step in identifying the authentic physiological role of RND efflux pumps is to determine the effect of physico-chemical factors on efflux pump activity and expression of genes that encode them. A common physical factor affecting the composition and physiology of bacterial cells is incubation temperature (Cebrián et al. 2008; Denich et al. 2003), which influences bacterial cell membrane fatty acid (FA) composition (Denich et al. 2003; Kim et al. 2001). Altered membrane FA composition is an adaptation mechanism used by bacteria to compensate for changes in membrane fluidity caused by physiological or biochemical stress. Fluidity of the membrane affects the interaction of lipids and proteins (including RND efflux pumps) anchored in the membrane and in turn permeation and transport of hydrophobic molecules across the membrane [Denich et al. 2003; Phillips et al. 2009; Sikkema et al. 1995). Changes in the resiliency of cells grown at different temperatures to various environmental stresses have been reported (Cebrián et al. 2008). However, increased resistance to antibiotics, environmental stresses, or membrane-damaging agents has not previously been linked to the effect of growth temperature on increased activity of efflux pumps or expression of their genes.

This work describes the identification of an alternative physiological role for EmhABC, a RND efflux pump in *Pseudomonas fluorescens* LP6a. This efflux pump is a good model for investigating a physiological role for

RND-type efflux pumps because it extrudes PAHs considered non-toxic to the cells as well as hydrophobic antibiotics (Bugg et al. 2000) and dyes (Hearn et al. 2003). EmhABC expression is not induced by its PAH substrates (Hearn et al. 2003). PAH transport can be monitored in the absence of PAH metabolism (Hearn et al. 2003) by using strain cLP6a, a cured strain of *P. fluorescens* LP6a lacking the PAH catabolic plasmid pLP6a (Foght and Westlake 1996). Comparing the properties of cLP6a with its *emhB* disruption mutant, strain cLP6a-1 (Hearn et al. 2003) allows inference of a physiological role for the RND efflux pump EmhABC based on the effects of growth temperature, antibiotics or PAHs on its activity and expression in relation to membrane FA changes.

2.2. Methods

2.2.1. Bacterial strains and growth conditions

P. fluorescens cLP6a is a cured strain of the wild type *P. fluorescens* strain LP6a that lacks the catabolic plasmid pLP6a (Foght and Westlake 1996) and cannot metabolize PAHs. Strain cLP6a-1 is an *emhB* disruption mutant of the cured strain (Hearn et al. 2003). Strains were grown to stationary phase (unless otherwise indicated) in 100 ml of trypticase soy broth (TSB) (Difco) with gyratory shaking at 200 rpm at 10°C, 28°C (the optimal growth temperature; Foght and Westlake 1991) or 35°C. TSB inoculated with strain cLP6a-1 contained kanamycin (Sigma) at 25 µg ml^{-1} to maintain the gene disruption. Growth was measured as

optical density at 600 nm (OD_{600}) using an Ultrospec 3100 pro UV/Visible spectrophotometer (GE Healthcare Bio-Sciences), diluting the TSB blank and culture sample with distilled water as necessary. Naphthalene and phenanthrene were added at a final concentration of 5 mmol l^{-1}, either dissolved in N,N-dimethylformamide (ACS grade, Anachemia) and added to cultures used for RNA extraction or added as a suspension of crystals to cultures used for FA extraction.

2.2.2 Phenanthrene efflux assay

Efflux of [9-^{14}C]phenanthrene (96.5% radiochemical purity; Amersham) was determined using a rapid centrifugation method (Bugg et al. 2000) conducted at room temperature (~22°C). The final concentration of radiolabeled plus unlabeled phenanthrene in the assay medium was 6.4 µM, which corresponds to 90% of its aqueous solubility limit at that temperature and ensures that insoluble phenanthrene does not confound measurement of cell-associated radiolabel. *P. fluorescens* cLP6a and cLP6a-1 cells were harvested by centrifugation, washed once with potassium phosphate buffer [pH 7] and re-suspended in the same buffer at room temperature at an OD_{600} of 1.0. Cell suspensions were used immediately in the rapid assay to prevent long-term FA composition changes, and phenanthrene efflux was measured over a period of only 25 min. At time zero radiolabelled phenanthrene was added to the cell suspension and thereafter samples were withdrawn at timed intervals, collecting the cells by using a microfuge. The concentration of

phenanthrene in the cell pellet (µmol/g) was calculated from the amount of ^{14}C in the pellet fraction, the initial phenanthrene concentration and the cell dry weight, as previously described by Bugg et al. (2000). Sodium azide (Fisher Scientific) was added 9 min into the assay to a final concentration of 120 mM as an inhibitor of active transport (Bugg et al. 2000). All efflux assays were performed using independent triplicate cultures. Steady state concentrations pre- and post-azide addition were calculated and statistically evaluated by analysis of variance (ANOVA) in Excel 2007.

2.2.3 Antibiotic sensitivity assays

The minimum inhibitory concentration (MIC), the lowest concentration of antibiotic that inhibits growth, was measured as turbidity (OD_{600}) using a Powerwave XS spectrophotometer (BioTek). The MICs of tetracycline, streptomycin, nalidixic acid, erythromycin and chloramphenicol were determined using the microtiter broth dilution method (Wiegand et al. 2008) for *P. fluorescens* cLP6a and cLP6a-1 grown at 10°C, 28°C or 35°C.

2.2.4 RNA extraction

P. fluorescens cLP6a cells were grown in TSB to logarithmic, stationary or death phase at 28°C; to stationary phase at 10°C, 28°C or 35°C; or to stationary phase in the presence of antibiotics (chloramphenicol or tetracycline at ¼ MIC) or PAHs (naphthalene or phenanthrene at 5 mmol l^{-1}). At point of harvest, 10 ml of culture was stopped by adding 1.25 ml of ice-cold ethanol/phenol solution (5% water-

saturated phenol, in ethanol). Total RNA was immediately extracted from the harvested cultures using MasterPure^{TM} RNA Purification Kit (Epicentre Biotechnologies) according to the manufacturer's instructions. Total RNA recovered was dissolved in 100 µl of nuclease-free water and treated with 10 µl of 10× DNase I buffer and 10 units of RNase-free DNase I (Ambion): the reaction was incubated at 37°C for 30 min, stopped with 5 µl of 50 mM EDTA [pH 8] and then 1 µl of SUPERase•In (Ambion) was added before storage at -80°C. The purity and concentration of the RNA extracted from each culture sample was determined using an Agilent 2100 bioanalyzer (Agilent Technologies).

2.2.5 Reverse-transcription-PCR (RT-PCR)

A RNA-primer hybridization mix containing 2 µl DNase-treated total RNA and 10 ng/µl random hexamer primers (Invitrogen) was incubated in a thermocycler at 70°C for 10 min followed by 25°C for 10 min. The 60 µl cDNA synthesis mixture contained the RNA-primer mix, 0.5 mM dNTP mix, 1× first strand buffer (Invitrogen), 10 mM dithiothreitol, 0.5 U/µl SUPERase•In (Ambion) and 6.7 U/µl SuperScript III reverse transcriptase (Invitrogen). The mixture was incubated at 25°C for 10 min, 37°C for 60 min, 42°C for 60 min and then at 70°C for 10 min to inactivate the SuperScript III. cDNA was stored at -80°C until used for real-time PCR.

2.2.6 Primer design for quantitative real-time PCR (qPCR)

Primers were designed for qPCR using Primer Express® Software v3.0, which considers factors such as amplicon size, homology with other genes, secondary structure and the estimated duplex melting temperature (T_m). Primers were designed using partial sequences retrieved from GenBank (*www.ncbi.nlm.nih.gov/genbank/*) for *emhA* (AAQ92180), *emhB* (AAQ92181) and *emhC* (AAQ92182) of *P. fluorescens* cLP6a (Hearn et al. 2006) and the 16S rRNA gene of *P. fluorescens* pf0-1 (NC_007492) (Silby et al. 2009), the latter being used as the endogenous control. Primer pairs designed for each gene are listed in Table 2.1.

Table 2.1 Primers for qPCR analysis

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
emhA	CGGTGAGCCGTCAGGAATAC	TTGATCTGGGCGCTTTGC
emhB	GTCCCACTGGCGATTTCC	CCGTGATCATACCGCCAATAA
emhC	GATCGCCTGGCGCAACT	CTTTCGCAGTCTGCTCATTCC
16S rRNA	GGAGACTGCCGGTGACAAACT	TGTAGCCCAGGCCGTAAGG

2.2.7 RT-qPCR

qPCR of cDNA was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Each 10-µl RT-qPCR reaction mixture containing 2.5 µl cDNA and 0.4 µM of each corresponding primer specific for target genes or the endogenous control was incubated with a reaction mixture (Molecular Biology Services Unit, Edmonton, Canada) comprising

5 µl 2× qPCR reaction mix with SYBR Green (Molecular Probes) as the detection dye and ROX (Invitrogen) as a normalizing dye. The PCR conditions consisted of a denaturation cycle at 95°C for 2 min, followed by 40 cycles at 95°C for 30 s and 60°C for 1 min, and a dissociation cycle at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and then 60°C for 15 s. The melting curve generated at the end of real-time PCR cycles was analysed to confirm the absence of non-specific double stranded DNA-SYBR Green hybrids.

RT-qPCR data analysis was performed using the gene expression study function of the ABI 7500 software v2.0 (Applied Biosystems). The fluorescence of SYBR Green is measured against ROX at the end of each PCR cycle in the ABI 7500 Fast Real-Time PCR System. The comparative C_T method ($2^{-\Delta\Delta CT}$) was used to calculate the relative quantities of nucleic acid sequence of target genes in each sample (Wong and Medrano 2005). C_T (threshold cycle) is the fractional cycle number at which the SYBR Green fluorescence passes the baseline signal (Wong and Medrano 2005). The expression levels of target genes were normalized against that of the 16S rRNA gene (endogenous control). RNA obtained from *P. fluorescens* cLP6a cultures grown at 28°C to stationary phase was used as the calibrator sample in this study. Statistical analysis of data was performed using ANOVA (Excel 2007).

2.2.8 Membrane integrity assay

Membrane integrity of *P. fluorescens* cLP6a cells grown to

stationary phase at 10°C, 28°C or 35°C was determined using a modification of the method described by Niven and Mulholland (1998). Cell samples (1 ml) were harvested by centrifugation, re-suspended in 1 ml of phosphate-buffered saline and adjusted to an OD_{600} of 1.0. Propidium iodide (PI; Invitrogen), either alone or with the membrane-disrupting agent cetyltrimethylammonium bromide (CTAB; Sigma), were added to final concentrations of 30 µmol l^{-1} and 1 µmol l^{-1} respectively; untreated cells were included as parallel controls. After 30 min incubation at room temperature, fluorescence of 100-µl cell samples was measured in a 96-well microplate using a Synergy HT Multi-mode Microplate Reader (BioTek) at excitation and emission wavelengths of 500 nm and 600 nm respectively.

2.2.9 Phospholipid fatty acid (FA) extraction and identification

Total cell lipids were extracted using the Bligh-Dyer method (Bligh and Dyer 1959) modified by White and Ringelberg (1998) from 10 mg lyophilized cLP6a or cLP6a-1 cells grown to stationary phase at different temperatures or in the presence of antibiotics (at 1/4 MIC) or PAHs (5 mmol l^{-1}). Fatty acid methyl esters (FAME) were prepared from extracted total lipids using mild alkaline methanolysis (Guckert et al. 1985), dried under a stream of N_2 and re-dissolved in 500 µl chloroform (HPLC grade, Fisher Scientific). FAME were analysed by gas chromatography with mass spectrometry (GC-MS) on an Agilent 6890N GC with a model 5973 inert mass selective detector (Agilent) fitted with an Agilent HP-5MS capillary

column (30 m × 0.25 mm ID, 0.25 µm film thickness; J + W Scientific). Helium was used as the carrier gas with a temperature program of 150°C (1 min) increasing to 190°C at 1.5°C min^{-1}, then 25°C min^{-1} to 290°C (held for 4 min). Sample peaks were compared to Bacterial Acid Methyl Ester Mix standards (Supelco, Sigma Aldrich) and quantified by calculating individual FAME peak areas as a percentage of the total FAME in each sample (Londry et al. 2004).

2.2.10 Free FA assay

P. fluorescens strains cLP6a and cLP6a-1 cultures grown to stationary phase at 10°C, 28°C or 35°C were harvested by centrifugation. The culture supernatants were filtered using a 0.22 µm Millex-GS filter unit (Millipore), then 50 µl of the filtrate was assayed for free FA using a free fatty acid quantification kit (Abcam) according to the manufacturer's protocol.

2.3 Results

2.3.1 EmhABC enhances growth at supra-optimal temperature

The growth curves for *P. fluorescens* strains were determined at 10°C, 28°C or 35°C to allow sampling at the appropriate phase of growth in subsequent studies. The optimum growth temperature for wild type *P. fluorescens* LP6a is 28°C (Foght and Westlake 1991), 10°C is a growth-permissive sub-optimal temperature, and 35°C is ~2°C below the maximum growth temperature of *P. fluorescens* LP6a wild type. Strains

cLP6a and cLP6a-1 grown in seed cultures at 28°C were transferred to fresh medium and incubated at 10°C, 28°C or 35°C and growth was monitored for 48 h. The growth curves of cLP6a and cLP6a-1, measured as OD_{600}, were similar to each other at 10°C (Fig. 2.1a) and at 28°C (Fig.

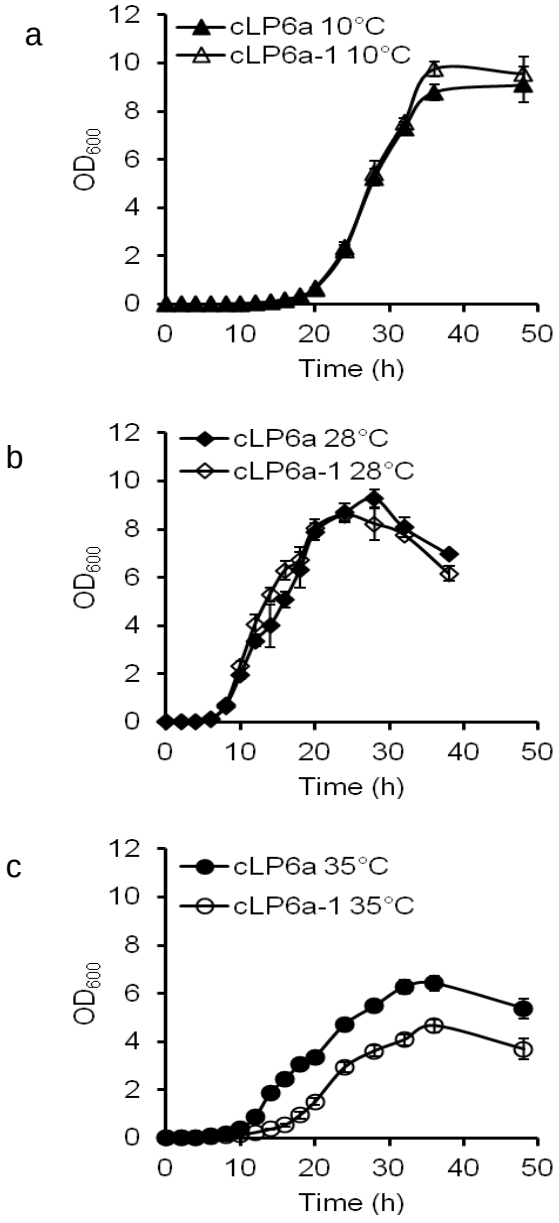

Figure 2.1 Growth of *P. fluorescens* strains cLP6a and cLP6a-1 at (a) 10°C, (b) 28°C or (c) 35°C determined as OD_{600}. Each data point is the mean of three independent cultures, and error bars, where visible, indicate the standard deviation.

2.1b). The lag phases of both cLP6a and cLP6a-1 were longer at 10°C than at 28°C but the maximum OD_{600} achieved was greater at 10°C. The maximum OD_{600} achieved by cLP6a and cLP6a-1 was lower at 35°C and growth of the two strains was dissimilar (Fig. 2.1c). The growth yield for strain cLP6a-1 at 35°C was about half that measured at 10°C and 28°C, and ~70% that of strain cLP6a at 35°C. Thus, disruption of *emhABC* in strain cLP6a-1 impaired its growth rate and cell yield at the supra-optimal temperature.

2.3.2 Phenanthrene efflux by EmhABC is affected by incubation temperature

To measure activity of the EmhABC efflux pump, a rapid efflux assay (Bugg et al. 2000) was performed using ^{14}C-phenanthrene. In the efflux assay, suspensions of cLP6a and cLP6a-1 harvested at stationary phase were incubated with ^{14}C-phenanthrene at a concentration below its aqueous solubility limit, to avoid any effects of dissolution on phenanthrene bioavailability. Partitioning of phenanthrene into the cells is very rapid, achieving steady state in less than 1 min (Bugg et al. 2000). At timed intervals, the radiolabel associated with the cell pellet is measured, and the steady state concentration is the sum of efflux and partitioning of phenanthrene. A significant increase in the concentration of phenanthrene associated with the cell pellet after addition of sodium azide indicates inhibition of active efflux, resulting in phenanthrene accumulation in the cell. A constant high concentration of phenanthrene in the pellet both

before and after azide addition indicates absence of efflux. Therefore, the relative activity of EmhABC at different incubation temperatures can be determined by comparing the difference between the steady state cell-associated phenanthrene concentrations during active efflux (pre-azide addition for strain cLP6a) and in the absence of efflux (in strain cLP6a-1 and post-azide addition for strain cLP6a); the greater the difference between phenanthrene concentrations in the pellets, the greater the efflux pump activity and consequently the more phenanthrene extruded from the cells. Importantly, because the centrifugation assay is so rapid (~25 min duration), the observed effects must be due to existing efflux pumps and membrane fatty acid (FA) composition rather than being influenced by induction of *emhABC* transcription or long-term membrane modifications through *de novo* synthesis of FA.

Because incubation temperature affects FA composition and fluidity of membranes, which in turn can affect protein-lipid interactions and integral membrane protein activity (Denich et al. 2003), the effect of growth temperature over a 25°C range on subsequent phenanthrene efflux activity was determined. The cell-associated phenanthrene prior to azide addition was 1.34 ± 0.19 µmol/g, 1.93 ± 0.34 µmol/g and 2.30 ± 0.36 µmol/g in cLP6a cells grown at 10°C, 28°C and 35°C respectively, indicating reduced efflux activity with increasing growth temperature. Consistent with previous work (Hearn et al. 2003), cLP6a cells grown at 28°C exhibited active efflux of phenanthrene (Fig. 2.2a): the steady state

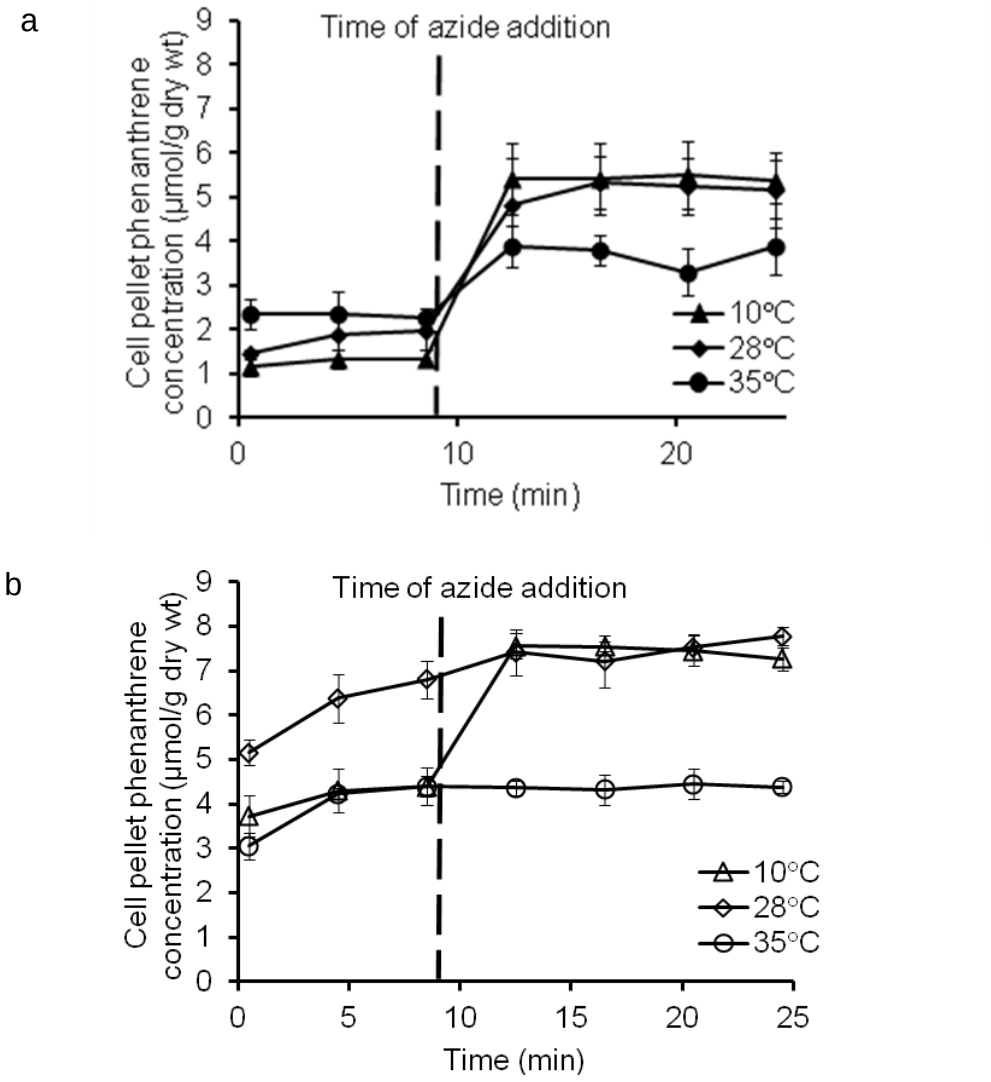

Figure 2.2 Partitioning of phenanthrene into the cell pellet of *P. fluorescens* strains, determined using a rapid efflux assay: (a) strain cLP6a grown at 10°C, 28°C or 35°C; (b) strain cLP6a-1 grown at 10°C, 28°C or 35°C. The vertical dashed line indicates the addition of azide (120 mM). Each data point is the mean of three independent experiments, and error bars, where visible, indicate the standard deviation.

concentrations of phenanthrene associated with the cell pellet before (1.93 ± 0.34 µmol/g) and after (5.28 ± 0.56 µmol/g) azide addition were

significantly different ($P < 0.0001$).

Efflux assays were also performed with the *emhB* disruption strain cLP6a-1 (Fig. 2.2b) to determine the steady state concentration of phenanthrene in the absence of efflux in the cells. As expected, there was no evidence of phenanthrene efflux by mutant cLP6a-1 at 28°C and 35°C, as the steady state concentrations of cell-associated phenanthrene were unchanged before and after azide addition. Notably, the cell-associated phenanthrene prior to azide addition was significantly greater in cLP6a-1 cells grown at 28°C (6.60 ± 0.50 µmol/g) than in the parallel cLP6a cells (1.93 ± 0.34 µmol/g; $P < 0.0001$) (Fig 2.2). Thus, EmhABC is the sole efflux system responsible for phenanthrene efflux in cLP6a cells grown at 28°C and 35°C. The cell-associated phenanthrene concentration in cLP6a-1 cells grown at 35°C before azide addition (4.32 ± 0.19 µmol/g) was significantly lower ($P < 0.0001$) than in cells grown at 28°C (6.60 ± 0.50 µmol/g; Fig. 2.2b), suggesting that phenanthrene partitioning into the cells was affected by changes in membrane FA composition induced by the incubation temperature. Unexpectedly, in cLP6a-1 cells grown at 10°C (Fig 2.2b) the cell-associated phenanthrene concentrations pre- (4.35 ± 0.42 µmol/g) and post- (7.50 ± 0.16 µmol/g) azide addition were significantly different ($P < 0.0001$), consistent with efflux subsequently inhibited by azide. This observation suggests the activity of another phenanthrene efflux pump(s) present and active at 10°C but not at 28°C. A second efflux pump expressed or active at low temperature would also

explain why cLP6a cells grown at 10°C accumulated the lowest measured concentration of cell-associated phenanthrene prior to azide addition (Fig. 2.2a): this could result from the combined activity of EmhB plus the postulated alternate efflux pump at the low temperature.

The difference in cell phenanthrene concentration in the presence and absence of efflux in cLP6a grown at 10°C (6.18 ± 0.002 µmol/g) was significantly greater ($P < 0.002$) than in cLP6a cells grown at 28°C (5.46 ± 0.03 µmol/g). Because a putative pump was likely induced at 10°C in addition to EmhB (Fig. 2.2b), the actual difference in cell pellet phenanthrene concentration due to the activity of EmhB in strain cLP6a grown at this temperature (3.01 ± 0.07 µmol/g) was significantly lower ($P < 0.001$) than in cells grown at 28°C. Similarly, the difference in phenanthrene concentrations for strain cLP6a grown at 35°C (2.07 ± 0.06 µmol/g) was less than in cells grown at 28°C. These results indicate that the activity of EmhB was reduced due to sub- or supra optimal incubation temperature. Therefore, incubation temperature affects phenanthrene efflux by the EmhB efflux pump.

2.3.3 Incubation temperature affects sensitivity to antibiotics

The effect of incubation temperature on antibiotic efflux by EmhABC was investigated to confirm the phenanthrene efflux assays. The sensitivity of cLP6a and cLP6a-1 cells grown at 10°C, 28°C or 35°C to various antibiotics was measured indirectly as MICs to test the effect of

temperature on efflux of known antibiotic substrates of the EmhABC pump (Hearn et al. 2003; 2006). As expected, the *emhB* mutant strain (cLP6a-1)

Table 2.2 Antibiotic sensitivity of P. fluorescens strains cLP6a and cLP6a-1 incubated at different temperatures. Antibiotic sensitivity was measured as minimum inhibitory concentration (MIC). Values shown are representative data from two independent experiments.

P. fluorescens strains	Incubation temperature	MIC (μg ml^{-1}) [a]				
		AP	CL	ER	NL	TE
cLP6a	10°C	512	64	128	32	2
	28°C	512	32	128	32	2
	35°C	256	8	64	32	1
cLP6a-1	10°C	512	4	32	2	0.125
	28°C	512	1	8	<1	0.125
	35°C	512	<0.5	8	<1	<0.063

[a], AP, ampicillin; CL, chloramphenicol; ER, erythromycin; NL, nalidixic acid; TE, tetracycline

was more sensitive to such antibiotics than strain cLP6a grown at a comparable incubation temperature (Table 2.2), exhibiting a ≥16-fold difference in MIC for chloramphenicol, nalidixic acid and tetracycline, and a 4- to 8-fold difference for erythromycin. Both strains showed similar sensitivity to ampicillin, which is not a substrate of EmhABC (Hearn et al. 2003; 2006). Smaller differences in MIC values (≤8-fold, or no difference) were observed within a single strain incubated at different temperatures for some antibiotics.

2.3.4 *emhABC* expression is affected by incubation temperature and growth phase

Changes in the activity of EmhABC in cLP6a cells grown at different temperatures could reflect differential expression of *emhABC*, differential EmhABC translation or changes in the membrane physiology of the cells as a result of deviation from the normal growth temperature. Thus, the effect of incubation temperature on the expression of *emhABC* and on the cell membrane physiology was determined. It is assumed that the *emhABC* genes form an operon based on their homology to the *ttgABC* and *mexAB-OprM* efflux operons (Hearn et al. 2003). Expression of the *emhABC* genes in cLP6a cells incubated at different temperatures and grown to different phases was determined using RT-qPCR to identify the condition(s) that induce *emhABC* transcription. The reference level of expression (i.e., calibrator) was defined as that exhibited by cLP6a cells grown to stationary phase at 28°C. Expression at 28°C was dependent on growth phase: *emhABC* genes were induced ~20–35 fold in log phase cells, and ~6-fold in death phase cells (Fig. 2.3). Sub- and supra-optimal incubation temperature also increased expression ~10-fold at 10°C and ~32-fold at 35°C in stationary phase cells. The presence of tetracycline in the growth medium at 28°C induced *emhABC* by ~10-fold. Induction levels obtained for all these conditions were significantly different ($P < 0.005$) from the calibrator. In each case, except for logarithmic growth, the three

emhABC genes were expressed at equivalent levels, but during log phase their expression followed the trend *emhA>B>C.*

Expression of *emhABC* genes did not increase in stationary phase cells incubated at 28°C in the presence of chloramphenicol, naphthalene or phenanthrene although chloramphenicol and phenanthrene are known

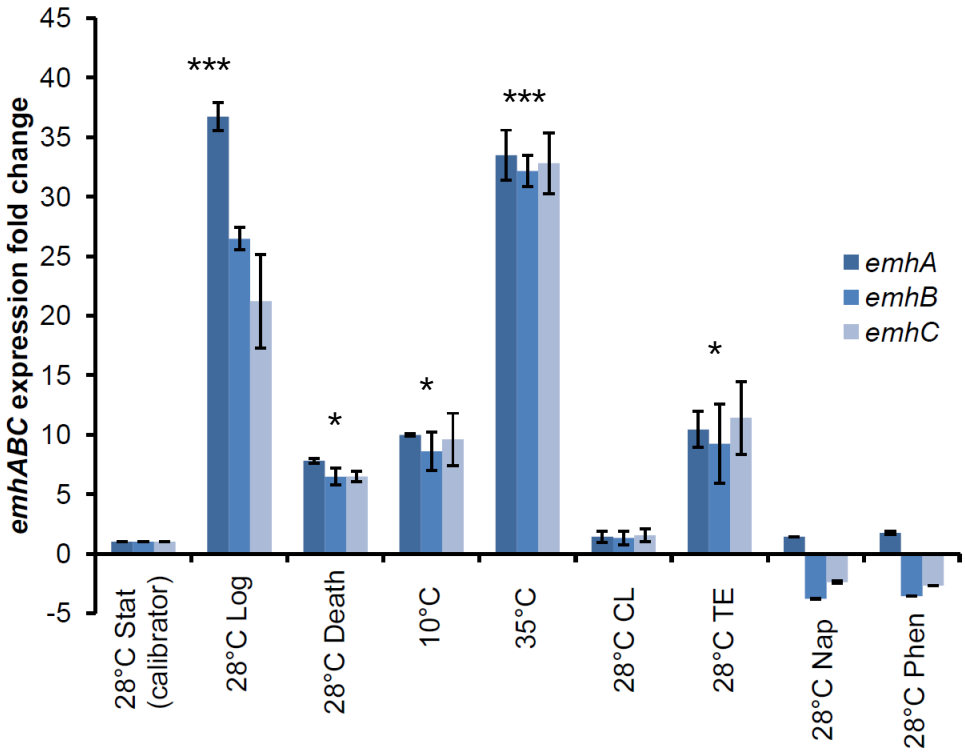

Figure 2.3 Expression of *emhABC* in *P. fluorescens* strain cLP6a grown to stationary (Stat), logarithmic (Log) or death phase at 28°C; grown to stationary phase at 10°C or 35°C; grown to stationary phase at 28°C in the presence of chloramphenicol (CL) or tetracycline (TE) at ¼ MIC; or grown to stationary phase at 28°C in the presence of naphthalene (Nap) or phenanthrene (Phen) at 5 mmol l^{-1}, determined using RT-qPCR. The values shown are the fold-difference in expression of *emhABC* compared to expression levels in cells grown to stationary phase at 28°C (calibrator = 1). Each bar represents the mean of two independent experiments performed in duplicate. Error bars, where visible, indicate the average deviation. Asterisks indicate significant differences.

substrates of EmhABC efflux pump. This is consistent with the hypothesis that PAHs and antibiotics are not primary substrates of resistance-

nodulation-division (RND) efflux pumps (Piddock 2006; Poole 2008). The observation by Hearn et al. (2003) that *emhABC* genes are not induced by PAHs was confirmed by the current study. Conversely, antibiotics such as tetracycline and chloramphenicol that inhibit ribosomal function were shown to induce the expression of *mexY*, which encodes the MexY efflux pump in *P. aeruginosa* PAO1, but their effect on expression was concentration-dependent (Jeannot et al. 2005). Induction of *emhABC* by tetracycline but not chloramphenicol (Fig. 2.3) may likewise depend on concentration. Because single sub-lethal concentrations of antibiotics were tested in this study we cannot make any conclusions about the effect of chloramphenicol on *emhABC* expression. Dimethylformamide, the water-miscible solvent used to add the PAHs, did not affect expression of *emhABC* genes in parallel control incubations.

2.3.5 Incubation temperature affects cLP6a membrane integrity

Because the activity of EmhABC was low but the expression of the *emhABC* was high in cLP6a cells grown at 35°C compared to other incubation temperatures, it was hypothesized that membrane integrity and (or) changes in membrane FA components might be responsible for these observations. To test the hypothesis, cell membrane integrity was determined using fluorescent dyes to determine the effect of incubation temperature on membrane permeability. Propidium iodide (PI) is a fluorescent reporter molecule that cannot cross intact cell membranes

(Niven and Mulholland 1998). Therefore, cell fluorescence in the presence of PI only occurs if membrane integrity is compromised, allowing PI to penetrate and interact with intracellular DNA. Cetyltrimethylammonium bromide (CTAB) is a cationic surfactant that can permeabilize bacterial cell membranes and thus increase PI penetration. The fluorescence value of cells exposed to PI with CTAB treatment or without CTAB treatment represents, respectively, the total number of cells (with artificially induced membrane permeability) and the number of cells naturally exhibiting compromised membrane integrity (Niven and Mulholland 1998). A permeability index can be calculated as the percentage of the net fluorescence value of PI-treated cells in the absence of CTAB relative to that in its presence. In Fig. 2.4 the permeability index of cLP6a cells grown

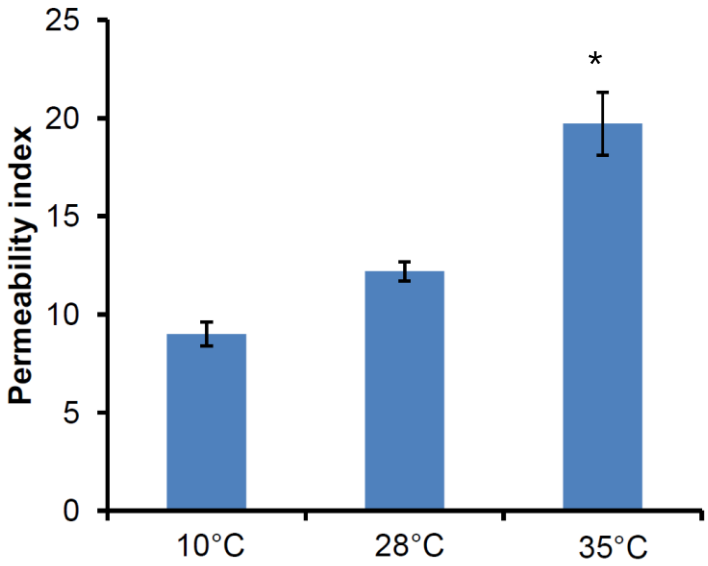

Figure 2.4 The permeability index of *P. fluorescens* cLP6a cells grown to stationary phase at 10°C, 28°C or 35°C. See text for definition of permeability index. Each bar represents the mean of three culture sub-samples. Asterisks indicate significant differences.

to stationary phase increased with higher incubation temperature: cells grown at 10°C, 28°C or 35°C had permeability indices of approx. 9%, 12% and 20% respectively. This indicates that, as anticipated, cLP6a cells exhibit increasingly compromised membrane integrity when grown at 35°C, just below the maximum permissive growth temperature.

2.3.6 Membrane FA content is modified in response to incubation temperature

Temperature is well known to cause modification of membrane FA content (Denich et al. 2003; Kim et al. 2001). Therefore, the membrane FA profiles of strain cLP6a grown to stationary phase at 10°C, 28°C or 35°C in the presence of PAHs or antibiotics were quantified to determine the effect of temperature on cell membrane FA composition (Table 2.3). Strain cLP6a grown at 28°C in the absence of PAHs and antibiotics was used as a reference. Generally, incubation temperature caused greater changes in the proportions of saturated-, unsaturated- and cyclopropane-FA than the other conditions tested. Compared to 28°C, cells grown at 10°C responded by decreasing the total saturated membrane FA by half to ~20%, decreasing cyclopropane-FA from 43% to 7% and concomitantly increasing total unsaturated FA from 14% to 72%, primarily represented by the *cis*-isomers of 16:1Δ9 and 18:1Δ9. Cells grown at 35°C responded with slight increases in total saturated and cyclopropane-FA and a 4-fold decrease in total unsaturated FA. In the presence of tetracycline, cLP6a cells responded with a ~2-fold increase in unsaturated membrane FA and

Table 2.3 FA composition of P. fluorescens strain cLP6a under different growth conditions.Strain cLP6a cultures were grown to stationary phase at 10°C, 28°C or 35°C, or grown at 28°C in the presence of PAHs (naphthalene or phenanthrene, at 5 mmol l-1) or antibio tics (tetracycline or chloramphenicol, at ¼ MIC). FA contents are expressed as the mean weight % of total FA detected in two measurements; deviation from the mean was typically <1% and at most 3% of the measured values.

	FAs as % of total FA detected[a]													
Growth conditions	**14:0**	**15:0**	**16:0**	**16:1Δ9c**	**16:1Δ9t**	**17:0**	**cy17**	**18:0**	**18:1Δ9c**	**18:1Δ9t**	**cy19**	**Total Saturated FAs**	**Total Unsaturated FAs**	**Total Cyclo-FAs**
10°C	0.2	0.2	19.9	34.0	7.0	0.3	6.6	0.3	30.5	0.7	0.4	**20.9**	**72.2**	**7.0**
28°C	1.0	0.2	40.4	4.6	1.6	0.3	40.0	1.2	7.6	ND[b]	3.1	**43.1**	**13.8**	**43.1**
35°C	0.6	0.2	44.6	1.3	0.1	0.3	44.1	1.9	2.1	0.1	4.9	**47.6**	**3.6**	**49.0**
28°C with naphthalene	0.6	0.1	40.8	5.5	3.2	0.2	36.5	1.2	9.3	0.3	2.3	**42.9**	**18.3**	**38.8**
28°C with phenanthrene	0.7	0.2	40.1	4.7	1.9	0.3	39.7	1.2	7.9	ND	3.3	**42.5**	**14.5**	**43.0**
28°C with tetracycline	1.0	0.2	40.3	14.5	ND	0.3	32.5	1.0	8.6	ND	1.6	**42.8**	**23.1**	**34.1**
28°C with chloramphenicol	1.1	0.2	41.0	6.6	ND	0.4	40.1	1.3	6.2	ND	3.1	**44.0**	**12.8**	**43.2**

[a], FA nomenclature: number of carbons; saturation (:0); mono-unsaturation (:1); position of double bond calculated from the carboxyl end (Δ9); *cis*- (c) or *trans*- (t) isomer; cyclopropyl ring (cy)
[b], not detected

a ~25% decrease in total cyclopropane-FA but unchanged total saturated membrane FA. There were no major changes in the proportions of different membrane FA in cells incubated with chloramphenicol, naphthalene or phenanthrene. Consistent with observations of *emhABC* gene induction, tetracycline but not chloramphenicol induced major changes in membrane FA content (although both antibiotics are substrates of EmhABC), possibly due to the sub-inhibitory concentration of chloramphenicol used in the assay or because tetracycline is a better substrate of EmhABC efflux pump. In contrast, the PAHs naphthalene and phenanthrene did not induce major FA changes likely because cLP6a is adapted to growth on PAHs, having been isolated from a hydrocarbon-contaminated soil (Foght and Westlake 1996).

2.3.7 Free FA are substrates of EmhABC

I investigated the possibility that free FA released from membranes damaged by stress or undergoing rapid phospholipid replacement are substrates of the EmhABC efflux pump. The concentration of free FA was determined in the cell-free medium of strains cLP6a and cLP6a-1 grown at 10°C, 28°C or 35°C to stationary phase. The concentrations of free FA in the cell-free medium of cLP6a and cLP6a-1 cultures incubated at 10°C or 28°C (Fig. 2.5) were not significantly different ($P < 0.4$ or $P < 0.8$ respectively). However, there was a significant difference ($P < 0.04$) in the concentration of free FA in the medium of cLP6a and cLP6a-1 cultures

incubated at 35°C. Higher concentrations of free FA were observed in the medium of cLP6a cultures grown at 35°C in the presence of a functional

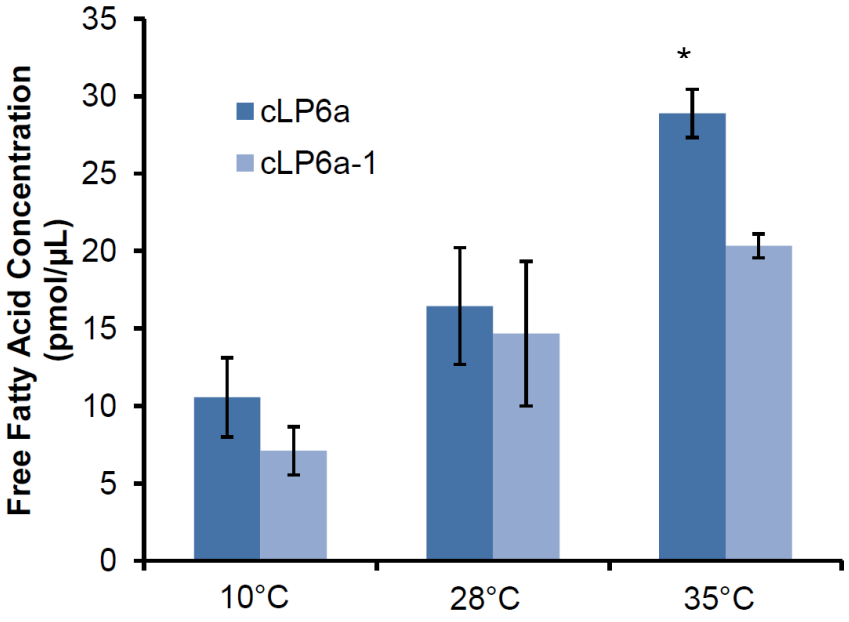

Figure 2.5 Free FA concentration in filtered medium from cLP6a and cLP6a-1 cultures grown to stationary phase at 10°C, 28°C or 35°C. Each bar represents the mean of two independent experiments, and error bars, where visible, indicate the average deviation. Asterisks indicate significant differences.

EmhABC pump compared to cultures of cLP6a -1 lacking EmhB, consistent with the involvement of EmhABC in the transport of FA originating from membranes under stress or rapid turnover.

2.4 Discussion

Efflux pumps of the resistance-nodulation-division (RND) superfamily are common in Gram negative bacteria (Nikaido and Takatsuka 2009; Poole 2008) and are well studied for their role in

antibiotic resistance and solvent tolerance in many *Pseudomonas* species (Blair and Piddock 2009; Rodriguez-Herva et al. 2007). However, these may not be the native or dominant physiological functions of RND pumps in bacteria. Piddock (2006) and Poole (2008), among others, have suggested that RND pumps fulfill other crucial roles, including management of diverse physico-chemical and biochemical stresses, quorum sensing and virulence. One of the stress-responsive roles proposed for RND efflux pumps such as MexCD-OprJ in *Pseudomonas aeruginosa* (Fraud et al. 2008; Poole 2008; Stickland et al. 2010) is the export of membrane constituents released by FA replacement due to natural turnover of membrane components during cell growth or resulting from membrane damage. Results obtained in this study are consistent with that proposal: EmhABC appears to play a role in efflux of replaced membrane FA in response to temperature-induced membrane perturbation, in addition to its demonstrated function of transporting hydrophobic antibiotics, dyes and PAHs (Hearn et al. 2003). Reciprocally, because RND efflux pumps are membrane-associated protein complexes, EmhABC activity may in turn be influenced by modulation of FA content in response to membrane stressors like temperature and hydrophobic compounds (Denich et al. 2003) that partition into lipid bilayers. As expected, results obtained indicate that temperature affects cell growth, FA content (measured as fatty acid methyl esters) and membrane integrity (measured as permeability index). In addition, it is now shown that

temperature affects expression and activity of the EmhABC RND efflux pump (measured by using RT-qPCR, phenanthrene efflux and antibiotic MIC assays).

The FA content of cLP6a followed the expected trends at 10°C and at 35°C, shifting towards unsaturation and saturation respectively (Denich et al. 2003; Zhang and Rock 2008). The FA content of the membrane affected the partitioning of phenanthrene into the membrane, since cLP6a-1 cells grown at 35°C contained lower fractions of phenanthrene in the absence of active efflux compared to those grown at 28°C. This observation is consistent with the rationale that saturated FA pack closely, hindering partitioning of hydrophobic molecules like PAHs into the lipid bilayer (Denich et al. 2003) whereas angular *cis*-unsaturated FA pack more loosely, facilitating partitioning. The observed changes in FA with temperature are also consistent with results from the membrane integrity assay in which the permeability index increased with temperature.

Growth temperature also affected EmhABC activity in cLP6a, possibly indirectly through membrane perturbation including the modulation of FA. cLP6a cells having high unsaturated FA content (i.e., 72% in cells grown at 10°C) and greater membrane integrity had higher efflux activity than cells with lower proportions of unsaturated FA (i.e., 14% at 28°C or 4 % at 35°C) and increased permeability. This observation suggests that increased unsaturated FA content may allow efficient or stable association of the three protein components of RND efflux pumps,

which spans two membranes and the periplasm.

The enhanced phenanthrene efflux observed in cLP6a at 10°C is consistent with the additive effect of EmhABC with a postulated alternate efflux pump that is active at 10°C. The presence of an alternate pump in *P. fluorescens* is not unexpected, as multiple efflux pumps have been identified in other *Pseudomonas* species (Kieboom and de Bont 2001; Poole 2008) and additional efflux pumps were invoked by Hearn et al. (2003) to explain anthracene and fluoranthene efflux in *P. fluorescens* strain cLP6a.

The induction of *emhABC* genes was observed in cLP6a cells exhibiting major changes in membrane FA composition due to sub-optimal growth conditions, namely at 10°C, 35°C and in the presence of tetracycline. Expression was also increased in logarithmic phase cells, which undergo rapid synthesis and turnover of FA, and in death phase cells that experience membrane deterioration. The relationship between induction of *emhABC* genes and membrane FA modulation indicates that the EmhABC efflux pump may be involved in the extrusion of replaced membrane FA as a result of membrane turnover. This possibility is further supported by the higher concentration of free FA in the medium of cLP6a cultures grown at 35°C concomitant with high membrane permeability and over-expression of *emhABC* genes. Comparable results were obtained recently by Stickland et al. (2010) who reported that over-expression of *mexCD-oprJ* efflux genes in *P. aeruginosa* leads to up-regulation of FA

secretion and fitness impairment. Over-expression of *emhABC* genes in cLP6a cells grown at 35°C may be explained either as compensation for reduced activity of EmhABC (caused by the modulation of the FA content) or may be due to increased membrane permeability and membrane FA turnover. According to Denich et al. (2003), damage to the membrane is still possible even with modulation of membrane FA quantity or composition to maintain fluidity and integrity. This conclusion is supported by the observation of similarly high levels of *emhABC* over-expression in log phase cells. Such cells may have compromised cell membranes due to rapid phospholipid synthesis and turnover since membrane integrity is temporarily affected by physical cell wall reconstruction at the sites of cell division during the log phase of growth (Mailaender et al. 2004; Müller and Nebe-von-Caron 2010). It is unclear why there was differential expression of the three *emhABC* genes in log phase cells (*emhA>B>C*), although stability of the transcripts may differ as a result of rapid cell growth. The effect on membrane integrity was confirmed by the higher permeability index at 35°C. Similarly, the reduced cell yields and growth rates at 35°C compared to 10°C or 28°C, along with altered FA content, are consistent with compromised cell membranes at the higher temperature. The negative effects of the compromised membrane on growth are muted by the presence and activity of EmhABC, allowing cLP6a cells to out-grow cLP6a-1 at supra-optimal temperature.

The discovery that EmhABC activity influences growth of *P.*

fluorescens cLP6a (and by extension wild type LP6a) at supra-optimal temperature suggests a role for efflux in temperature adaptation in the environment, and may apply to other Gram-negative species. For example, *P. aeruginosa* and *Salmonella* strains lacking RND efflux pumps are unable to colonize and infect their hosts (Hirakata et al. 2002; Nishino et al. 2009), which may in part result from an inability to adapt to host temperatures higher than the external environment. Temperature also may affect efflux-mediated antibiotic resistance although the effect on MIC was not pronounced in *P. fluorescens* cLP6a. It will also be interesting to examine whether temperature-sensitive efflux of antibiotics is a general phenomenon in other Gram-negative bacteria. Because bacterial cells are commonly exposed to temperature changes in the environment, we propose that RND efflux pumps in Gram-negative bacteria may play a major role in management of temperature-induced membrane damage.

This study focussed on modifications to the FA portion of membrane lipids since phospholipid head group modification is typically less dynamic and critical in bacteria (reviewed by Denich et al. 2003), but it is possible that head group composition also changed in response to temperature, PAHs and/or antibiotics. Other indirect effects such as decreased proton motive force resulting from damaged membranes could also be factors. Such possibilities are incentives for clarifying the natural physiological roles of RND efflux pumps in Gram-negative bacteria in anticipation of devising new methods for combating antibiotic resistance or

improving hydrocarbon transformation for bioremediation or biocatalytic processing of hydrophobic substrates.

The alternative and likely the primary physiological role of EmhABC in *P. fluorescens* cLP6a is the efflux of membrane FA replaced as a result of adaptation to membrane stress caused by physico-chemical stressors (See Fig. 6.1). Efflux of incidental substrates such as hydrophobic antibiotics, PAHs or dyes may be a consequence of membrane stress.

2.5 Literature cited

Blair JMA, Piddock LJV (2009) Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Curr Opin Microbiol 12:512-519

Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911-917

Bugg T, Foght JM, Pickard MA, Gray MR (2000) Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol 66:5387-5392

Cebrián G, Sagarzazu N, Pagán R, Condón S, Manas P (2008) Resistance of *Escherichia coli* grown at different temperatures to various environmental stresses. J Appl Microbiol 105:271-278

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Methods 52:149-182

Foght JM, Westlake DWS (1991) Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. Can J Microbiol 37:924-932

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353-366

Fraud S, Campigotto AJ, Chen Z, Poole K (2008) MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in

chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response sigma factor. Antimicrob Agents Chemother 52:4478-4482

Guckert JB, Antworth CP, Nichols PD, White DC (1985) Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol Lett 31:147-158

Hearn EM, Dennis JJ, Gray MR, Foght JM (2003) Identification and characterization of the *emh*ABC efflux system for polycyclic aromatic hydrocarbons in *Pseudomonas fluorescens* cLP6a. J Bacteriol 185:6233-6240

Hearn EM, Gray MR, Foght JM (2006) Mutations in the central cavity and periplasmic domain affect efflux activity of the resistance-nodulation-division pump EmhB from *Pseudomonas fluorescens* cLP6a. J Bacteriol 188:115-123

Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, Kohno S, Kamihira S, Hancock REW, Speert DP (2002) Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. J Exp Med 196:109-118

Jeannot K, Sobel ML, Garch FE, Poole K, Plésiat P (2005) Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. J Bacteriol 187:5341-5346

Kieboom J, de Bont JAM (2001) Identification and molecular characterization of an efflux system involved in *Pseudomonas putida* S12 multidrug resistance. Microbiol 147:43-51

Kieboom J, Dennis JJ, de Bont JAM, Zylstra GJ (1998) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85-91

Kim IS, Lee H, Trevors JT (2001) Effects of 2,2´,5,5´,-tetrachlorobiphenyl and biphenyl on cell membranes of *Ralstonia eutropha* H850. FEMS Microbiol Lett 200:17-24

Lin JT, Connelly MB, Amolo C, Otani S, Yaver DS (2005) Global transcriptional response of *Bacillus subtilis* to treatment with subinhibitory concentrations of antibiotics that inhibit protein synthesis. Antimicrob Agents Chemother 49:1915-1926

Londry KL, Jahnke LL, Des Marais DJ (2004) Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. Appl Environ Microbiol 70:745-751

Mailaender C, Reiling N, Engelhardt H, Bossmann S, Ehlers S, Niederweis M (2004) The MspA porin promotes growth and increases antibiotic susceptibility of both *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis*. Microbiol 150:853-864

Morita Y, Sobel ML, Poole K (2006) Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: Involvement of the antibiotic-inducible PA5471 gene product. J Bacteriol 188:1847-1855

Müller S, Nebe-von-Caron G (2010) Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. FEMS Microbiol Rev 34:554-587

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

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

Nishino K, Nikaido E, Yamaguchi A (2009) Regulation and physiological function of multidrug efflux pumps in *Escherichia coli* and *Salmonella*. Biochim Biophys 1794:834-843

Niven GW, Mulholland F (1998) Cell membrane integrity and lysis in *Lactococcus lactis*: the detection of a population of permeable cells in post-logarithmic phase cultures. J Appl Microbiol 84:90-96

Phillips R, Ursell T, Wiggins P, Sens P (2009) Emerging roles for lipids in shaping membrane-protein function. Nature 459:379-385

Piddock LJV (2006) Multidrug-resistance efflux pumps – not just for resistance. Nature Rev Microbiol 4:629-636

Poole K (2008) Bacterial multidrug efflux pumps serve other functions. Microbe 3:179-185

Rodriguez-Herva JJ, Garcia V, Hurtado A, Segura A, Ramos JL (2007) The *ttgGHI* solvent efflux pump operon of *Pseudomonas putida* DOT-T1E is located on a large self-transmissible plasmid. Environ Microbiol 9:1550-1561

Sikkema J, de Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222

Silby MW, Cerdeno-Tarraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, Zhang X-X, Moon CD, Gehrig SM, Godfrey SAC, Knight CG, Malone JG, Robinson Z, Spiers AJ, Harris S, Challis GL, Yaxley AM, Harris D, Seeger K, Murphy L, Rutter S, Squares R, Quail MA, Saunders E, Mavromatis K, Brettin TS, Bentley SD,

Hothersall J, Stephens E, Thomas CM, Parkhill J, Levy SB, Rainey PB, Thomson NR (2009) Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol 10:R51

Stickland HG, Davenport PW, Lilley KS, Griffin JL, Welch M (2010) Mutation of *nfxB* causes global changes in the physiology and metabolism of *Pseudomonas aeruginosa*. J Prot Res 9:2957-2967

Webber MA, Bailey AM, Blair JMA, Morgan E, Stevens MP, Hinton JCD, Ivens A, Wain J, Piddock LJV (2009) The global consequence of disruption of the AcrAB-TolC efflux pump in *Salmonella enterica* includes reduced expression of SPI-1 and other attributes required to infect the host. J Bacteriol 191:4276-4285

White DC, Ringelberg DB (1998) Signature lipid biomarker analysis. In: Burlage RS, Atlas R, Stahl D, Geesey G, Sayler G (eds) Techniques in microbial ecology. Oxford University Press, New York, pp 255-272

Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protocols 3:163-175

Wong ML, Medrano JF(2005) Real-time PCR for mRNA quantitation. BioTech 39:75-85

Zhang Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nature Rev Microbiol 6:222-233

3. The EmhABC efflux pump decreases efficiency of phenanthrene biodegradation by *Pseudomonas fluorescens* strain LP6a[2]

3.1 Introduction

Phenanthrene is a low-molecular-weight polycyclic aromatic hydrocarbon (PAH) (Mallick et al. 2011) considered to be non- or slightly-toxic to hydrocarbon-degrading bacterial cells because of its low bioavailability (Sikkema et al. 1995). Phenanthrene is a common PAH pollutant in soil, largely introduced into the environment along with other PAHs through fossil fuel extraction, processing, transport and use (Cerniglia 1992; Wammer and Peters 2005). There is need for efficient methods of removing PAHs from contaminated soils because of their toxicity and potential for bioaccumulation. Bioremediation using natural or genetically modified microorganisms in a controlled environment (e.g., in an ex situ bioreactor) with appropriate nutritional conditions can be a cost-effective and nonhazardous option for cleaning PAH-contaminated soils (Daugulis 2001; Mohn et al. 2001). Thus, bioremediation has great potential to replace physico-chemical remediation technologies such as incineration and solvent extraction (Mallick et al. 2011; Samanta et al. 2002; Van Hamme et al. 2003). It is therefore desirable that bacterial

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species employed for the remediation of contaminated environments degrade the pollutant efficiently. Bacterial transformation of hydrocarbons for biocatalysis or biorefining (Foght 2004) is another potential technology in which efficient microbial catabolism of the hydrocarbon substrate is desirable.

Phenanthrene is a substrate of the EmhABC efflux pump of *Pseudomonas fluorescens* strain LP6a (Bugg et al. 2000; Hearn et al. 2003) and other efflux pumps in *Pseudomonas* species (Hearn et al. 2003), which suggests that PAH efflux may be a common phenomenon in PAH-degrading strains. In Chapter 2, it was shown that the EmhABC efflux pump is not induced by phenanthrene (Adebusuyi and Foght 2011; Hearn et al. 2003) however, the efflux of phenanthrene via this pump may affect the rate at which it is degraded. The efficiency of PAH biodegradation depends on environmental factors such as bioavailability of the substrate (Cerniglia 1992), temperature, pH and salinity (Ulrich et al. 2009) and cell properties such as adhesion to hydrocarbons (Abbasnezhad et al. 2011a; 2011b) but the effect of efflux on efficiency of PAH biodegradation has not been studied previously.

Phenanthrene efflux can be examined independently of its catabolism in *P. fluorescens* LP6a because the genes encoding EmhABC are located on the chromosome (Hearn et al. 2003) whereas the PAH catabolic genes are located on a plasmid (Foght and Westlake 1996). Because efflux of a substrate may reduce its availability for catabolism by

cytoplasmic enzymes, it was hypothesized that phenanthrene biodegradation would be greater in an *emhB* mutant than in the wild type. This study determines how the efflux of phenanthrene affects the rate and extent of its degradation by comparing wild-type *P. fluorescens* strain LP6a and a mutant with a disrupted *emhB* gene (strain WEN; wild type PAH catabolism, efflux negative). Whether metabolites of phenanthrene and dibenzothiophene are substrates of the EmhB efflux pump was also determined to understand how efflux might influence PAH biodegradation and the fate of PAH metabolites during bioremediation, and to extend the known range of substrates of the EmhB efflux pump.

3.2 Materials and methods

3.2.1 Chemicals

Phenanthrene (Fig. 3.1a) (98% pure) and *o*-terphenyl (>99% pure) were purchased from Aldrich Chemical Co. [9-^{14}C]Phenanthrene (96.5% radiochemical purity ; 19.3 mCi mmol^{-1}) and 2-aminobenzoic acid were purchased from Amersham (Arlington Heights, IL, USA) and Analar (BDH Ltd, Poole, England) respectively. [U-^{14}C]D-glucose was purchased from New England Nuclear (Boston, MA, USA), *N,N*-dimethylformamide (ACS grade) from Anachemia (Montreal, QC, Canada), dibenzothiophene (DBT; 98% pure) (Fig. 3.1b) from Fluka Chemika (Sigma – Aldrich, Steinheim, Switzerland) and dichloromethane (HPLC grade) was purchased from Fisher Scientific Co.

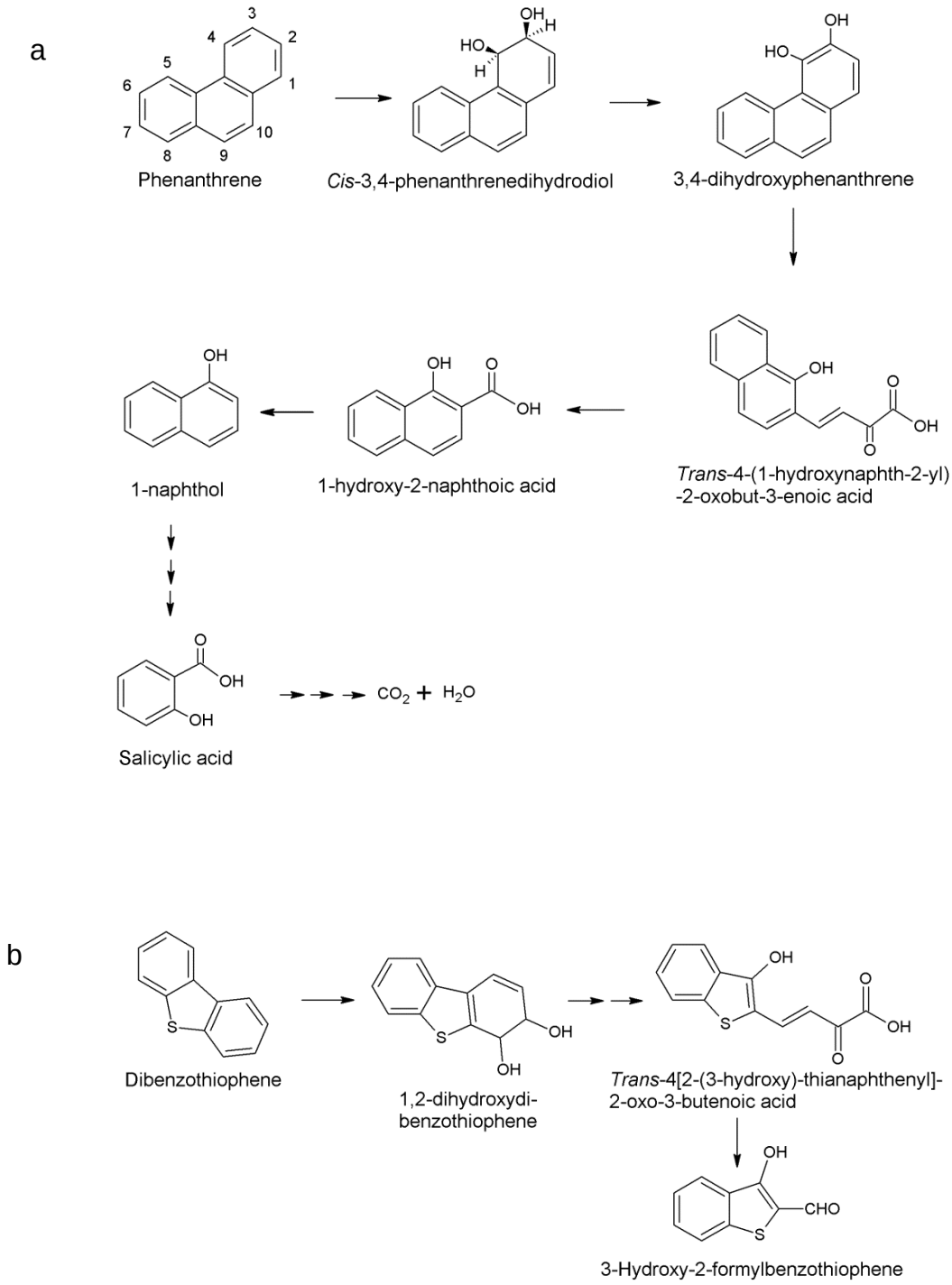

Figure 3.1 Proposed degradation pathway of a, phenanthrene (Mallick et al. 2011) and b, dibenzothiophene (Bressler and Fedorak 2001) in *P. fluorescens.*

3.2.2 Bacterial strains and growth conditions

Table 3.1 lists the bacterial strains used in this study. *P. fluorescens* LP6a wild-type strain contains the 63 kb PAH catabolic plasmid pLP6a (Foght and Westlake 1996) and chromosomally-encoded *emhABC* genes (Hearn et al. 2003) that are expressed constitutively but are also inducible by some antibiotics (Chapter 2; Adebusuyi and Foght 2011). Two mutants were used as negative controls for biodegradation assays: cured strain cLP6a, which cannot metabolize PAHs (Foght and Westlake 1996) but retains EmhABC activity (Bugg et al. 2000); and strain cLP6a-1, an *emhB* disruption mutant of the cured strain (Hearn et al. 2003). To construct the mutant strain WEN (wild type PAH catabolism, efflux negative) that carries pLP6a but has a disrupted *emhB* gene, plasmid pLP6a was purified from LP6a cells using the method of Casse et al. (1979) and introduced into cLP6a-1 by electroporation using a Gene Pulser (Bio-Rad Laboratories,

Table 3.1 Bacterial strains used in this study

***Pseudomonas fluorescens* strains**	**Characteristics**	**Reference or source**
LP6a	Wild-type containing PAH catabolic plasmid pLP6a, *emhABC*$^{+}$	Foght and Westlake (1991)
WEN	Km^{r} *emhB*$^{-}$ transposon mutant containing pLP6a	This study
cLP6a	Cured LP6a strain lacking pLP6a, *emhABC*$^{+}$	Foght and Westlake (1996)
cLP6a-1	Km^{r} *emhB*$^{-}$ transposon mutant lacking pLP6a	Hearn et al. (2003)

Mississauga, Ontario). Mutants were selected on Bushnell Haas agar (BH [see below] plus 1.5% w/v Bacto Agar [Difco Laboratories, Detroit, MI]) containing 25 µg ml^{-1} kanamycin and vapour phase naphthalene as the sole carbon source. The presence of pLP6a in WEN was confirmed by extracting the plasmid and comparing it to the restriction pattern and fragment sizes of the plasmid in LP6a.

3.2.3 Phenanthrene biodegradation assay

Each 250 ml Erlenmeyer flask used for biodegradation experiments contained 25 ml Bushnell Haas (BH) medium [g l^{-1}]: magnesium sulphate 0.2; calcium chloride 0.02; potassium hydrogen monophosphate 1.0; potassium dihydrogen phosphate 1.0; ammonium nitrate 1.0; ferric chloride 0.05; final pH 7 – 7.2. After heat-sterilization, 50 µl 2-aminobenzoate dissolved in 95 % ethanol and 50 µl phenanthrene dissolved in dimethylformamide were added to the medium to yield final concentrations of 0.5 mM and 2.8 mmol l^{-1} (500 mg l^{-1}) respectively. As determined in Chapter 2, dimethylformamide at the concentration used does not induce the EmhABC efflux pump (Adebusuyi and Foght 2011). 2-Aminobenzoate, a non-metabolized inducer of the PAH catabolic genes in *P. fluorescens* LP6a (Foght 2004), was added to decrease the lag phase and enhance the rate of PAH biodegradation. Inocula for biodegradation assays were grown in 100 ml trypticase soy broth (TSB; Difco) at 28°C with 200 rpm gyratory shaking. Overnight cultures were harvested by centrifugation at 10,000 × *g* for 5 min and washed once with 0.1 M

potassium phosphate buffer (pH 7). The harvested cells were re-suspended in the same buffer then re-adjusted to an optical density at 600 nm (OD_{600}) of 3.0 and inoculated into sterile medium at 25 µl per flask. TSB or BH media inoculated with strain WEN or cLP6a-1 contained kanamycin (Sigma Chemical Co.) at 25 µg ml^{-1} to maintain the gene disruption.

Cultures of *P. fluorescens* strains LP6a, WEN and cLP6a incubated with phenanthrene as sole carbon source were sacrificed at intervals and biodegradation was monitored by extracting and quantifying residual phenanthrene using gas chromatography with mass spectrometry (GC-MS). At the point of sampling, 250 µl 4N H_2SO_4 was added to culture flasks to achieve pH < 1, then 100 µl of *o*-terphenyl dissolved in dichloromethane was added to each flask to a final concentration of 1.4 mM before extraction of phenanthrene using 10 ml dichloromethane. The solvent extract was dried over sodium sulphate. Aliquots of the solvent extract were transferred to screw-cap glass vials with Teflon liners. Phenanthrene concentrations in each sample extract were quantified on an Agilent 6890N GC with a model 5973 inert mass selective detector (Agilent, Palo Alto, CA) fitted with an Agilent HP-5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness; J + W Scientific, Folsom, CA). Helium was used as the carrier gas with a temperature program of 90°C (1 min) increasing to 280°C at 10°C min^{-1} (held for 5 min). The phenanthrene peak area was compared to the peak of *o*-terphenyl, which

served as both an extraction and surrogate standard for phenanthrene quantification.

3.2.4 Mineralization and transformation of radiolabelled phenanthrene and glucose

Mineralization and metabolite production by *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1 incubated with radiolabeled substrates were measured as cumulative $^{14}CO_2$ evolution and ^{14}C-water-soluble products as described by Ulrich et al. (2009). Experiments were performed using biometer flasks containing 25 ml BH medium, 250 mg l^{-1} ^{12}C-phenanthrene or 0.1% ^{12}C-glucose, 0.5 mM 2-aminobenzoate and 25 µL of inoculum suspension (OD_{600} = 3.0). Each flask also contained either [9-^{14}C]phenanthrene or [U-^{14}C]D-glucose to provide ~100,000 disintegrations per minute (dpm). The side arm of each biometer flask contained 10 ml 1 N KOH as a CO_2 trap. Flasks were incubated at 22°C with gyratory shaking at 100 rpm. Mineralization experiments were performed in triplicate except for controls (cLP6a and cLP6a-1), which were performed as single cultures. Abiotic (No cells) and killed cell (heat killed LP6a cells) controls were also performed using the same method as single cultures to account for radiolabel sorption to glassware and partitioning into cells, respectively.

Cumulative $^{14}CO_2$ trapped in the KOH in the side arm of biometer flasks was measured as described by Ulrich et al. (2009). Water-soluble phenanthrene metabolites were measured in supernatant samples from

the culture medium as follows: At intervals, 1-ml culture samples were collected and centrifuged at 13,000 × *g* for 10 min to remove insoluble residual ^{14}C-phenanthrene and cells. A 0.5-ml aliquot of the clarified supernatant was transferred to scintillation vials containing 10 ml ACS Fluor (Amersham Biosciences, UK Ltd). Samples were dark-adapted for 30 min to reduce chemiluminescence before counting with a Beckman LS3801 liquid scintillation counter. The percentage of label associated with the supernatant or KOH fraction over time was calculated relative to the initial radiolabel added to the culture (Abbasnezhad et al. 2011a) after correcting for background radiation (31 dpm, using samples of the aqueous phase and KOH sampled at time zero).

3.2.5 Phenanthrene metabolite efflux

Strains LP6a, WEN and cLP6a were grown to stationary phase at 28°C in TSB medium, harvested by centrifugation at 10,000 × *g* for 5 min and washed once with 0.1 M potassium phosphate buffer. Harvested cells were resuspended in 20 ml of buffer and incubated at 28°C with 0.5 mM 2-aminobenzoate and 2.8 mmol l^{-1} phenanthrene. After 3 h incubation, the cultures were harvested by centrifugation and the supernatants were filtered using a 0.22 µm pore size Millex-GS filter unit (Millipore). Supernatant samples (15 ml) were extracted as described for the phenanthrene biodegradation assay. The extracts were derivatized by trimethylsilylation using N, O-bis(trimethylsilyl)-trifluoroacetamide (Pierce Chemical Co; Rockford, IL, USA) according to manufacturer's instructions

and analyzed using GC-MS. The peak corresponding to the phenanthrene early open ring metabolite (Fig 3.1a) was presumptively identified in the gas chromatogram based on the appropriate molecular ion and fragment mass spectrum.

3.2.6 DBT metabolite efflux

Strains LP6a and WEN were grown, harvested and washed as described above, and resuspended in 20 ml 0.1 M potassium phosphate buffer in 125 ml screw cap flasks at a final OD_{600} of 3. At time zero, 2-aminobenzoate and DBT were added to LP6a and WEN cultures to achieve a final concentration of 0.5 mM and 5 mmol l^{-1} respectively. Sodium azide, an inhibitor of active transport and therefore of EmhABC activity (Bugg et al. 2000), was added to replicate cell suspensions at a final concentration of 30 mM either at time zero or after 60 min incubation. No azide was added to parallel suspensions. Culture samples (1 ml) were collected every 30 min and clarified by centrifugation at 10,000 × *g* for 2 min. The absorbance of the supernatant was measured using an Ultrospec 3100 pro UV-visible spectrophotometer (GE Healthcare) at 472 nm and 390 nm, corresponding respectively to the absorption maxima of the DBT intermediate metabolite *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (HTOB) and the dead-end product of DBT metabolism (3-hydroxy-2-formylbenzothiophene) (Bressler and Fedorak 2001; Foght and Westlake 1996; Kodama et al. 1973) (Fig. 3.1b). The molar absorption coefficient of HTOB is ~31,300 M^{-1} cm^{-1} at 472 nm (Foght and Semple,

unpublished results) and the coefficient of 3-hydroxyformylbenzothiophene is 12,400 M^{-1} cm^{-1} at 390 nm (Bressler and Fedorak 2001).

3.3 Results

3.3.1 EmhABC activity decreases mineralization and transformation of phenanthrene

A preliminary experiment was conducted to determine whether EmhABC activity affected the rate and/or extent of phenanthrene biodegradation, monitored by extracting residual phenanthrene from sacrificed replicate cultures and quantifying normalized peak areas using GC-MS analysis. Within 6 d of incubation the *emhB*$^-$ mutant strain WEN had degraded 7.8 ± 0.3 mg of the initial 12.5 mg phenanthrene added per culture (500 mg L^{-1}) compared to 5.4 ± 0.5 mg in LP6a cultures, an increase of 44% over the wild type. Whereas strain WEN degraded more phenanthrene, the growth of strain LP6a on phenanthrene was slightly more efficient during the logarithmic phase of growth than that of strain WEN. The control strain lacking PAH catabolism (cLP6a) did not grow on nor degrade phenanthrene (< 0.02 mg loss), as expected. The inference was that EmhB activity improved growth during rapid cell division in the presence of phenanthrene (as previously observed in chapter one; Adebusuyi and Foght 2011) but decreased the total amount of phenanthrene degraded, warranting further examination of the effect of efflux on biodegradation.

Notably, GC-MS analysis of residual substrate indicates only that phenanthrene has been altered sufficiently to decrease chromatogram peak area, but cannot distinguish between complete oxidation to CO_2 (mineralization) or partial oxidization to metabolites (transformation). To determine the fate of the biodegraded phenanthrene, LP6a, WEN and the control strains cLP6a and cLP6a-1 were incubated with ^{14}C-phenanthrene. Mineralization was measured as cumulative $^{14}CO_2$ production and transformation was measured as accumulation of water-soluble radiolabel (i.e., polar metabolites) in the culture supernatant (Fig. 3.2). Consistent with previous observations (Abbasnezhad et al. 2011a; Foght and Westlake 1991), *P. fluorescens* LP6a exhibited a low degree of phenanthrene mineralization. However, the maximum rate of phenanthrene mineralization was enhanced in the absence of efflux in WEN cultures (1.07 ± 0.01% h-1) compared to strain LP6a (0.39 ± 0.04% h-1), resulting in a significantly ($P < 0.001$) greater production of $^{14}CO_2$ by WEN (8.0 ± 0.7%) than LP6a (3.5 ± 0.7%) by 12 h of incubation (Fig. 3.2a). This effect is likely not due to differences between the strains in phenanthrene uptake since partitioning into the cells is extremely rapid (within seconds; chapter 1, Fig. 2.2; Bugg et al. 2000; Hearn et al. 2003). Transformation of phenanthrene to water-soluble metabolites exceeded the proportion mineralized, as expected (Foght and Westlake 1991, 1996), and the maximum amount of radiolabel recovered in the aqueous phase was greater in the WEN culture (64 ± 1%) than in the LP6a culture (29 ±

5%) (Fig. 3.2b). Partitioning of polar metabolites into the supernatant of WEN cultures in the absence of efflux is consistent with the observations of Bugg et al. (2000) that inhibition of energy-dependent efflux by LP6a cells did not affect accumulation of phenanthrene metabolites in the culture supernatant. The appearance of ^{14}C-metabolites in the *emhB*$^{-}$ WEN culture supernatant is presumably due to diffusion and/or the presence of an additional efflux pump, as discussed below. Phenanthrene was neither transformed nor mineralized by control strains cLP6a and cLP6a-1, which had levels of $^{14}CO_2$ (0.3%) and aqueous phase radiolabel (~8%) equivalent to abiotic and killed cell controls. The latter radiolabel fraction includes the water-soluble fraction of ^{14}C-phenanthrene (~0.5% of initial dpm added, based on phenanthrene aqueous solubility of 1.18 mg l^{-1} [Eastcott et al. 1988]). Notably, metabolism of the unknown radiolabeled contaminant(s) in the [9-^{14}C]phenanthrene does not contribute to either $^{14}CO_2$ or polar metabolite fractions in LP6a or WEN cultures, since there was no evidence of metabolism by either cLP6a or cLP6a-1 cultures.

To determine whether the effect of EmhABC activity on degradation was specific to phenanthrene, the cultures were grown with [U-^{14}C]D-glucose, which is not known to be a substrate of this or any other RND efflux pump. All strains tested (LP6a, WEN, cLP6a-1 and cLP6a-1)

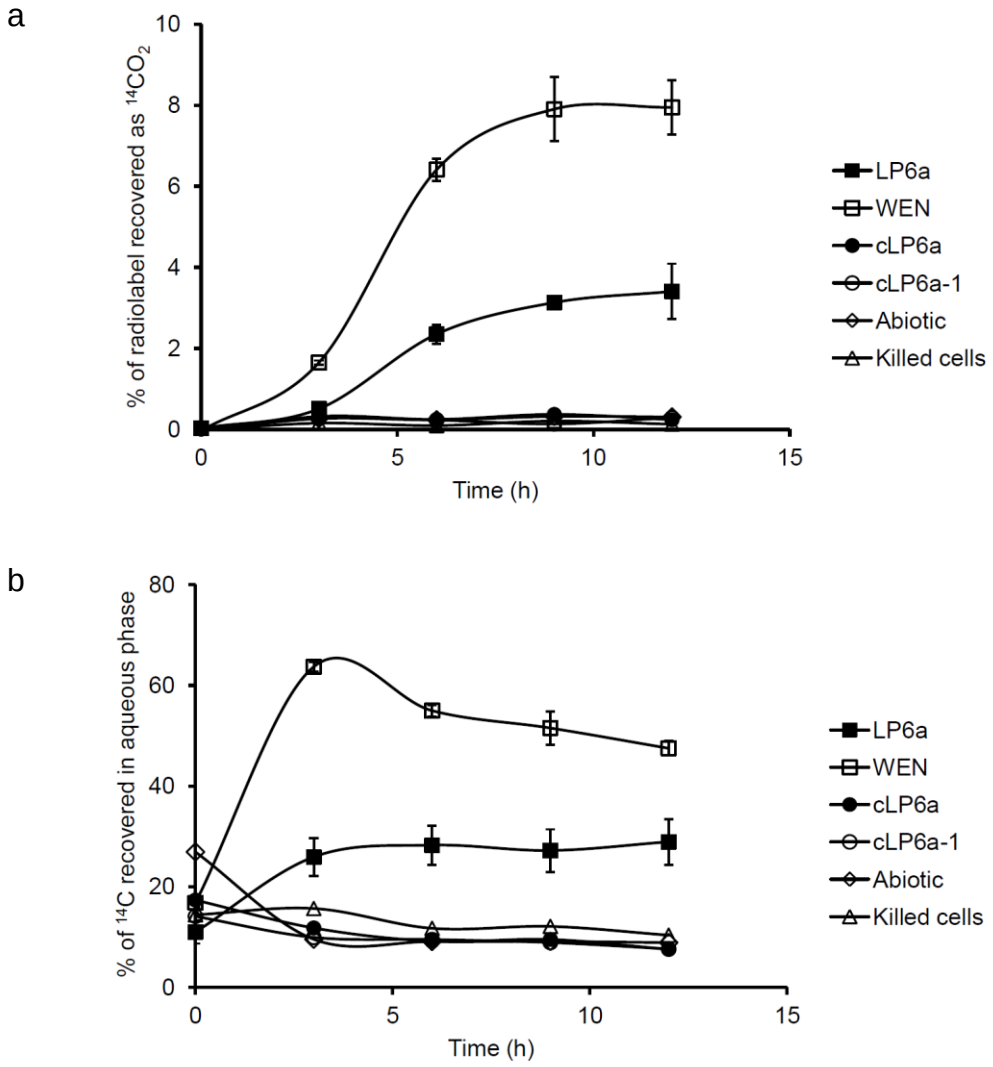

Figure 3.2 Mineralization and transformation of phenanthrene by *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1 and two negative controls (abiotic and killed cell incubations). *P. fluorescens* strains were incubated in medium containing ~100,000 dpm [9-^{14}C]phenanthrene at 22°C and induced with 0.5 mM 2-aminobenzoate. The percentage of initial ^{14}C recovered as: (a) $^{14}CO_2$ and (b) polar products in the aqueous culture supernatant (with minor contribution from soluble phenanthrene, ~0.5%, detected in the abiotic and killed cell control cultures) is shown. Each data point represents the mean of three independent experiments except for single controls; error bars, where visible, indicate the standard deviation.

mineralized the radiolabeled glucose at a similar rate (~2.8% h^{-1}) and to a similar extent (~20%) (Fig. 3.3a), and depletion of the water-soluble

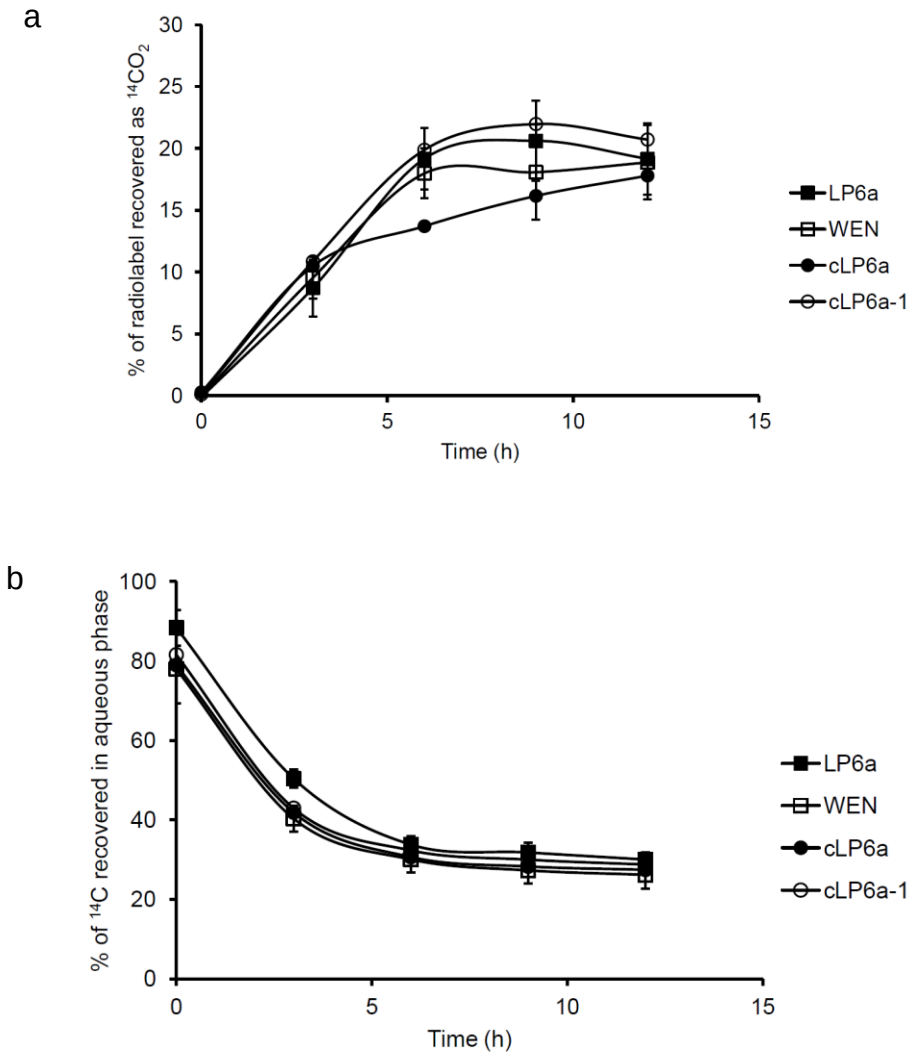

Figure 3.3 Mineralization and transformation of [U-14C]D-glucose by *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1. *P. fluorescens* strains were grown in medium containing ~100,000 dpm [U-^{14}C]D-glucose at 22°C. The percentage of ^{14}C recovered as (a) $^{14}CO_2$ and (b) water-soluble radiolabel in the culture supernatant (primarily as ^{14}C-glucose, presumably with minor contributions by polar metabolites) is shown. Each data point represents the mean of three independent experiments except for single controls; error bars, where visible, indicate the standard deviation.

substrate was equivalent in all cultures (Fig. 3.3b). Therefore, disruption of the EmhB efflux pump does not have a general inhibitory effect on

degradation of a compound such as glucose that is not a substrate of the pump, but does increase degradation of phenanthrene, an EmhABC substrate.

3.3.2 EmhABC extrudes certain phenanthrene and dibenzothiophene pathway metabolites

Based on the results of mineralization assays it was unclear whether the difference in the rate and extent of phenanthrene degradation measured for LP6a and WEN cultures was due solely to phenanthrene efflux or whether efflux of metabolite(s) might also contribute to the observed phenotypes. Therefore efflux of *trans*-4-(1-hydroxynaphth-2-yl)-2-oxo-3-butenoic acid (Fig. 3.1a), an early open-ring metabolite in the phenanthrene pathway used by *P. fluorescens* LP6a (Foght and Westlake 1996) was examined. Its efflux to the aqueous medium by strain LP6a but not by WEN was confirmed by extraction, derivatization and GC-MS analysis of supernatants from resting cell suspensions incubated with phenanthrene. The LP6a culture supernatant yielded a GC-MS peak corresponding to the double trimethylsilyl derivative of *trans*-4-(1-hydroxynaphth-2-yl)-2-oxo-3-butenoic acid (parent ion at m/z 388 and fragment major ions at m/z 271, 229, 147 and 73). No corresponding peak was observed in the efflux negative WEN strain despite phenanthrene catabolic activity, or in the efflux positive cLP6a strain lacking phenanthrene catabolism. Based on this evidence, it was revealed that a substantial portion of the radiolabel detected in the aqueous phase of the

LP6a culture described above corresponds to the phenanthrene open-ring metabolite *trans*-4-(1-hydroxynaphth-2-yl)-2-oxo-3-butenoic acid, whereas the greater proportion of water-soluble radiolabel in the WEN culture represents later metabolites in the phenanthrene catabolic pathway (Fig 3.1a).

Dibenzothiophene (DBT; Fig3.1b) is a polycyclic heteroaromatic compound that is co-metabolized by wild type *P. fluorescens* LP6a using the same upper pathway enzymes as phenanthrene but does not serve as a growth substrate, being only partially transformed to 3-hydroxy-2-formyl-benzothiophene (Foght and Westlake 1996). Unfortunately, DBT is not commercially available in radiolabeled form, precluding conventional efflux assays (Bugg et al. 2000), but its partially oxidized intermediates are highly coloured (Kodama et al. 1973), making it ideal for spectrophotometric analysis. Therefore, to further characterize the substrate range of EmhABC for aromatic metabolites, suspensions of LP6a and WEN cells incubated with 5 mmol l^{-1} DBT were monitored for accumulation of metabolites in the supernatant, specifically the intermediate *trans*-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (HTOB, an analog of the phenanthrene open-ring metabolite) and the dead-end product 3-hydroxy-2-formyl-benzothiophene (Fig. 3.1b). Sodium azide was added at different time points to some replicate cell suspensions to differentiate between proton-driven efflux and passive diffusion.

LP6a extruded HTOB to the aqueous phase in an energy-dependent process that was inhibited by addition of azide at time zero or after 60 min of incubation (Fig. 3.4a). Unexpectedly, the *emhB*$^{-}$ WEN strain also exported HTOB in the absence of azide (Fig. 3.4b), suggesting that an alternate efflux pump is responsible for extruding this metabolite from the *emhB* mutant. In the absence of azide the rate of HTOB efflux by LP6a was >50% faster than its efflux by WEN (0.033 ± 0.003 AU min^{-1} versus 0.020 ± 0.001 AU min^{-1}), and LP6a accumulated approximately twice as much HTOB in the supernatant within 4 h (Fig. 3.4a and 3.4b). This observation can be explained by the combined activity of both the EmhABC and an alternate pump in LP6a versus the alternate pump alone in WEN. Once active efflux was halted by addition of azide, there was no further increase in HTOB in the culture supernatants of LP6a and WEN (Fig. 3.4a and 3.4b), suggesting that diffusion of this DBT intermediate is not significant during the 4-h incubation period. The lack of diffusion of HTOB was consistent with the presence of this metabolite in its deprotonated form at culture medium and intracellular pHs well above the probable pKa, in the range of 2-3, by analogy to pyruvate. In contrast 3-hydroxy-2-formylbenzothiophene, the dead-end metabolite of DBT, accumulated in the culture supernatants of both LP6a and WEN cultures

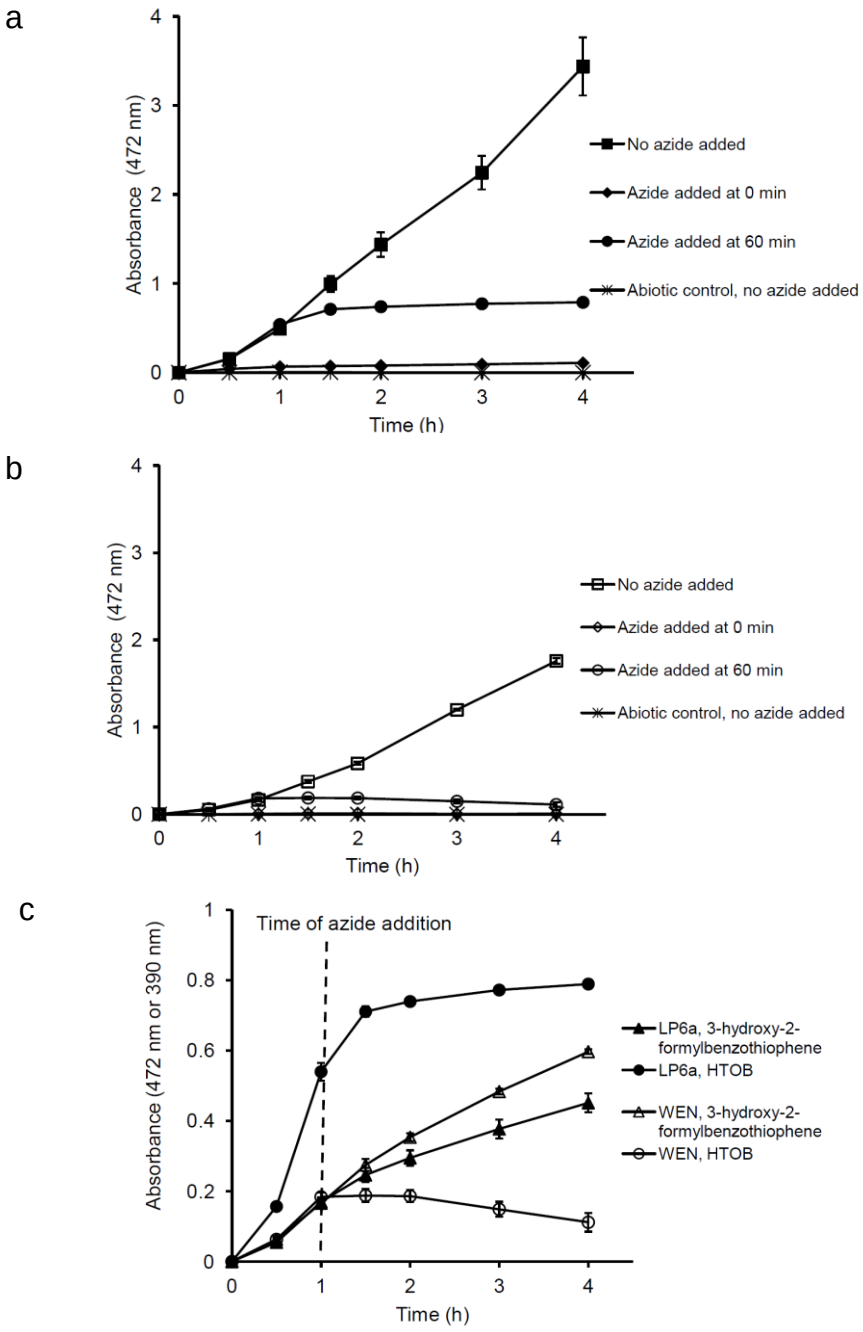

Figure 3.4 Extracellular accumulation of the DBT early open ring metabolite *trans*-4[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (HTOB) or the dead-end metabolite 3-hydroxy-2-formylbenzothiophene by LP6a or WEN cultures, measured spectrophotometrically at 472 or 390 nm, respectively. Each data point represents the mean of three replicate subsamples and error bars, where visible, indicate the standard deviation. (a) Extracellular accumulation of HTOB in LP6a cultures, with or without azide addition as indicated. (b) Extracellular accumulation of HTOB in WEN cultures, with or without azide addition as indicated. (c) Extracellular accumulation of HTOB (circles) or 3-hydroxy-2-formylbenzothiophene (triangles) by LP6a (closed symbols) or WEN cultures (open symbols) with addition of azide at 60 min (HTOB data redrawn from panels a and b)

at similar rates, which continued even after azide addition (Fig.3.4c). This indicates that 3-hydroxy-2-formylbenzothiophene is not a substrate of either EmhABC or the putative alternate efflux pump. Furthermore, its appearance in the supernatant is consistent with passive diffusion. This result also suggests that azide did not inhibit the DBT catabolic pathway, since the end-product continued to accumulate extracellularly for at least 3h after azide addition at approximately the same rate as before azide addition.

Longer-term incubations were conducted to examine the fate of the DBT metabolites. Samples collected at 24 h and 48 h from the LP6a and WEN cell suspensions incubated with DBT in the absence of azide showed that HTOB and 3-hydroxy-2-formylbenzothiophene continued to accumulate in the supernatant and represented ~4–10% each of the initial DBT present by 24 h (Table 3.2). Consistent with the short-term incubations (Fig. 3.4a and 3.4b), the HTOB concentration in LP6a supernatants was approximately twice that in WEN, but 2-formyl-3-benzothiophene accumulation was similar in both culture supernatants. The HTOB concentrations in the supernatants were stable between 24 and 48 h, indicating that HTOB was not re-internalized for further metabolism. A slight decrease in 2-formyl-3-benzothiophene concentration was observed between 24h and 48 h, which may be due to abiotic dimerization to thioindigo (Bressler and Fedorak 2001).

Table 3.2 Proportion of DBT measured in culture supernatant as metabolites, detected by spectrophotometry and calculated using molar absorption coefficients (see Methods).

		DBT metabolites detected in culture supernatant			
		trans-4-[2-(3-hydroxy)-thianaphthenyl]-2-oxo-3-butenoic acid (HTOB)		3-hydroxy-2-formyl-benzothiophene	
P. fluorescens strain	Incubation time	A_{472} [a]	% of DBT [b]	A_{390} [a]	% of DBT [b]
LP6a	24 h	14.9	9.5	4.0	6.4
	48 h	14.5	9.3	3.5	5.7
WEN	24 h	7.4	4.8	3.3	5.4
	48 h	7.2	4.6	2.5	4.1

[a] Absorption units at maximum absorption wavelength
[b] based on 5 mmol L^{-1} DBT added at time zero

3.4 Discussion

The aerobic biodegradation of PAHs and limitations of the process are well documented (e.g., Cerniglia 1992; Abbasnezhad et al. 2011b). While it has been shown that biodegradation is a better option for the removal of hydrocarbon pollutants in the environment than physico-chemical methods such as incineration, limitations to biodegradation remain a barrier to process optimization, for example in ex situ bioreactors (Daugulis 2001). To date, bacterial features that may affect degradation of hydrocarbons, other than the regulation of catabolic gene expression and enzyme specificity, have received little attention (Abbasnezhad et al. 2011a; Van Hamme et al. 2003).

RND efflux pumps are common in Gram-negative bacteria and have been associated with solvent tolerance in *Pseudomonas* species (Poole 2008; Ramos et al. 1998), many of which are known for their ability to degrade hydrocarbon compounds (Van Hamme et al. 2003). Efflux of toxic substrates such as toluene was assumed to improve biodegradation whereas efflux of PAH growth substrates that are considered non- or slightly-toxic to hydrocarbon-degrading bacterial cells should follow the opposite trend. The assumption that efflux of toxic substrates should improve biodegradation of the toxic substrate toluene was not supported by the evidence presented by Hernandez et al. (2009). They reported that the activity of the TtgGHI efflux pump in *P. putida* DOT-T1E had no effect on degradation of toluene, although it confers tolerance to toluene (Hernandez et al. 2009). Following the discovery of PAH efflux via a RND efflux pump in *P. fluorescens* LP6a and other PAH-degrading strains (Bugg et al. 2000; Hearn et al. 2003), how the presence of a RND efflux pump affects the biodegradation of PAH substrates was investigated. Here the first evidence that a RND efflux pump involved in the extrusion of PAHs reduces the rate and extent of their biodegradation by decreasing the intracellular concentration of the parent compound as well as some of its metabolites was presented. It was also proposed that an alternate, unknown efflux pump is involved in the extrusion of at least one metabolite of dibenzothiophene. *Pseudomonas* species are well-known for harbouring multiple efflux pumps (Kieboom and de Bont 2001; Poole

2008), consistent with the latter proposal. Whether this putative alternative pump is the same as that proposed for fluoranthene efflux (Hearn et al. 2003) or fatty acids in Chapter 2 (Adebusuyi and Foght 2011) in wild type *P. fluorescens* LP6a is yet to be tested.

This study also has expanded the known substrate range of the EmhABC pump to include open-ring metabolites of phenanthrene and dibenzothiophene, but not the dead-end metabolite of DBT transformation. The consequence of effluxing a growth substrate such as phenanthrene and its metabolite(s) is decreased biodegradation efficiency, as shown by decreased mineralization of ^{14}C-phenanthrene. *P. fluorescens* LP6a expends energy (via the proton gradient) to efflux phenanthrene and at least one of its metabolites to the extracellular medium, even though they are growth substrates. This efflux in wild type *P. fluorescens* appears to be counter-productive and superfluous for PAH degradation, since WEN cells unable to efflux phenanthrene were able to utilize it at a faster rate than the wild type and tolerated the presence of phenanthrene (i.e., apparently experienced no toxic effects due to its partitioning into the cell membrane). Conversely, the pump imparts a selective advantage through resistance to hydrophobic toxins; e.g., strain WEN with a disrupted *emhB* gene is more sensitive to hydrophobic antibiotics such as chloramphenicol, which is a substrate of the EmhABC efflux, compared to the wild-type strain LP6a with an intact *emhB* gene as shown in Chapter 2 (Adebusuyi and Foght 2011). The location of PAH catabolic genes on a plasmid versus the

chromosomal *emhABC* genes suggests that PAH biodegradation is dispensable whereas efflux by EmhABC is a core function. Thus, rather than being a primary role for EmhABC, efflux of PAHs may be a consequence of the broad substrate-range of EmhB due to the hydrophobic amino acids in the presumed active site of the EmhB protein (Hearn et al. 2006). The better growth of strain LP6a versus WEN during the logarithmic phase of growth when cells are rapidly dividing corroborates the previously proposed physiological function of the EmhABC efflux pump in Chapter 2 as a mechanism to mitigate membrane damage (Adebusuyi and Foght 2011). Supporting this interpretation, in addition to antibiotics, RND pumps have been associated with efflux of structurally unrelated substrates such as quorum sensing molecules, bile salts and fatty acids (Poole 2008; Adebusuyi and Foght 2011). The energetic cost to the wild type strain of effluxing metabolites of a non-growth substrate such as DBT (and possibly DBT itself, although this is untested) is unknown. Efflux is however not always associated with fitness cost (Olivares et al. 2012). It is possible that a benefit of this efflux is export of toxic intermediates, but also possible that it is a futile non-specific activity.

The results of this study show that phenanthrene is transformed by wild type *P. fluorescens* primarily to water-soluble products of unknown toxicity that will be more mobile in an aqueous phase than the parent compound. These products would not be detected by GC-MS in a

contaminated environment undergoing bioremediation unless suitable extraction conditions and derivatization were used: if depletion of the parent compound was the only criterion assessed during bioremediation, the extent of pollutant degradation would be greatly over-estimated. The role of efflux pumps in decreasing the efficiency of complete oxidation of hydrophobic organic contaminants and increasing the export of partially oxidized metabolites has environmental implications as this activity could contribute to accumulation of toxic mobile hydrocarbon metabolites as well as increased biological oxygen demand down-gradient of the contamination source. This phenomenon also has consequences for ex situ bioremediation treatments such as bioreactors (Daugulis 2001) and biopiles (Mohn et al. 2001), among others, because aqueous effluents and leachates could harbour significant concentrations of pathway intermediates and dead-end metabolites. The magnitude of the impact of metabolite efflux is shown by the efflux of HTOB, which amounts to about 10% of DBT metabolized by *P. fluorescens* LP6a (Table 2.2), and apparently does not re-enter the cells via active or passive transport for further transformation.

The significance of this study is that it reveals yet another factor affecting efficient bioremediation of environmental pollutants by influencing the rate, extent, production and export of biodegradation products. This is also a consideration in the selection or manipulation of strains that may be

used for transformation of hydrocarbon (or other) substrates in bioconversion or biorefining (Foght 2004).

3.5 Literature cited

Abbasnezhad H, Foght JM, Gray MR (2011a) Adhesion to the hydrocarbon phase increases phenanthrene degradation by *Pseudomonas fluorescens* LP6a. Biodegradation 22:485-496

Abbasnezhad H, Gray M, Foght JM (2011b) Influence of adhesion on aerobic biodegradation and bioremediation of liquid hydrocarbons. Appl Microbiol Biotechnol 92:653–675

Adebusuyi AA, Foght JM (2011) An alternative physiological role for the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a. BMC Microbiol 11:252

Bressler DC, Fedorak PM (2001) Purification, stability and mineralization of 3-hydroxy-2-formylbenzothiophene, a metabolite of dibenzothiophene. Appl Environ Microbiol 67:821-826

Bugg T, Foght JM, Pickard MA, Gray MR (2000) Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol 66:5387-5392

Casse F, Boucher C, Julliot JS, Michel M, Denarie J (1979) Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J Gen Microbiol 113:229-242 doi:10.1099/00221287-113-2-229

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351-368

Daugulis AJ (2001) Two-phase partitioning bioreactors: a new technology platform for destroying xenobiotics. Trends Biotechnol 19:457-462

Eastcott L, Shiu WY, Mackay D (1988) Environmentally relevant physical-chemical properties of hydrocarbons: a review of data and development of simple correlations. Oil and Chemical Pollution 4:191-216

Foght JM (2004) Whole-cell bio-processing of aromatic compounds in crude oil and fuels. In: Vazquez-Duhalt R, Quintero-Ramirez R

(eds) Petroleum biotechnology: developments and perspectives, vol 151. Elsevier, Amsterdam, pp 145-175

Foght JM, Westlake DWS (1991) Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. Can J Microbiol 37:924-932

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353-366

Hearn EM, Dennis JJ, Gray MR, Foght JM (2003) Identification and characterization of the *emh*ABC efflux system for polycyclic aromatic hydrocarbons in *Pseudomonas fluorescens* cLP6a. J Bacteriol 185:6233-6240

Hearn EM, Gray MR, Foght JM (2006) Mutations in the central cavity and periplasmic domain affect efflux activity of the resistance-nodulation-division pump EmhB from *Pseudomonas fluorescens* cLP6a. J Bacteriol 188**:**115-123

Hernandez M, Gouveia J, Segura A, Munoz R, Villaverde S (2009) Addressing the role of the extrusion pump-bearing pGRT1 plasmid in toluene biodegradation by *Pseudomonas putida* DOT-T1E under real case scenarios. Water Sci Technol 60:9

Kieboom J, de Bont JAM, (2001) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 multidrug resistance. Microbiology 147:43-51

Kieboom J, Dennis JJ, de Bont JAM, Zylstra GJ (1998) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85-91

Kodama K, Umehara K, Shimizu K, Nakatani S, Minoda Y, Yamada K (1973) Identification of microbial products from dibenzothiophene ant its proposed oxidation pathway. Agric Biol Chem 37:45-50

Mallick S, Chakraborty J, Dutta TK (2011) Role of oxygenases in guiding diverse metabolic pathways in the bacterial degradation of low-molecular-weight polycyclic aromatic hydrocarbons: A review. Crit Revs Microbiol 37:64-90

Mohn WW, Radziminski CZ, Fortin MC, Reimer KJ (2001) On site bioremediation of hydrocarbon-contaminated Arctic tundra soils in

inoculated biopiles. Appl Microbiol Biotechnol 57:242-247

Olivares J, Alverez-Ortega C, Linares JF, Rojo F, Kohler T, Martinez JL (2012) Overproduction of the multidrug efflux pump MexEF-OprN does not impair *Pseudomonas aeruginosa* fitness in competition tests, but produces specific changes in bacterial regulatory networks. Environ Microbiol doi:10.1111/j.1462-2920.2012.02727.x

Poole K (2008). Bacterial multidrug efflux pumps serve other functions. Microbe 3:179-185

Ramos J-L, Duque E, Godoy P, Segura A (1998) Efflux pumps involved in toluene tolerance in *Pseudomonas putida* DOT-T1E. J Bacteriol 180:3323-3329

Samanta SK, Singh OV, Jain RK (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol 20:243-247

Sikkema J, De Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222

Ulrich A, Guigard S, Foght J, Semple K, Pooley K, Armstrong J, Biggar K (2009) Effect of salt on aerobic biodegradation of petroleum hydrocarbons in contaminated ground water. Biodegradation 20:27-38

Van Hamme JD, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Revs 67:503-549

Wammer KH, Peters CA (2005) Polycyclic aromatic hydrocarbon biodegradation rates: a structure-based study. Environ Sci Technol 39:2571- 2578

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

4. EmhABC efflux pump is involved in naphthalene tolerance but not efflux in *Pseudomonas fluorescens* LP6a

4.1 Introduction

Three major mechanisms are used by Gram-negative bacteria to tolerate the presence of toxic compounds, namely modification of cell membrane composition to overcome the initial shock of the toxic compound partitioning into membranes, extrusion of the compound via efflux pumps and metabolism of the toxic compound to less toxic metabolites (Huertas et al. 2000; Ramos et al. 2002). The effective removal of PAHs, which are high priority pollutants (U.S. EPA 2002), from contaminated sites via bioremediation is desirable. *Pseudomonas fluorescens* LP6a, isolated from petroleum condensate-contaminated soil (Foght and Westlake 1996) was selected as the model organism for this study because (1) it can metabolize a wide variety of polycyclic aromatic hydrocarbons (PAHs) (Chapter 3; Adebusuyi et al. 2012; Foght and Westlake 1996), (2) it effluxes certain PAHs by using the EmhABC efflux pump (Bugg et al. 2000; Hearn et al. 2003), (3) it modifies its membrane fatty acid (FA) composition when stressed (Chapter 2; Adebusuyi and Foght 2011) and (4) a suite of catabolic and efflux mutants is available for study (Foght and Westlake 1996; Hearn et al. 2003). The wild-type strain LP6a can metabolize a wide variety of PAHs because it harbours a catabolic plasmid, pLP6a, carrying naphthalene catabolism genes

homologous to well studied plasmid NAH7 (Foght and Westlake 1996), whereas the EmhABC efflux pump is chromosomally-encoded (Hearn et al. 2003).

The tri-cyclic PAHs phenanthrene and anthracene are both degraded and effluxed by LP6a, whereas the bi-cyclic PAH naphthalene, although degraded by this strain, is not a substrate of the EmhABC efflux pump (Bugg et al. 2000; Hearn et al. 2003). Although the reason for this substrate selectivity in EmhABC is unknown, specific amino residues in the periplasmic domain of RND efflux proteins such as AcrB and MexB are involved in substrate binding (Murakami et al. 2006; Sennhauser et al. 2009) and such binding sites in EmhB for naphthalene may not exist. The octanol-water partition coefficient (log K_{ow}) of a compound correlates with the degree of hydrophobicity and toxicity of that compound (Sikkema et al. 1994; 1995). Hydrocarbon compounds having log K_{ow} between 2 – 4 such as toluene (log K_{ow} = 2.5), are highly toxic to bacterial cells (Osborne et al. 1990; Sikkema et al. 1995) because they increase membrane fluidity, disrupt bilayer stability and membrane structure, cause membrane swelling and reduce the normal functioning of membrane associated proteins. Naphthalene has a log K_{ow} of 3.3, which should allow it to partition into the cell membrane more effectively than phenanthrene with a log K_{ow} of 4.5 (Patton et al. 1984). Therefore naphthalene is potentially more toxic to bacteria than phenanthrene (Ramos et al. 2002), possibly causing membrane stress requiring modification of membrane fatty acid

composition. Supporting this proposal, *P. fluorescens* strain cLP6a, a cured mutant of LP6a unable to metabolize PAHs, had increased membrane unsaturated fatty acid (FA) composition, especially *trans*-unsaturated FAs (UFAs), when exposed to naphthalene (Chapter 2; Adebusuyi and Foght 2011).

It was shown in Chapter 2 that in addition to PAH and antibiotic efflux, EmhABC has an additional role in mitigating membrane damage in *P. fluorescens* LP6a and is involved in the transport of replaced membrane FAs (Adebusuyi and Foght 2011). There is evidence that the presence of EmhABC decreases the efficiency of phenanthrene degradation in strain LP6a because of the efflux of phenanthrene and its early open-ring metabolite (Chapter 3; Adebusuyi et al. 2012). Here, the role of EmhABC in *P. fluorescens* LP6a tolerance to naphthalene and how its presence affects the efficiency of naphthalene biodegradation was investigated.

4.2 Materials and methods

4.2.1 Bacterial strains and growth conditions

Bacterial strains used for this study are listed in Table 4.1. Cured strain *P. fluorescens* cLP6a (Foght and Westlake 1996) and its *emhB* disruption mutant cLP6a-1 (Hearn et al. 2003), both incapable of PAH metabolism, were used for electron microscopy, efflux, growth, membrane

FA composition and membrane permeability assays. Wild-type *P. fluorescens* LP6a (Foght and Westlake 1991; 1996) and the wild-

Table 4.1 Bacterial strains used in this study.

***Pseudomonas fluorescens* strains**	**Characteristics**	**Reference**
LP6a	Wild-type containing PAH catabolic plasmid pLP6a, *emhABC*$^{+}$	Foght and Westlake (1996)
WEN	Transposon mutant of LP6a containing pLP6a, *emhB*$^{-}$, Km^{r}	Adebusuyi et al. (2012)
cLP6a	Cured LP6a strain lacking pLP6a, *emhABC*$^{+}$	Foght and Westlake (1996)
cLP6a-1	Transposon mutant of LP6a lacking pLP6a, *emhB*$^{-}$, Km^{r}	Hearn et al. (2003)

type EmhB disruption mutant (WEN; Adebusuyi et al. 2012), both of which are capable of utilizing PAHs such as naphthalene and phenanthrene as carbon source, were used for naphthalene biodegradation, FA composition and membrane permeability assays. *P. fluorescens* LP6a has been deposited with the University of Alberta Microfungus Collection and Herbarium as *Pseudomonas fluorescens* UAMH 11620. All strains were grown to stationary phase on trypticase soy broth (TSB; Difco Laboratories, Detroit, MI) with 200 rpm shaking at 28°C, the optimum growth temperature (Foght and Westlake 1996). Crystals of naphthalene were added to TSB cultures used for electron microscopy, growth,

membrane FA composition and membrane permeability assays to achieve a final concentration of 5 mmol L^{-1} unless otherwise stated. Gene disruption in strains cLP6a-1 and WEN was maintained with 25 µg ml^{-1} kanamycin (Sigma Chemical Co., St. Louis, Mo).

4.2.2 Rapid efflux assays

Efflux of [U-^{14}C]naphthalene (Sigma Chemical Co.) was determined using the rapid centrifugation method performed at ~ 22°C (Bugg et al. 2000). Washed cell pellets of strains cLP6a and cLP6a-1 were re-suspended in 0.1 M potassium phosphate buffer pH [7.0] to an optical density at 600 nm (OD_{600}) of 1.0. Efflux assay buffer contained ^{14}C-naphthalene plus unlabeled naphthalene at a final concentration of 120 µM and ~ 100 000 disintegrations per minute (dpm). This concentration of naphthalene corresponds to ~ 53% of its aqueous solubility at 22°C to ensure complete dissolution. Efflux assays were performed in 35 ml serum bottles sealed with Teflon stoppers to eliminate volatility losses and sampling was accomplished using 18-gauge needles (Bugg et al. 2000). The energy inhibitor sodium azide (Fisher Scientific Co.) was added 9 min into the assay to a final concentration of 120 mM. All efflux assays were performed using three independent cultures.

4.2.3 PAH biodegradation assay

Degradation of naphthalene (0.32 mg ml^{-1}) and phenanthrene (0.5 mg ml^{-1}) by *P. fluorescens* strains LP6a and WEN were assayed as previously described in Chapter 3 (Adebusuyi et al. 2012).

4.2.4 Growth assays

Growth of strains LP6a, WEN, cLP6a and cLP6a-1 in TSB with or without PAHs was measured as protein concentration using the bicinchoninic acid microplate assay (Pierce, Rockford, IL) rather than optical density, to avoid spectrophotometric interference by crystals of the PAH substrate. For bidegradation assays, LP6a strains were grown in Bushnell Haas medium [g l^{-1}]: magnesium sulphate 0.2; calcium chloride 0.02; potassium hydrogen monophosphate 1.0; potassium dihydrogen phosphate 1.0; ammonium nitrate 1.0; ferric chloride 0.05; final pH 7 – 7.2 with naphthalene or phenanthrene as sole carbon source. Cell pellets were lysed by re-suspending in 0.1 ml of 0.1 M sterile NaOH and incubating at 60°C for 30 min then the lysed cell samples were assayed for protein according to manufacturer's instructions. Absorbance of the assay liquid at 562 nm was determined using a PowerWave XS Microplate Spectrophotometer (BioTek, Winooski, VT).

4.2.5 Phospholipid fatty acid (FA) extraction and identification

Total cell lipid extraction, fatty acid methyl ester (FAME) preparation and FA analysis of strains cLP6a and cLP6a-1 cultures grown to stationary phase at 28°C in the absence or presence of 5 mmol L^{-1} PAHs were performed as previously described in Chapter 2 (Adebusuyi and Foght 2011).

4.2.6 Transmission electron microscopy

P. fluorescens strains cLP6a and cLP6a-1 were grown in 50 ml TSB medium at 28°C to stationary phase in the presence or absence of 5 mmol L^{-1} naphthalene, added as crystals. Cells were harvested by centrifugation for 2 min at 5,000 × *g* and washed once with 0.1 M potassium phosphate buffer (pH 7.0). Cell debris due to cell lysis was not observed in the electron micrographs of strains cLP6a and cLP6a-1 processed using this method. Cell samples were fixed in 2% glutaraldehyde for 2 h and then washed three times in 0.1 M potassium phosphate buffer (pH 7.2). Samples were post-fixed with 1 % osmium tetroxide (OsO_4 in the same buffer) for 2 h and washed twice. Dehydration was achieved using a graded series of ethanol washes (50%, 70%, 90% and 3 changes of absolute ethanol). After dehydration, samples were embedded in Spurr low viscosity embedding resin (Electron Microscopy Sciences, Hatfield, PA), sectioned using ULTRACUT E (Reichert-Jung Ultramicrotome) then stained with uranyl acetate and lead citrate. Thin sectioned samples were examined using a Philips Morgagni 268 transmission electron microscope (Philips-FEI, Hillsboro, Oregon, USA) operating at 80 kV with Gatan Orius CCD camera.

4.2.7 Membrane permeability assay

Membrane permeability of *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1 grown to stationary phase at 28°C in TSB in the presence of 5 mmol L^{-1} PAHs was determined as previously described

(Chapter 2: Adebusuyi and Foght 2011). Briefly, harvested *P. fluorescens* strains suspended in 1 ml of phosphate-buffered saline were adjusted to an OD_{600} of 1.0 then treated with 30 µmol l^{-1} propidium iodide (PI; Invitrogen), either alone or with 1 µmol l^{-1} cetyltrimethylammonium bromide (CTAB; Sigma). Untreated cells were included as parallel controls. After 30 min incubation at room temperature, fluorescence of 100-µl cell samples was measured in a 96-well microplate using a Synergy HT Multi-mode Microplate Reader (BioTek) at excitation and emission wavelengths of 530 nm and 590 nm respectively.

4.3 Results

4.3.1 Naphthalene is not a substrate of EmhABC

Efflux assays were performed using strains cLP6a with functional EmhB efflux pump and its *emhB* disruptant mutant cLP6a and 120 µM naphthalene instead of the 5.7 µM used by Bugg et al. (2000) to confirm that naphthalene is not a substrate of the EmhABC efflux pump. A significant increase in cell-associated ^{14}C-naphthalene after the addition sodium azide would indicate active efflux to the extracellular medium (Bugg et al. 2000; Hearn et al. 2003), however addition of sodium azide had no effect on the accumulation of naphthalene in strain cLP6a (Fig. 4.1), indicating lack of active efflux of naphthalene. Likewise, no significant change in radiolabel partitioning to the supernatant was observed after azide addition.

Disruption of *emhB* in strain cLP6a-1 also had no effect on the amount of ^{14}C-naphthalene that accumulated in the cell pellet fraction compared to cLP6a cells (Fig. 4.1). This confirms that naphthalene is not a

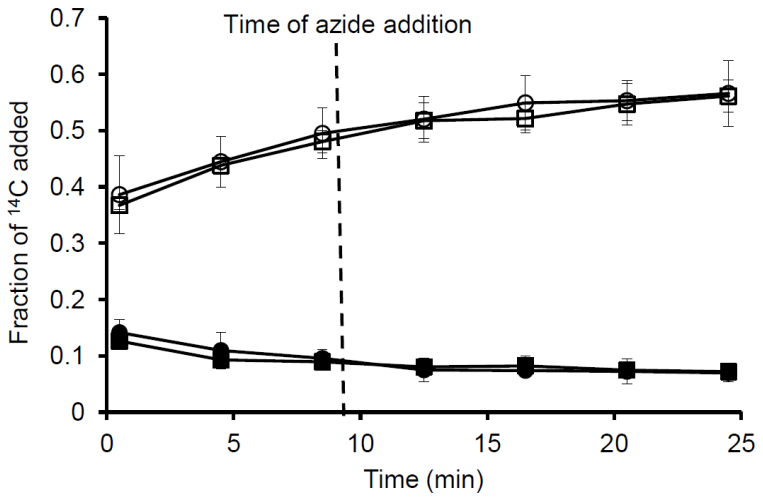

Figure 4.1 Fraction of [U-^{14}C]naphthalene in the cell pellet of *P. fluorescens* strains cLP6a and cLP6a-1 and supernatant fraction determined using a rapid efflux assay. Partitioning of [U-^{14}C]naphthalene for strain cLP6a (squares) or strain cLP6a-1 (circles) in cell pellet (closed symbols) and supernatant (open symbols). The vertical dashed line indicates the addition of azide (120 mM). Each data point is the mean of three independent experiments, and error bars, where visible, indicate the standard deviation.

substrate of the EmhABC efflux pump, in accordance with the conclusions of Bugg et al. (2000) and Hearn et al. (2003). In addition, there was no evidence of energy-dependent uptake of naphthalene in strains cLP6a and cLP6a-1 (Fig. 4.1) although Whitman et al. (1998) have previously reported this activity in other naphthalene-degrading *P. fluorescens* strains.

4.3.2 EmhABC mitigates naphthalene toxicity

Growth of *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1 in TSB medium was determined as increased protein concentration over time. All strains grew to similar density by 24 h (Fig. 4.2), which showed that the disruption of *emhB* in strains WEN and cLP6a-1 does not affect growth compared to the wild-type strain in the absence of naphthalene. Strains cLP6a and cLP6a-1 were also used to test the effect of

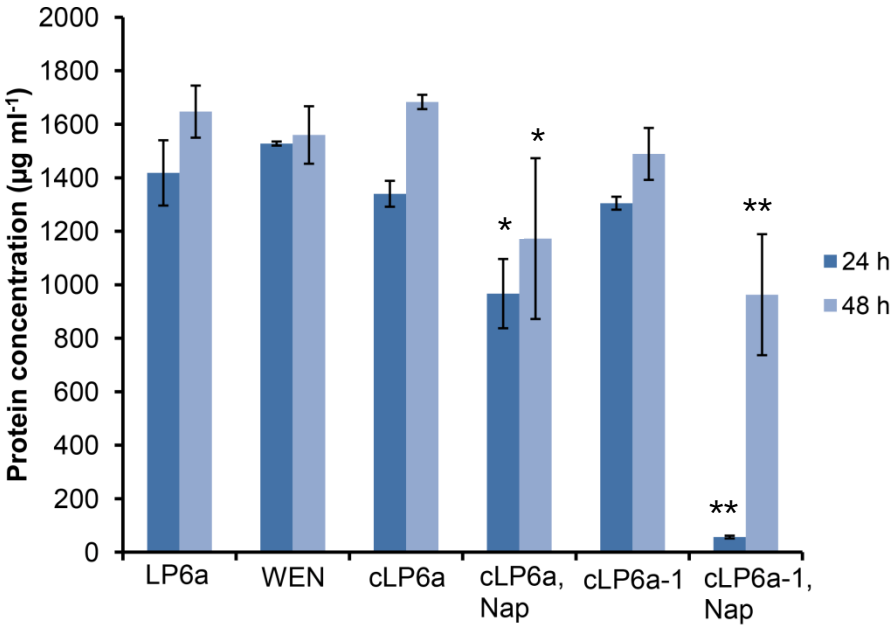

Figure 4.2 Growth of four *P. fluorescens* strains in TSB medium at 28°C measured as protein concentration at 24 h and 48 h in the presence (Nap) or absence of 5 mmol L^{-1} naphthalene. Each data point is the mean of three independent experiments and error bars, where visible, indicate the standard deviation. Asterisks indicate significant differences.

naphthalene in the absence of metabolism combined with presence or absence of a functional EmhB efflux pump. Both strains were grown in TSB broth in the presence of 5 mmol L^{-1} naphthalene. In the presence of naphthalene, by 24 h and 48 h of incubation the protein concentrations of strain cLP6a cultures were 28 ± 12% ($P < 0.02$) and 31 ± 17% ($P < 0.02$) less than in the absence of naphthalene, respectively. Growth of strain cLP6a-1 was inhibited to a greater extent in the presence of naphthalene: by 24 h incubation, cLP6a-1 cultures contained only 4% ($P < 0.0001$) of the protein concentration of the cultures incubated in the absence of naphthalene. The protein concentration in strain cLP6a-1 cultures incubated with naphthalene increased over the next 24 h of incubation (Fig. 4.2) reaching about 65 ± 12% of the protein concentration in cLP6a-1 control cultures. cLP6a and cLP6a-1 cultures incubated in the presence of naphthalene had statistically similar protein concentrations by 48 h of incubation ($P < 0.4$), suggesting that the deleterious effect of naphthalene on strain cLP6a is mitigated by the presence of the EmhABC efflux pump. However, since naphthalene is neither a substrate (Fig. 4.1) nor an inducer (Adebusuyi and Foght 2011) of this efflux pump it raises the question of how mitigation is achieved. The ability of strain cLP6a-1 to recover from the initial shock of the presence of naphthalene also suggests that it takes at least 24 h to achieve other form(s) of resistance against naphthalene versus that which is achieved rapidly in the presence of EmhABC.

4.3.3 *P. fluorescens* strains modify membrane composition and physiology in the presence of naphthalene

We determined the effect of naphthalene on membrane composition and physiology to understand how EmhABC functions to mitigate the toxic effect of naphthalene on *P. fluorescens* strain cLP6a. Parallel cultures incubated with phenanthrene, which has a higher log K_{ow} and is considered non-toxic, for comparison. Rather than analyzing at a selected incubation time,, cultures were grown to stationary phase to avoid dynamic changes associated with growth phase differences. The membrane FA composition of strains cLP6a and cLP6a-1 incubated in TSB at 28°C with or without PAHs was determined using fatty acid methyl ester (FAME) analysis. Because strains cLP6a and cLP6a-1 do not metabolize PAHs, any change in membrane composition and physiology is due to exposure to naphthalene or phenanthrene rather than metabolites. In the presence of naphthalene, strain cLP6a with a functional EmhB efflux pump exhibited only slight changes in its membrane FA content (Table 4.2), notably a two-fold increase in the *trans*-UFA content. Although the percentage of *trans*-UFA in *P. fluorescens* strains is low, this is consistent with adaptation to naphthalene by increasing membrane rigidity to reduce partitioning of naphthalene into the membrane. *Trans*-UFAs, similar to saturated-FAs, decrease membrane fluidity and provide resistance to solvents (Zhang and Rock 2008), whereas *cis*-UFAs cause looser packing of the membrane FA and thus increase membrane

Table 4.2 FA composition of *P. fluorescens* strains cLP6a and cLP6a-1 incubated in TSB at 28°C to stationary phase with or without 5 mmol L^{-1} naphthalene. FA contents are expressed as the mean weight % of total FA detected in two measurements. Deviation from the mean was typically <1% and at most 3% of the measured values.

	% of total fatty acid detected [a]					
	cLP6a[b]			cLP6a-1		
Fatty acid	28°C	28°C with naphthalene	28°C with phenanthrene	28°C	28°C with naphthalene	28°C with phenanthrene
14:0	1.0	0.6	0.7	0.6	0.3	0.6
15:0	0.2	0.1	0.2	0.1	0.1	0.1
16:0	40.4	40.8	40.1	39.7	36.6	38.8
16:1Δ9c	4.6	5.5	4.7	3.7	21.3	5.2
16:1Δ9t	1.6	3.2	1.9	2.1	5.7	2.9
17:0	0.3	0.2	0.3	0.1	0.7	0.4
cy17	40.0	36.5	39.7	40.4	8.5	36.8
18:0	1.2	1.2	1.2	1.3	5.3	1.5
18:1Δ9c	7.6	9.3	7.9	8.3	20.9	10.0
18:1Δ9t	ND[c]	0.3	ND	0.3	0.4	ND
cy19	3.1	2.3	3.3	3.5	0.3	3.8
Total saturated FA	43.1	42.9	42.5	41.8	43.0	41.0
Total cis-unsaturated FA	12.2	14.8	12.6	12.0	42.2	15.2
Total trans-unsaturated FA	1.6	3.5	1.9	2.4	6.1	2.9
Total cyclopropane FA	43.1	38.8	43.0	43.9	8.8	40.6

[a], FA nomenclature: number of carbons; saturation (:0); mono-unsaturation (:1); position of double bond calculated from the carboxyl end (Δ9); *cis* (c) or *trans*- (t) isomer; cyclopropyl ring (cy)
[b], Data for strain cLP6a from Adebusuyi and Foght (2011)
[c], not detected

fluidity (Denich et al. 2003; Zhang and Rock 2008). The other changes in membrane FA composition of strain cLP6a incubated in the presence of naphthalene are the slight but statistically significant increase in the total *cis*-UFAs from 12.2% to 14.8% ($P < 0.02$) and concomitanat decrease in the total cyclopropane-FAs content from 43.1% to 38.8% ($P < 0.001$). The FA profile of strains cLP6a and cLP6a-1 incubated with phenanthrene was similar to that of controls lacking phenanthrene.

Although there were only slight modifications in the FA composition of strain cLP6a-1 compared to strain cLP6a in the absence of naphthalene (Table 4.2), the difference between the strains in the presence of naphthalene was striking. *Cis*- and *trans*-UFAs increased ~ 3- to 4-fold in the *emhB* mutant strain cLP6a-1 in the presence of naphthalene, whereas there was a substantial decrease in both cyclopropane-FAs (Table 4.2). The total percentage of cyclopropane-FA decreased ~ 4-fold to 8.8% in strain cLP6a-1 compared to 38.8% in strain cLP6a. This indicates that naphthalene interferes with the synthesis of cyclopropane-FAs, which are formed by modifying existing *cis*-UFAs in *P. fluorescens* strains (Londry et al. 2004; Zhang and Rock 2008). Notably, EmhABC counteracts the inhibition of cyclopropane-FA formation by naphthalene, because strain cLP6a with functional EmhB formed cyclopropane-FA. Phenanthrene also caused a slight increase in *cis*-FAs with concomitant decrease in cyclopropane FAs in the *emhB*$^{-}$ mutant cLP6a-1 (Table 4.2). This suggests that although phenanthrene may not be inhibitory to LP6a strains like naphthalene, it also elicits membrane modification in these strains that is mitigated by the EmhABC efflux pump.

Transmission electron microscopy of thin-sectioned cells grown to stationary phase in TSB with naphthalene showed that naphthalene had no apparent effect on the structure of strain cLP6a compared to a control (Fig. 4.3a and 4.3b). This supports the FAME analysis results where only minor membrane FA changes occurred in this efflux-competent strain

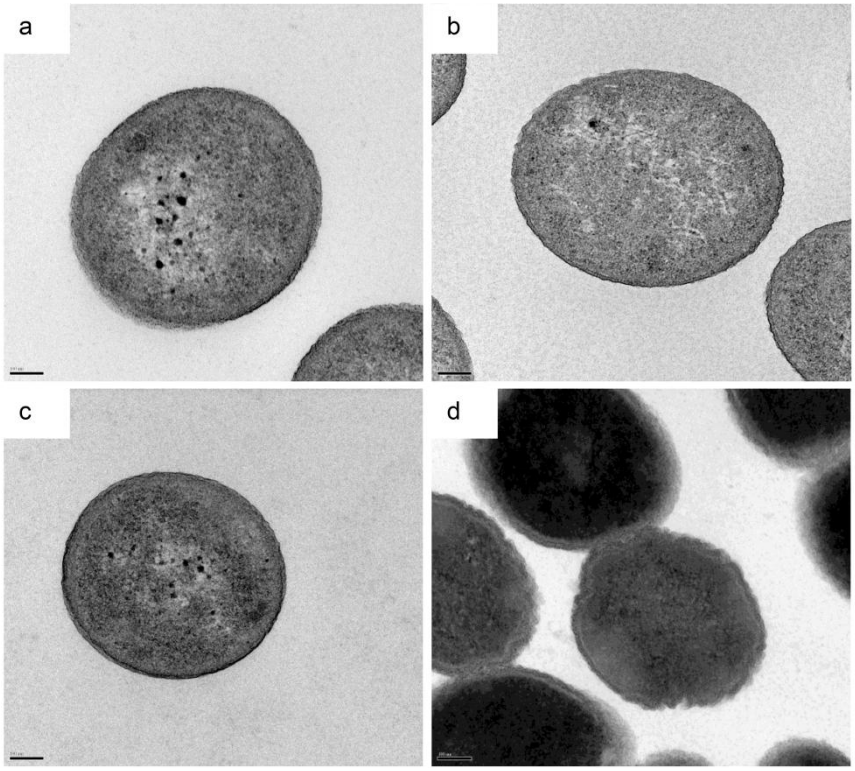

Figure 4.3 Transmission electron micrographs of *P. fluorescens* strains cLP6a and cLP6a-1 incubated in TSB medium to stationary phase at 28°C with or without 5 mmol L^{-1} naphthalene: (a) strain cLP6a without naphthalene (control), (b) strain cLP6a with naphthalene, (c) strain cLP6a-1 without naphthalene (control) and (d) strain cLP6a-1 with naphthalene. Scale bar on electron micrographs is 100 nm.

during incubation with naphthalene. Conversely, cLP6a-1 cells incubated with naphthalene exhibited a different cell surface, in agreement with major changes in their membrane FA composition (Fig. 4.3c and 4.3d). The membrane of thin-sectioned cLP6a-1 cells incubated with naphthalene (Fig. 4.3d) appeared thicker and convoluted compared to controls. This is consistent with the proposal that the EmhABC efflux is important to maintain membrane integrity in the presence of naphthalene.

4.3.4 Naphthalene affects membrane integrity in the absence of EmhB and metabolism

The membrane permeability of *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1 incubated in TSB with or without 5 mmol L^{-1} of PAHs was determined to confirm if the major changes in the membrane FA and membrane morphology in the absence of EmhB compromised membrane integrity. In the absence of PAH (controls), the membrane permeability index (a measure of the number of cells with compromised membrane in a culture sample; Chapter 2; Adebusuyi and Foght 2011) of all the strains were similar (~ 16%; $P = 0.05$). The permeability index of strains LP6a and cLP6a incubated with naphthalene was similar to controls (Fig. 4.4). Likewise, the permeability index of strain WEN, an *emhB*$^{-}$ mutant with the ability to degrade naphthalene, did not increase in the presence of naphthalene. Conversely, the membrane permeability of strain cLP6a-1 lacking the ability to degrade or efflux naphthalene increased greatly in the presence of naphthalene, with > 4-fold increase in permeability index compared to controls (Fig. 4.4). Increased membrane permeability of strain cLP6a-1 incubated with naphthalene is in agreement with the results of FAME analysis and electron microscopy. This indicates that efflux and degradation of naphthalene by LP6a strains confers tolerance to it and

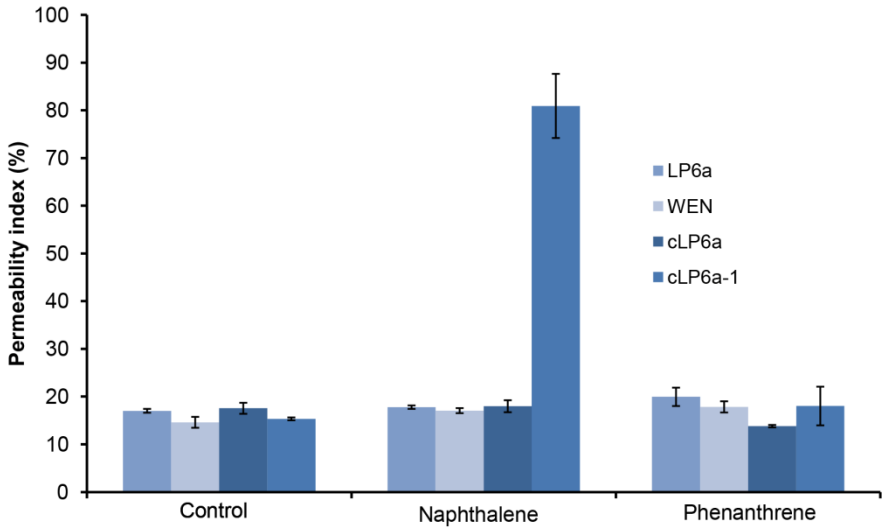

Figure 4.4 Membrane permeability indexes of *P. fluorescens* strains LP6a, WEN, cLP6a and cLP6a-1 incubated in TSB medium to stationary phase at 28°C with or without 5 mmol L^{-1} naphthalene or phenanthrene. Each data point is the mean of three independent experiments, and error bars, where visible, indicate the standard deviation.

that ability to degrade naphthalene in combination with membrane modification in the absence of efflux is sufficient to eventually overcome the deleterious effects of naphthalene on these strains. Also, the changed cell wall (Fig. 4.3d) and membrane FA composition (Table 4.2) combined with increased permeability (Fig 4.4) may allow increased penetration of the osmium tetroxide and lead citrate, resulting in darker cytoplasmic stains (Fig. 4.3d). As expected, the membrane permeability index of LP6a strains incubated with phenanthrene was similar to controls (Fig. 4.4).

4.3.5 Naphthalene metabolism obviates efflux

Naphthalene is a carbon substrate (Foght and Westlake 1996) and has been shown here to be inhibitory to *P. fluorescens* strains, as evident from the results of membrane FA composition (Table 4.2) and cell morphology (Fig. 4.3), but it is not a substrate of EmhABC efflux pump (Fig. 4.1) nor an inducer of emhABC expression (Adebusuyi and Foght 2011). Therefore, the difference in the efficiency of biodegradation of naphthalene and phenanthrene (an EmhABC substrate) was examined. Growth of strains LP6a and WEN was similar when phenanthrene was used as the sole carbon source (Fig. 4.5a) indicating that phenanthrene had little or no toxic effect on strain WEN with disrupted *emhB* gene. On the other hand, the growth of strain WEN was slower when naphthalene was the sole carbon source (Fig. 4.5b). Although WEN cells recovered from naphthalene inhibition by 96 h of incubation, reduced growth of the *emhB* disruptant mutant on naphthalene supports FAME analysis results that EmhABC is important to suppress the effect of naphthalene- (Adebusuyi and Foght 2011). The estimated rate of naphthalene degradation by wild-type strain LP6a with functional EmhB efflux pump and strain WEN with disrupted *emhB* was compared to that of phenanthrene in both strains (Fig. 4.5b). Naphthalene was degraded at a faster rate (7×10^{-3} mg ml^{-1} d^{-1}) than phenanthrene (3×10^{-3} mg ml^{-1} d^{-1}) by the wild-type strain LP6a. The rate of naphthalene degradation was calculated using data obtained at 24 h of incubation. Within 24 h, ~56% of

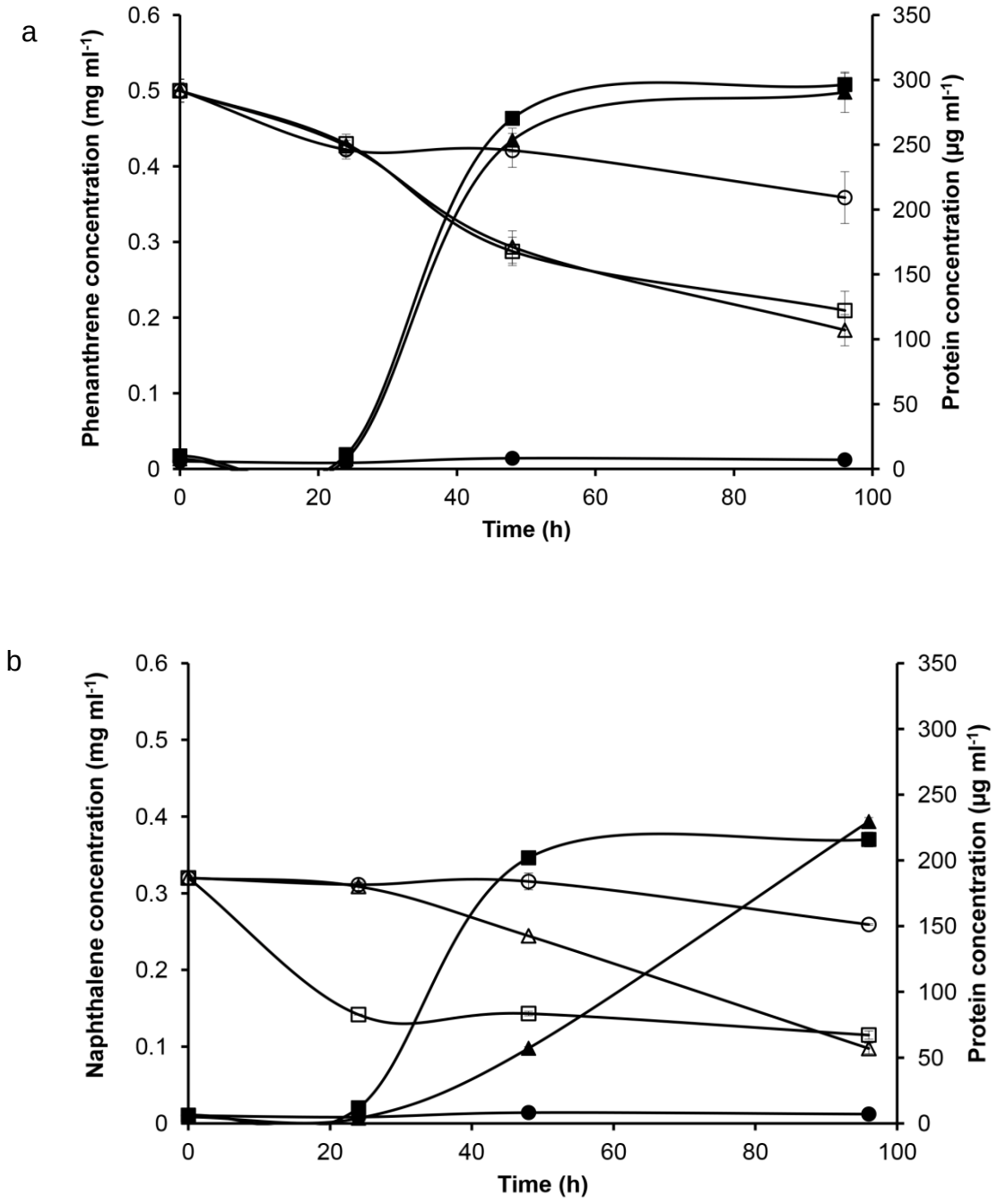

Figure 4.5 Growth on and metabolism of naphthalene and phenanthrene by *P. fluorescens* strains LP6a and WEN; (a) protein concentration (closed symbols) and phenanthrene degradation (open symbols) of abiotic controls, LP6a and WEN cultures grown at 28°C and (b) protein concentration (closed symbols) and naphthalene degradation (open symbols) of abiotic controls, LP6a and WEN cultures grown at 28°C. LP6a (squares), WEN (triangles) and abiotic (circles). Each data point is the mean of two pseudo-replicates, and error bars, where visible, indicate the standard deviation.

the naphthalene had been degraded, whereas only 14% of phenanthrene was degraded (Fig. 4.5b). This suggests that naphthalene concentration in the cells and the medium is greatly reduced by metabolism thus making the need for efflux redundant. Consistent with the results presented in Chapter 2, phenanthrene degradation is unhindered in strain WEN (Adebusuyi et al. 2012). In parallel with growth inhibition, naphthalene degradation was hindered in strain WEN compared to strain LP6a.

4.4 Discussion

Naphthalene, a priority environmental pollutant (U.S. EPA 2002) and component of petroleum and coal tar (Cerniglia 1992), can be degraded by many *Pseudomonas* species that carry naphthalene catabolic genes on a plasmid (Foght and Westlake 1991; 1996). *P. fluorescens* LP6a, isolated from petroleum-contaminated soil, can use naphthalene and other PAHs such as phenanthrene as sole source of carbon and energy (Foght and Westlake 1991). On the other hand, LP6a possesses a RND-type efflux pump, EmhABC, which transports phenanthrene and anthracene as well as hydrophobic antibiotics but not naphthalene (Bugg et al. 2000; Hearn et al. 2003). Substrate capture and binding has been shown to occur in periplasmic domains of RND efflux pumps but not in the transmembrane domains (Murakami et al. 2006; Sennhauser et al. 2009). Since hydrophobic compounds such as PAHs are expected to partition into the inner membrane, the mechanism of PAH

capture and selectivity by the EmhABC efflux pump is unknown. Because naphthalene is poetentially toxic to LP6a but not effluxed by EmhABC, the involvement of this pump in the tolerance to naphthalene was examined.

As expected, naphthalene is inhibitory to the *P. fluorescens* strains tested here, revealed as growth inhibition and altered cell membrane composition and morphology. In general, PAH compounds are assumed to be non- or slightly toxic to hydrocarbon-degrading bacteria (Sikkema et al. 1995) but their metabolites may be toxic to these bacteria. These observations were due to the effect of naphthalene and not its metabolites because the assays were performed using LP6a mutants incapable of metabolizing naphthalene. Although it has been previously shown that naphthalene cause changes in bacterial cell membrane fatty acid composition, most studies determining the toxicity of naphthalene to bacteria have been performed using strains capable of utilizing PAHs (e.g., Kahng and Kyoungphile 2002; Kallimanis et al. 2007; Mrozik et al. 2004). Use of PAH-degrading strains to determine PAH toxicity makes it difficult to discern the effect of the parent compound on the cell because the results are camouflaged by the effect of metabolites.

Results of FAME analysis, growth and membrane permeability assays of mutants of LP6a exposed to naphthalene presented in this study showed that the EmhABC efflux is indirectly involved in the mitigation of the deleterious effects of naphthalene. EmhABC is required by LP6a mutants for uninhibited growth in the presence of naphthalene and for

efficient metabolism of naphthalene. Increased membrane *trans*-UFAs in cLP6a and cLP6a-1 cells exposed to naphthalene is consistent with adaptation to the presence of a membrane stressor (Junker and Ramos 1999). Increased *trans*-UFA content decreases membrane fluidity in response to membrane stresses (Denich et al. 2003; Zhang and Rock 2008). This indicates that naphthalene had a fluidizing effect on *P. fluorescens* cLP6a membrane that wascounteracted by membrane FA changes.

Cyclopropane-FAs are formed by the addition of a methylene group across the double bond of pre-existing phospholipid *cis*-FAs by cyclopropane synthase (Pini et al. 2009) during transition from logarithmic to stationary phase of growth (Londry et al. 2004; Zhang and Rock 2008). Although naphthalene inhibited the growth of strain cLP6a-1, all membrane FAs were extracted from cultures in stationary phase, so changes in the percentage of cyclopropane-FAs reported here represent changes due to the presence of naphthalene rather than any effect of growth phase. Cyclopropane-FAs have been shown to confer resistance to acid in *Escherichia coli* (Chang and Cronan 1999), and to organic solvents and freeze drying in *P. putida* (Munoz-Rojas et al. 2006; Pini et al. 2009). They are known to generally enhance the viability of cells in harsh environments and play an important role in the ability of bacterial pathogens to survive in the environment (Zhang and Rock 2008). cLP6a-1, the mutant lacking EmhB and unable to metabolize naphthalene, was

particularly unable to convert *cis*-UFA to cyclopropane-FA (Table 4.2) causing significant change in its membrane morphology and membrane permeability (Fig. 4.3d) with growth inhibition. This indicates that naphthalene may have indirectly or directly reduced the expression of cyclopropane synthase and in turn the production of cyclopropane FAs, and that cyclopropane FAs are important for maintaining membrane integrity of LP6a strains in the presence of membrane stressors. The exact role of EmhABC in the process of *cis*-FA conversion to cyclopropane-FA is unknown although EmhABC has been previously associated with free fatty acid transport (Adebusuyi and Foght 2011). This infers that cyclopropane-FAs are involved in the survival of LP6a in its natural environment and that *cis*-UFA may be substrates of the EmhABC efflux pump. Other RND efflux pumps such as AcrAB-TolC in *Escherichia coli* are also involved in FA transport (Nikaido 1996). Replaced or damaged membrane FAs due to natural membrane turnover or adaptation to membrane stressors are either re-cycled or eliminated to prevent accumulation and toxic effects in bacterial cells (Zhang and Rock 2008). Alternatively, RND efflux pumps may be involved in the transport of membrane lipids such as hopanoids required for stress resistance in the outer membrane of Gram-negative bacteria (Doughty et al. 2011). This supports the idea that RND efflux pumps play other role(s) in Gram-negative bacteria related to the environment and that antibiotics, solvents, or PAHs are incidental

substrates of these efflux pumps (Adebusuyi and Foght 2011; Martinez et al. 2009).

In LP6a, a combination of membrane modification, efflux of replaced or damaged FAs, and metabolism is involved in overcoming the deleterious effect of naphthalene. Efflux is likely an indispensable mechanism in LP6a because genes that encode the EmhABC efflux pump are located on the chromosome (Bugg et al. 2000; Hearn et al. 2003), whereas the ability to metabolize PAHs is disposable since the PAH catabolic genes are located on a plasmid (Foght and Westlake 1996).

In Chapter 2, it was shown that the EmhABC decreases the efficiency of phenanthrene degradation in strain LP6a due to efflux of phenanthrene as well as its metabolite(s) (Adebusuyi et al. 2012). In contrast, EmhABC is important for efficient degradation of naphthalene because it mitigates the deleterious effect of naphthalene on the cell membrane to enhance growth and metabolism. This implies that RND-type efflux pumps present in other bacterial species that degrade naphthalene (and possibly other hydrocarbons that affect bacterial cell membrane) may be equally important for efficient the degradation. Selection of bacterial strains with or without a hydrocarbon extruding RND efflux pump for use in bioremediation or biocatalytic process involving hydrocarbons may depend on the target compound and the type of process since it is important to use microbes that degrade and also exhibit tolerance to the pollutants they degrade (Fillet et al. 2012).

4.5 Literature cited

Adebusuyi AA, Fogh JM (2011) An alternative physiological role for the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a. BMC Microbiol 11:252

Adebusuyi AA, Smith AY, Gray MR, Fogh JM (2012) The EmhABC efflux pump decreases the efficiency of phenanthrene biodegradation by *Pseudomonas fluorescens* strain LP6a. Appl Microbiol Biotechnol DOI 10.1007/s00253-012-3932-4

Bugg T, Foght JM, Pickard MA, Gray MR (2000) Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol 66**:**5387-5392

Bullard JW, Champlin FR, Burkus J, Millar SY, Conrad RS (2011) Triclosan-induced modification of unsaturated fatty acid metabolism and growth in *Pseudomonas aeruginosa* PAO1. Curr Microbiol 62:697-702

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3:351-368

Chang YY, Cronan Jr. JE (1999) Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. Mol Microbiol 33:249-259

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Meth 52:149-182

Dominguez-Cuevas P, Gonzalez-Pastor J-E, Marques S, Ramos J-L, de Lorenzo V (2006) Transcriptional tradeoff between metabolic and stress-response programs in *Pseudomonas putida* KT2440 cells exposed to toluene. The J Biol Chem 281:11981-11991

Doughty DM, Coleman ML, Hunter RC, Sessions AL, Summons RE, Newman DK (2011) The RND-family transporter, HpnN, is required for hopanoids localization to the outer membrane of *Rhodopseudomonas palustris* TIE-1. PNAS 108:E1045-E1051

Fillet S, Daniels C, Pini C, Krell T, Duque E, Bernal P, Segura A, Lu D, Zhang X, Ramos J-L (2012) Transcriptional control of the main aromatic hydrocarbon efflux pump in *Pseudomonas* Environ Microbiol Rep 4:158-167

Foght JM, Westlake DWS (1991) Cross hybridization of plasmid and genomic DNA from aromatic and polycyclic aromatic hydrocarbon degrading bacteria. Can J Microbiol 37:924-932

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353-366

Hearn EM, Dennis JJ, Gray MR, Foght JM (2003) Identification and characterization of the *emh*ABC efflux system for polycyclic aromatic hydrocarbons in *Pseudomonas fluorescens* cLP6a. J Bacteriol 185:6233-6240

Huertas M-J, Duque E, Molina L, Rossello-Mora R, Mosqueda G, Godoy P, Christensen B, Molin S, Ramos J-L (2000) Tolerance to sudden organic solvent shocks by soil bacteria and characterization of *Pseudomonas putida* strains isolated from toluene polluted sites. Environ Sci Technol 34:3395-3400

Junker F, Ramos JL (1999) Involvement of the *cis/trans* isomerase Cti in solvent resistance of *Pseudomonas putida* DOT-T1E. J Bacteriol 181: 5693-5700

Kahng H-Y, Kyoungphile N (2002) Molecular characteristics of *Pseudomonas rhodesiae* strain KK1 in response to phenanthrene. J Microbiol Biotechnol 12:729-734

Kallimanis A, Frillingos S, Drainas C, Koukkou AI (2007) Taxonomic identification, phenanthrene uptake activity, and membrane lipid alterations of the PAH degrading *Arthrobacter* sp. strain Sphe3. Appl Micrbiol Biotechnol 76:709-717

Londry KL, Jahnke LL, Des Marais DJ (2004) Stable carbon isotope ratios of lipid biomarkers of sulfate-reducing bacteria. Appl Environ Microbiol 70:745-751

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

Mrozik A, Piotrowska-Seget Z, Labuzek S (2004) Changes in whole cell-derived fatty acids induced by naphthalene in bacteria form genus *Pseudomonas*. Microbiol Res 159: 87-95

Munoz-Rojas J, Bernal P, Duque Estrella, Godoy P, Segura A, Ramos J-L (2006) Involvement of cyclopropane fatty acids in the response of *Pseudomonas putida* KT2440 to freeze-drying. Appl Environ Microbiol 72:472-477

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

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

Patton JS, Stone B, Papa C, Abramowitz R, Yalkowsky SH (1984) Solubility of fatty acids and other hydrophobic molecules in liquid trioleoylglycerol. J Lipid Res 25:189-197

Pini C-V, Bernal P, Godoy P, Ramos J-L, Segura A (2009) Cyclopropane fatty acids are involved in organic solvent tolerance but not in acid stress resistance in *Pseudomonas putida* DOT-T1E. Microbiol Biotechnol 2:253-261

Ramos J-L, Duque E, Gallegos M-T, Godoy P, Ramos-Gonzalez MI, Rojas A, Teran W, Segura A (2002) Mechanisms of solvent tolerance in Gram-negative bacteria. Annu Rev Microbiol 56:743-768

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

Sikkema J, De Bont JAM, Poolman B (1995) Mechanisms of membrane toxicity of hydrocarbons. Microbiol Rev 59:201-222

U.S. EPA (2002) National recommended water quality criteria: 2002. EPA-822-R-02-047:33

Whitman BE, Lueking DR, Mihelcic JR (1998) Naphthalene uptake by a *Pseudomonas fluorescens* isolate. Can J Microbiol 44:1086-1093

Zhang, Y-M, Rock CO (2008) Membrane lipid homeostasis in bacteria. Nature Rev Microbiol 6: 222-233

5. Chloramphenicol efflux and the induction of the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a[3]

5.1 Introduction

Chloramphenicol is a broad-spectrum antibiotic whose use has been limited to topical treatment of bacterial infections due to toxicity to humans (Aakra et al. 2010; Schwarz et al. 2004; Xaplanteri et al. 2003). Recently there has been renewed interest in the use of chloramphenicol in treating certain bacterial infections because it is effective against some multidrug-resistant bacteria pathogens (Fernández et al. 2012; Maviglia at al. 2009). Chloramphenicol inhibits protein synthesis in bacteria by blocking the peptidyltransferase function of the 50S ribosomal subunit (Aakra et al. 2010; Drainas et al. 1987; Xaplanteri et al. 2003). Bacteria have developed several mechanisms to reduce uptake or intracellular concentration of chloramphenicol including enzymatic inactivation by acetylation (Aakra et al. 2010; Fernández et al. 2012), modification of its target site, decreased outer membrane permeability (Schwarz et al. 2004) and active efflux into the extracellular medium (Adebusuyi and Foght 2011; Jeannot et al. 2005).

Uptake of chloramphenicol by bacterial cells occurs via an energy-dependent process but is dependent on the growth medium, growth phase of the cells, temperature and pH (Abdel-Sayed 1987; Burns and Smith

[3] A version of this chapter has been submitted for publication.

1987). Chloramphenicol is a substrate of most efflux pumps of the resistance-nodulation-division (RND) superfamily but the physico-chemical factors that affect its efflux have not been examined. Antibiotics that associate with or disrupt ribosomes, such as chloramphenicol and tetracycline, have been reported to induce expression of RND efflux pumps such as TtgABC in *Pseudomonas putida* DOT-T1E (Teran et al. 2003) and MexXY in *P. aeruginosa* (Jeannot et al. 2005; Poole 2008). Contrary to the proposed model of RND efflux pump induction by ribosome-disrupting antibiotics (Jeannot et al. 2005), chloramphenicol did not induce expression of the EmhABC efflux pump (Adebusuyi and Foght 2011).

It was revealed in Chapter 2 that growth phase, incubation temperature and tetracycline induced the *emhABC* genes encoding the EmhABC efflux pump (Adebusuyi and Foght 2011). This induction correlated with major changes in the membrane fatty acid composition and membrane damage and turnover during growth, supporting the proposal that EmhABC is involved in the mitigation of membrane damage in this strain.

The activity and expression of RND efflux pumps, which are major determinants of antibiotic resistance, may be influenced by the bacterial environment (Martinez et al. 2009) given that they play other physiological roles in bacteria (Adebusuyi and Foght 2011; Piddock 2006; Poole 2008). Three different physico-chemical parameters were selected to determine

effects of membrane modulation and proton potential on chloramphenicol efflux by EmhABC: (a) temperature, for its effect on membrane fatty acid composition; (b) pH, for its effect on the proton motive force; and (c) Mg^{2+}, for its effect on outer membrane stability. The effect of chloramphenicol and tetracycline on the cell envelope of *P. fluorescens* strains was also compared to determine why chloramphenicol is not an inducer of *emhABC* expression. Identification of factors influencing induction or determining why a particular antibiotic induces expression of a RND efflux pump will be useful in developing new strategies to combat antibiotic resistance in pathogenic bacteria.

5.2 Materials and methods

5.2.1 Bacterial strains and growth conditions

P. fluorescens cLP6a (Foght and Westlake 1996) and its EmhB disruption mutant cLP6a-1 (Hearn et al. 2003) were used in this study. *P. fluorescens* strains were grown to stationary phase on trypticase soy broth (TSB; Difco Laboratories, Detroit, MI) at 10°C, 28°C or 35°C with 200 rpm shaking. Gene disruption in strain cLP6a-1 was maintained with 25 µg ml^{-1} kanamycin (Sigma Chemical Co.).

5.2.2 Rapid efflux assays

The efflux of chloramphenicol D-threo [dichloroacetyl-1-^{14}C] (99% radiochemical purity; American Radiolabeled Chemicals, Inc. St. Louis, MO) was determined using the rapid centrifugation method performed at

~22°C (Bugg et al. 2000). To test the effect of incubation temperature on the activity of the EmhABC efflux pump, strains cLP6a and cLP6a-1 grown to stationary phase at 10°C, 28°C or 35°C were used for efflux assays. Strains cLP6a and cLP6a-1 were re-suspended to an optical density at 600 nm (OD_{600}) of 1.0 in 0.1 M potassium phosphate buffer pH 7 or pH 5.8, or pH 7 in the presence of 5 mM $MgCl_2$ to test the effect of temperature, pH and divalent cation, respectively, on chloramphenicol transport by the EmhABC efflux pump. The buffer used for efflux assays contained 20 µl of ^{14}C- chloramphenicol to give ~ 100 000 disintegrations per minute (dpm) and a final concentration of 0.073 µM. The energy inhibitor, sodium azide (Fisher Scientific Co.) was added to the cell suspension 9 min into the assay to a final concentration of 120 mM. All efflux assays were performed using three independent cultures. Rapid efflux assays were performed over a period of only 25 min to prevent long-term changes in membrane fatty acids composition. The concentration of chloramphenicol associated with the cell pellet fraction was calculated based on the fractional amount of ^{14}C associated with the cell pellet at each time point, the initial concentration of ^{14}C-chlorophenicol added and the dry weight of *P. fluorescens* strains corresponding to OD_{600} of 1.0, as previously described by Bugg et al. (2000).

5.2.3 RT-qPCR

Total RNA was extracted from harvested cultures of *P. fluorescens* cLP6a grown to stationary phase at 28°C in TSB medium supplemented

with 5mM $MgCl_2$ or with a starting pH of 5.8. Total RNA extraction, reverse –transcription PCR (RT-PCR) and quantitative real-time PCR (qPCR) were performed as previously described in Chapter 2 and Adebusuyi and Foght (2011). A *P. fluorescens* cLP6a culture grown to stationary phase in TSB at 28°C (the optimum growth temperature for cLP6a) was used as the calibrator sample (control) and the 16S rRNA gene of *P. fluorescens* pf0-1 (NC_007492) (Silby et al. 2009) was used as the endogenous control. Statistical analysis of data was performed using ANOVA (Excel 2007).

5.2.4 Antibiotic sensitivity assays

The minimum inhibitory concentrations (MICs) of tetracycline, streptomycin, and chloramphenicol were determined using the microtiter broth dilution method (Wiegand et al 2008) for *P. fluorescens* cLP6a and cLP6a-1 grown at 10°C, 28°C (with or without 5 mM $MgCl_2$, or starting pH of 5.8), or at 35°C.

5.2.5 Phospholipid fatty acid (FA) extraction and identification

Total cell lipid extraction, fatty acid methyl ester (FAME) preparation and analysis of cLP6a and cLP6a-1 cultures grown to stationary phase at 28°C in the absence or presence of chloramphenicol or tetracycline (at 1/4 MIC) were performed as previously described in Chapter 2 and Adebusuyi and Foght (2011).

5.2.6 Membrane permeability assay

The membrane integrity of *P. fluorescens* cLP6a and cLP6a-1 cells grown to stationary phase at 28°C in the absence or presence of ¼ MIC

chloramphenicol or tetracycline was determined using a modification (Adebusuyi and Foght 2011) of the method described by Niven and Mulholland (1998).

5.3 Results

5.3.1 Physico-chemical factors affect chloramphenicol efflux

Rapid efflux assays were performed to determine the effect of incubation temperature, presence of the divalent cation Mg^{2+} and pH on the efflux of chloramphenicol by the EmhABC efflux pump. The results of this 25 min long assay represent short-term effects of the physico-chemical factors tested on the activity/expression of the EmhABC efflux pump. Change in concentration of chloramphenicol in the cell pellet fraction due to efflux (Fig. 5.1a) is calculated as the difference between steady state concentration of ^{14}C-chloramphenicol associated with the cell pellet fraction in the absence of efflux (strain cLP6a-1, after azide addition) and in the presence of efflux (strain cLP6a, before azide addition) as described by Adebusuyi and Foght (2011). The higher the change in chloramphenicol concentration, the lower the concentration of chloramphenicol associated with the cell pellet fraction. This value is normalized to the dry weight of the cells in each assay. The concentration of chloramphenicol associated with the cell pellet fraction due to efflux decreased in cells grown at 10°C (Fig. 5.1b) compared to 28°C, the

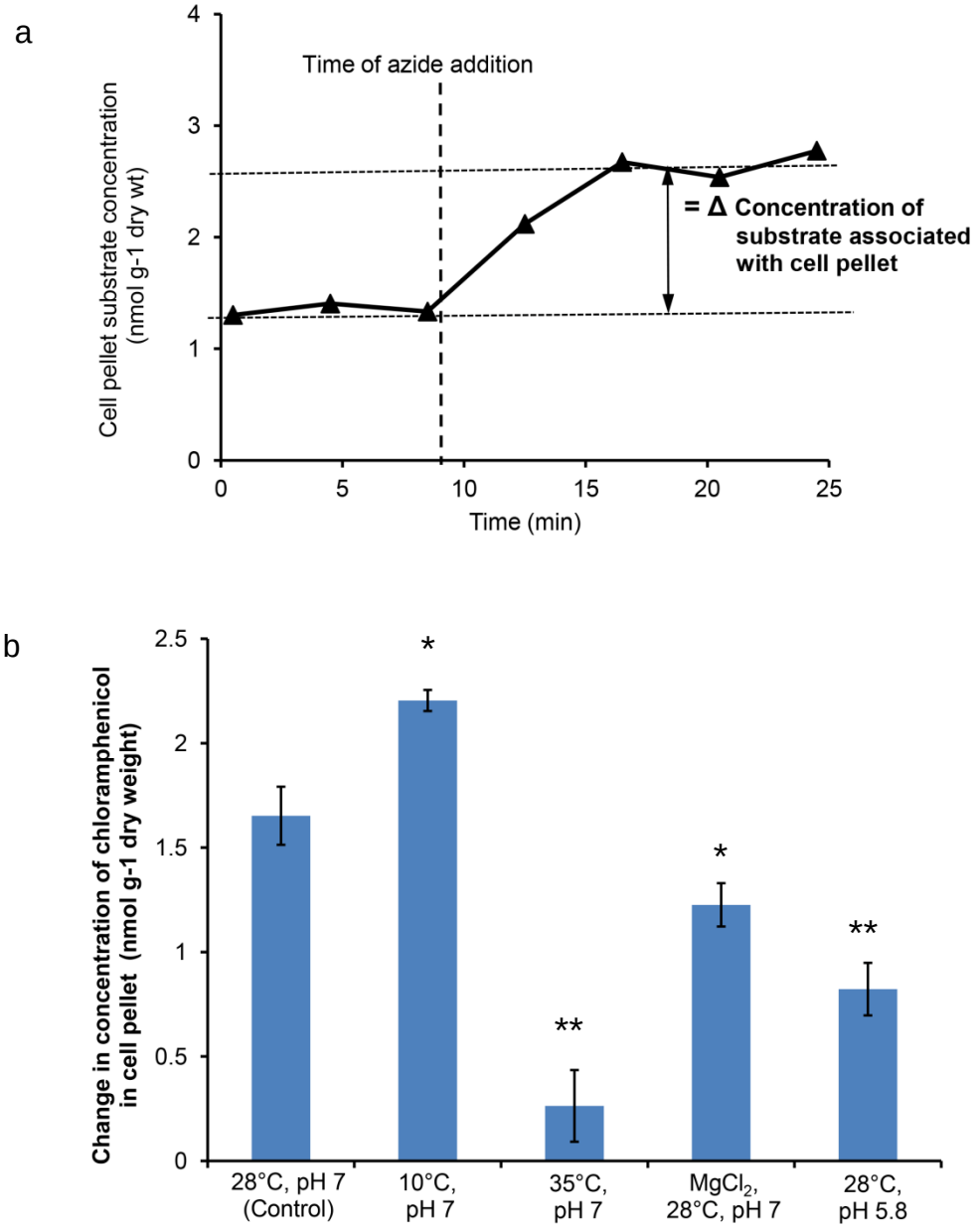

Figure 5.1 Rapid efflux assay for chloramphenicol in *P. fluorescens* cLP6a; (a) sample calculation for concentration of chloramphenicol effluxed; (b) concentration of chloramphenicol effluxed by *P. fluorescens* cLP6a under different conditions, determined using a rapid efflux assay. 28°C, 10°C and 35°C: cells grown at 28°C (the optimum growth temperature), 10°C or 35°C respectively, then harvested and re-suspended in 0.1 M phosphate buffer at pH 7; $MgCl_2$: cells grown at 28°C then re-suspended in 0.1 M phosphate buffer at pH 7 + 5 mM $MgCl_2$; pH 5.8: cells grown at 28°C then re-suspended in 0.1 M phosphate buffer at pH 5.8. Each bar is the mean of three independent experiments, and error bars, where visible, indicate the standard deviation. Asterisks indicate significant differences.

optimum growth temperature of *P. fluorescens* strains (Foght and Westlake 1996). A calculated mass of 2.20 ± 0.14 nmol of chloramphenicol g^{-1} cells (dry weight) was removed by strain cLP6a grown at 10°C, which was significantly different ($P < 0.004$) and about 33 ± 4 % greater than that removed by cells grown at 28°C (1.65 ± 0.05 nmol g^{-1}). The increased concentration of chloramphenicol removed by cells grown at 10°C may be due to the activity of the EmhABC efflux pump in combination with that of another postulated RND pump activated at this temperature, as suggested in Chapter 2 for phenanthrene efflux in the same strain (Adebusuyi and Foght 2011). Conversely, change in the concentration of chloramphenicol associated with strain cLP6a grown at 35°C was very low (0.26 ± 0.17 nmol g^{-1}) and significantly less (~ 84%; $P < 0.008$) than that of control cells grown at 28°C. This indicates that incubation temperature affects chloramphenicol efflux by the EmhABC efflux pump in strain cLP6a.

Presence of the divalent cation Mg^{2+} (5 mM) or low pH [5.8] in the efflux assay medium decreased the concentration of chloramphenicol associated to the cell pellet by 26 ± 4 % or 51 ± 6% respectively. In the presence of 5 mM $MgCl_2$, 1.23 ± 0.10 nmol g^{-1} of chloramphenicol was changed due to efflux, which significantly ($P < 0.009$) differs from the change by the control containing no $MgCl_2$ (Fig. 5.1b). Likewise, only 0.82 ± 0.13% nmol g^{-1} ($P < 0.007$) of chloramphenicol was changed due to efflux when transport assays were performed in phosphate buffer pH 5.8

compared to pH 7. Clearly, these results showed that the efflux of chloramphenicol by the EmhABC efflux pump is affected by the physico-chemical factors tested and in turn the concentration of chloramphenicol that accumulate in cLP6a cells and possibly its antimicrobial efficacy.

5.3.2 Physico-chemical factors induce *emhABC* expression

Because changes in the concentration of chloramphenicol effluxed by the EmhABC efflux pump under different conditions may be due to differential transcription of the *emhABC* genes, the effect of physico-chemical factors on *emhABC* transcription was determined. In a previous chapter (Chapter 2; Adebusuyi and Foght 2011), *emhABC* genes were shown to be induced in *P. fluorescens* strain cLP6a grown at 10°C and 35°C compared to a control at the optimum growth temperature of 28°C. Decreased activity of EmhABC in cLP6a cells grown at 10°C or 35°C was probably compensated by increased expression of the *emhABC*. Similarly, in this study the *emhABC* genes were induced ~8-fold in the presence of $MgCl_2$ and ~2-fold when the growth medium was slightly acidic [pH 5.8] compared to the control ($P < 0.003$; Fig. 5.2). There was no significant difference in the expression of *emhABC* genes in the presence of 5 mM $MgCl_2$ ($P > 0.1$) or at pH 5.8 ($P > 0.5$). This suggests that decreased EmhABC activity due to environmental conditions induces expression of the *emhABC* operon.

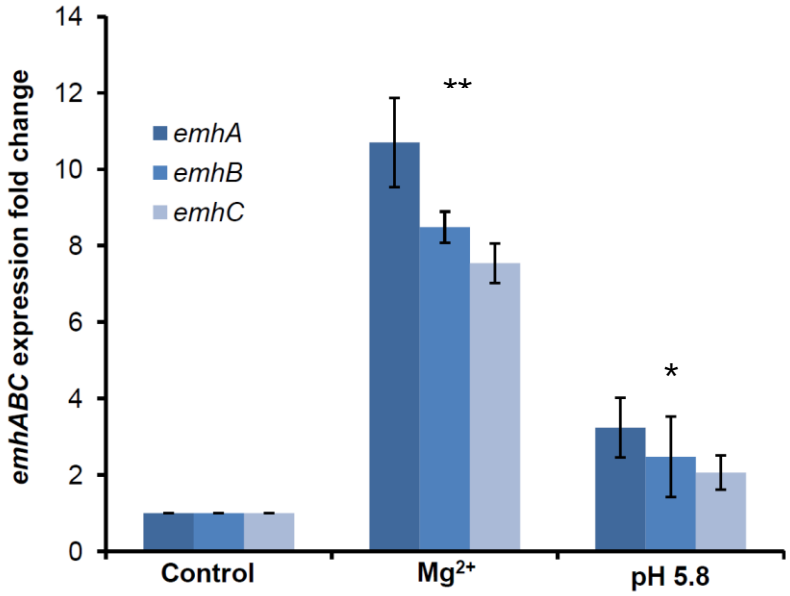

Figure 5.2 Expression of *emhABC* in *P. fluorescens* cLP6a grown to stationary phase at 28°C in the presence of 5 mM $MgCl_2$ or at pH 5.8, determined using RT-qPCR. The values shown are the fold-difference in expression of *emhABC* compared to expression levels in cells grown to stationary phase at 28°C (control = 1). Each bar represents the mean of two independent experiments performed in duplicate. Error bars, where visible, indicate the standard deviation. Asterisks indicate significant differences.

5.3.3 Sensitivity of *P. fluorescens* strains to antibiotics is influenced by physico-chemical factors

The effect of physico-chemical factors affecting chloramphenicol efflux on its antimicrobial efficacy was determined using the MIC assay. Sensitivity of strains cLP6a and cLP6a-1 to tetracycline, another substrate of the EmhABC efflux, and to streptomycin, a non-EmhABC substrate (Hearn et al. 2003) was also determined. As expected, strain cLP6a with active efflux was less sensitive to chloramphenicol and tetracycline

compared to strain cLP6a-1, which lacks EmhB, exhibiting ≥ 4-fold differences in the MICs of the antibiotics (Table 5.1). Decreasing the

Table 5.1 Antibiotic sensitivity of *P. fluorescens* strains cLP6a and cLP6a-1 incubated at different temperatures at pH 7, at 28°C at pH 5.8, and with or without MgCl2 at 28°C, pH 7. Antibiotic sensitivity was measured as minimum inhibitory concentration (MIC).Values shown are representative data from two independent experiments.

P. fluorescens				MIC (µg ml^{-1}) [a]		
strain	**Temperature**	**pH**	**$MgCl_2$**	**ST**	**CL**	**TE**
cLP6a	10°C	7	< 0.02 mM	8	128	4
	28°C	7	< 0.02 mM	4	16	2
	35°C	7	< 0.02 mM	4	8	0.5
	28°C	7	5 mM	8	64	16
	28°C	5.8	< 0.02 mM	4	16	0.125
cLP6a-1	10°C	7	< 0.02 mM	4	16	2
	28°C	7	< 0.02 mM	2	4	0.5
	35°C	7	< 0.02 mM	2	2	<0.063
	28°C	7	5 mM	4	8	4
	28°C	5.8	< 0.02 mM	2	<0.5	<0.063

[a], ST, streptomycin; CL, chloramphenicol; TE, tetracycline

incubation temperature to sub-optimal (10°C) caused significant decrease in the sensitivity of strains cLP6a and cLP6a-1 to chloramphenicol (4 fold increase in MIC; Table 5.1). Significant increase in resistance of strain cLP6a-1 (lacking EmhB) grown at 10°C to chloramphenicol and tetracycline is consistent with the presence of an alternative antibiotic efflux pump induced at this incubation temperature (Adebusuyi and Foght 2011). There was no significant difference in the MIC for tetracycline in strain cLP6a grown at 10°C compared to the control grown at 28°C. The

induction of the *emhABC* genes in cLP6a cells grown at 28°C may have decreased the sensitivity of this control culture to tetracycline. Growth at 35°C made *P. fluorescens* strains more sensitive to chloramphenicol and tetracycline. Increased membrane permeability of *P. fluorescens* strains grown at 35°C may explain the increased sensitivity of the strains at this temperature (Adebusuyi and Foght 2011). There was a 4- or 8-fold increase in MICs of chloramphenicol or tetracycline for strain cLP6a grown in TSB with added 5 mM $MgCl_2$. In contrast there was no significant difference in the sensitivity to chloramphenicol of strain cLP6a -1 grown in the presence of $MgCl_2$ compared to the control, whereas an 8-fold increase in tetracycline MIC was obtained for strain cLP6a-1 grown in the presence of Mg^{2+}. The decreased sensitivity of strains cLP6a and cLP6a-1 to chloramphenicol and tetracycline in the presence of Mg^{2+} may be explained as a combination of increased expression of the EmhABC efflux pump (Fig. 5.2) and the protection of the cell membrane by the divalent cation. Divalent cations such as Ca^{2+} and Mg^{2+} stabilize the cell membrane by interacting with negatively charged phospholipid polar head groups (Denich et al. 2003; Lee et al. 2007). Decreasing pH of the growth medium from ~7 to 5.8 had no effect on the sensitivity of strain cLP6a to chloramphenicol but did make the cells considerably (8-fold) more sensitive to tetracycline (Table 5.1). The overall trend observed for the MIC assays was that decrease in the efflux of chloramphenicol caused by physico-chemical factors appears to be offset by increased *emhABC*

expression. Under the test conditions, there was no significant difference in the susceptibility of *P. fluorescens* strains to streptomycin, which is not a substrate of EmhABC but a ribosome-interacting antibiotic (Busse et al. 1992; Hearn et al. 2003).

5.3.4 EmhABC activity mitigates the deleterious effect of chloramphenicol on cLP6a membrane

To determine why tetracycline and not chloramphenicol induces transcription of *emhABC*, the effect of these antibiotics on cell membrane permeability of strains cLP6a and cLP6a-1 was examined. Fatty acid methyl ester (FAME) analysis was used to determine the effect of antibiotics on membrane fatty acid composition. Cell membrane integrity was determined using a permeability assay performed with propidium iodide (PI, a fluorescent reporter molecule that cannot cross intact cell membranes) and cetyltrimethylammonium bromide (CTAB, a cationic surfactant that can permeabilize bacterial cell membranes and thus increase PI penetration), as described in Chapter 2 (Adebusuyi and Foght 2011). The permeability index of strains cLP6a and cLP6a-1 grown in the presence of chloramphenicol or tetracycline was determined as defined by Niven and Mulholland (1998).

Chloramphenicol caused only minor changes in the membrane fatty acid (FA) content of strain cLP6a (Table 5.2) and the membrane integrity of cLP6a was not affected compared to the control (Fig. 5.3). Conversely, a major change in the cell membrane FA and increased membrane

Table 5.2 FA composition of *P. fluorescens* strains cLP6a and cLP6a-1 grown to stationary phase at 28°C in the absence or presence of antibiotics (tetracycline or chloramphenicol, at ¼ MIC). FA contents are expressed as the mean weight % of total FA detected in two measurements; deviation from the mean was typically <1% and at most 3% of the measured values.

		FA classes as % of total FA detected		
***P. fluorescens* strains**	**Growth conditions**	**Total Saturated FAs**	**Total Unsaturated FAs**	**Total Cyclo-FAs**
cLP6a	28°C	42.1	14.9	43.0
	28°C with chloramphenicol[a]	44.0	12.8	43.2
	28°C with tetracycline[a]	42.8	23.1	34.1
cLP6a-1	28°C	41.4	16.3	42.3
	28°C with chloramphenicol	43.2	33.8	22.9
	28°C with tetracycline	42.2	25.0	32.7

[a], Data from Adebusuyi and Foght (2011)

permeability (~2.5-fold) was observed in the EmhB mutant strain cLP6a-1 grown in the presence of chloramphenicol. The total unsaturated FA in strain cLP6a-1 increased, whereas the percentage of cyclopropane FA decreased in the presence of chloramphenicol (Table 5.2). This is similar to the effect of naphthalene on strain cLP6a-1 (Chapter 4) and consistent with the down-regulation of the cyclopropane synthase in *P. putida* when exposed to chloramphenicol (Fernández et al. 2012). On the other hand, tetracycline caused an increase in total unsaturated FAs and

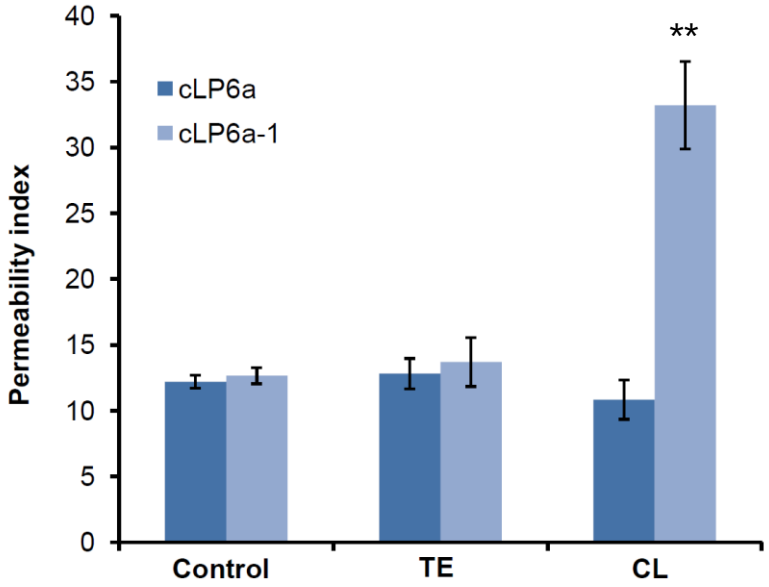

Figure 5.3 The permeability index of *P. fluorescens* strains cLP6a and cLP6a-1 grown to stationary phase at 28°C with or without ¼ MIC of chloramphenicol or tetracycline. Each bar represents the mean of three culture sub-samples. Asterisks indicate significant differences.

decrease in cyclopropane FAs in strains cLP6a and cLP6a-1 even though the permeability indices of both strains were similar to controls (Fig. 5.3). These results indicate that chloramphenicol affects the cell membrane in a different manner than tetracycline. The EmhABC efflux pump mitigated the permeabilizing action of chloramphenicol since strain cLP6a with a functional EmhABC was not affected whereas the EmhB disruptant mutant, cLP6a-1 had compromised membrane integrity.

5.4 Discussion

The bacterial cell membrane delineates bacteria cells from the environment, regulates movement of solutes in and out of the cell, and

stabilizes the structure of membrane-embedded proteins (Denich et al. 2003). Besides the changes in the cell membrane composition to prevent toxic hydrophobic chemicals from accumulating in the membrane (Denich et al 2003), RND efflux pumps, which are embedded in the cell membrane of most Gram-negative bacteria, are also engaged. This family of efflux pumps extrude mostly hydrophobic compounds (Martinez et al. 2009) that would otherwise accumulate and cause damage to the cell membrane. RND efflux pumps have been studied mainly because of their involvement in antibiotic resistance (Nikaido 1996; Zgurskaya and Nikaido 2000) and most studies are based on identification and characterization of new efflux pumps or identification of substrates that induce expression of an efflux pump (Hearn et al. 2003; Kieboom et al. 1998). This focus gives little insight to why efflux is important to bacterial survival in the natural environment, as suggested by their presence and conservation in most Gram-negative bacteria (Poole 2008).

Results presented here show that three different types of physico-chemical factors (temperature, pH and divalent cation) that affect the cell membrane physiology also influence the efflux and efficacy of chloramphenicol, as shown by the results of the rapid efflux and MIC assays. These physico-chemical factors are inducers of the *emhABC* genes that encode the EmhABC efflux pump. Deviation in incubation temperature from the optimum for a bacterial species is known to cause stress to the cell membrane and induction of *emhABC* expression

(Adebusuyi and Foght 2011; Denich et al. 2003). Induction of *emhABC* that encodes the proton-dependent EmhABC efflux pump (Hearn et al. 2003) by lower pH was expected and was observed. However, induction of *emhABC* expression by the divalent cation Mg^{2+}, which stabilizes bacterial cell membranes, was unexpected. Divalent cations previously have been associated with bacterial resistance to antibiotics and survival under harsh growth conditions (Lee et al 2007; Mao et al. 2001; Zgurskaya and Nikaido 1999). Although stabilization of the bacterial cell membrane has been the only mechanism used to explain the role of divalent cations in bacterial survival and antibiotic resistance, divalent cation induction of substrate efflux by RND efflux pumps (Zgurskaya and Nikaido 1999) or association of cations with efflux pump function (Mao et al. 2001) hint at an additional mechanism. This additional mechanism may be the induction (whether direct or indirect) of RND efflux pump genes as supported by the results obtained in this study.

The results of the MIC assay suggests that another efflux pump may be involved in chloramphenicol and tetracycline transport in both strains cLP6a and cLP6a-1 cells grown at 10°C as previously suggested in Chapter 2 (Adebusuyi and Foght 2011). Also important is the fact that efflux of chloramphenicol was very low in cLP6a cells grown at 35°C but had only slight effect on its efficacy at this temperature, which may be a complementary effect of the over-expression of the *emhABC* genes at this growth temperature (Chapter 2; Adebusuyi and Foght 2011).

Although chloramphenicol and tetracycline have been reported to induce RND efflux pumps in strains of *P. putida* (Teran et al. 2003) and *P. aeruginosa* (Jeannot et al. 2005; Poole 2008), other efflux pumps such as the MexAB-OprM in *P. syringiae* (Vargas et al. 2011) and EmhABC in strain cLP6a (Adebusuyi and Foght 2011) are not induced by chloramphenicol. Notably, experiments determining induction of these RND efflux pumps by chloramphenicol were performed under different conditions: induction seems to be dependent on the growth phase (Adebusuyi and Foght 2011; Jeannot et al. 2005; Vargas et al. 2011) of the cells used for gene expression assays. Tetracycline caused major changes in the membrane FA composition of *P. fluorescens* in both the presence and absence of the EmhABC, whereas the effect of chloramphenicol on the membrane was only apparent in the absence of EmhABC activity. This suggests that EmhABC helps counter the membrane-damaging effect of chloramphenicol and that the mode of action of chloramphenicol and tetracycline may be the determining factor for *emhABC* induction by tetracycline but not chloramphenicol. Although both chloramphenicol and tetracycline inhibit protein synthesis, they bind to different sites of the ribosome and possibly trigger specific bacterial responses (Fajardo and Martínez 2008). Moreover, tetracycline uptake and efflux is dependent on the presence of Mg^{2+} because it is transported across the membrane of Gram-negative bacteria such as *Escherichia coli* as a Mg^{2+}-chelate complex (McMurry et al. 1980; Yamaguchi et al. 1990).

Since the results of this study showed that Mg^{2+} is an inducer of *emhABC*, the induction of *emhABC* and probably other efflux pump genes by tetracycline (Adebusuyi and Foght 2011; Jeannot et al. 2005) may be due to increased intracellular concentration of Mg^{2+} as a consequence of tetracycline transport.

In summary, it is shown that the physico-chemical factors of temperature, pH and divalent cation affect the efflux of chloramphenicol in *P. fluorescens* cLP6a because they induce the expression of *emhABC*. This emphasizes the point that efflux pumps play other physiological roles in Gram-negative bacteria beyond antibiotic efflux (Adebusuyi and Foght 2011; Poole 2008). Knowledge of the other physiological roles is desirable to understand and combat antibiotic resistance. In clinical settings, these factors should be studied before determining the dosage of an antibiotic to be used for treating a bacterial infection. In addition, the action of an antibiotic on the cell envelope should be taken into account when developing new strategies to combat bacterial resistance.

5.5 Literature cited

Aakra A, Vebø H, Indahl U, Snipen L, Gjerstad Ø, Lunde M, Nes IF (2010) The response of *Enterococcus faecalis* V583 to chloramphenicol treatment. Int J Microbiol 2010:1-8 doi:10.1155/2010/483048

Abdel-Sayed S (1987) Transport of chloramphenicol into sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa*. J Antimicrob Chemother 19:7-20

Adebusuyi AA, Foght JM (2011) An alternative physiological role for the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a. BMC

Microbiol 11:252 doi:10.1186/1471-2180-11-252

Bugg T, Foght JM, Pickard MA, Gray MR (2000) Uptake and active efflux of polycyclic aromatic hydrocarbons by *Pseudomonas fluorescens* LP6a. Appl Environ Microbiol 66**:**5387-5392

Burns JL, Smith AL (1987) Chloramphenicol accumulation by *Haemophilus influenza*. Antimicrob Agents Chemother 31:686-690

Busse H-J, Wöstmann C, Bakker EP (1992) The bactericidal action of streptomycin: membrane permeabilization caused by the insertion of mistranslated proteins into the cytoplasmic membrane of Escherichia coli and subsequent caging of the antibiotic inside the cells due to degradation of these proteins. J Gen Microbiol 138:551-561

Denich TJ, Beaudette LA, Lee H, Trevors JT (2003) Effect of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. J Microbiol Meth 52:149-182

Drainas D, Kalpaxis DL, Coutsogeorgopoulos C (1987) Inhibition of ribosomal peptidyltransferase by chloramphenicol: kinetic studies. Eur J Biochem 164:53-68

Fajardo A, Martínez JL (2008) Antibiotics as signals that trigger specific bacterial responses. Curr Opinion Microbiol 11:161-167

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

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353-366

Hearn EM, Dennis JJ, Gray MR, Foght JM (2003) Identification and characterization of the *emh*ABC efflux system for polycyclic aromatic hydrocarbons in *Pseudomonas fluorescens* cLP6a. J Bacteriol 185:6233-6240

Jeannot K, Sobel ML, Garch FE, Poole K, Plesiat P (2005) Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. J Bacteriol 187:5341-5346

Kieboom J, Dennis JJ, de Bont JAM, Zylstra GJ (1998) Identification and molecular characterization of an efflux pump involved in *Pseudomonas putida* S12 solvent tolerance. J Biol Chem 273:85-91

Lee L-F, Chen Y-J, Kirby R, Chen C, Chen CW (2007) A multidrug efflux system is involved in colony growth in *Streptomyces lividans*. Microbiol 153:924-934

Mao M, Warren MS, Lee A, Mistry A, Lomovskaya O (2001) MexXY-OprM efflux pump is required for antagonism of aminoglycosides by divalent cations in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 45:2001-2007

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

Maviglia R, Nestorini R, Pennisi M (2009) Role of old antibiotics in multidrug resistant bacterial infections. Curr Drug Targets 10:895-905

McMurry L, Petrucci Jr. RE, Levy SB (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in *Escherichia coli*. PNAS 77:3974-3977

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

Niven GW, Mulholland F (1998) Cell membrane integrity and lysis in *Lactococcus lactis*: the detection of a population of permeable cells in post-logarithmic phase cultures. J Appl Microbiol 84:90-96

Piddock LJV (2006) Multidrug-resistance efflux pumps – not just for resistance. Nat Rev Microbiol 4**:**629-636

Poole K (2008) Bacterial multidrug efflux pumps serve other functions. Microbe 3:179-185

Schwarz S, Kehrenberg C, Doublet B, Cloeckaert A (2004) Molecular basis of bacterial resistance to chloramphenicol and florfenicol. FEMS Microbiol Revs 28:519-542

Silby MW, Cerdeno-Tarraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, Zhang X-X, Moon CD, Gehrig SM, Godfrey SAC, Knight CG, Malone JG, Robinson Z, Spiers AJ, Harris S, Challis

GL, Yaxley AM, Harris D, Seeger K, Murphy L, Rutter S, Squares R, Quail MA, Saunders E, Mavromatis K, Brettin TS, Bentley SD, Hothersall J, Stephens E, Thomas CM, Parkhill J, Levy SB, Rainey PB, Thomson NR (2009) Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*. Genome Biol 10:R51

Teran W, Felipe A, Segura A, Rojas A, Ramos J-L, Gallegos M-T (2003) Antibiotic-dependent induction of *Pseudomonas putida* DOT T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. Antimicrob Agents Chemother 47:3067-3072

Vargas P, Felipe A, Michán C, Gallegos M-T (2011) Induction of *Pseudomonas syringae* pv. Tomato DC3000 MexAB-OprM multidrug efflux pump by flavonoids is mediated by the repressor PmeR. Mol Plant-Microbe Interac 24:1207-1219

Wiegand I, Hilpert K, Hancock REW (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protocols 3**:**163-175

Xaplanteri MA, Andreou A, Dinos GP, Kalpaxis DL (2003) Effect of polyamines on the inhibition of peptidyltransferase by antibiotics: revisiting the mechanism of chloramphenicol action. Nucleic Acids Res 31:5074-5083

Yamaguchi A, Udagawa T, Sawai T (1990) Transport of divalent cations with tetracycline as mediated by the Transposon Tn10-encoded tetracycline resistance protein. The J Biol Chem 265:4809-4813

Zgurskaya HI, Nikaido H (1999) Bypassing the periplasm: Reconstitution of the AcrAB multidrug efflux pump of *Escherichia coli*. PNAS 96:7190-7195

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

6. Synthesis, conclusions and future perspective

6.1 Synthesis

The EmhABC efflux pump is a “win, lose” mechanism in *Pseudomonas fluorescens* LP6a. In Chapters 2, 4 and 5, the function of EmhABC to enhance LP6a survival in the presence of environmental stressors was highlighted. The function of the EmhABC pump is linked with the modulation of membrane fatty acids (FA), confirming an alternative “authentic” role for this pump in LP6a (Chapters 2 and 4). Efflux of replaced membrane FAs seems impossible based on the evidence that substrates are captured in the periplasmic domain of resistance-nodulation-division (RND) efflux pumps (Murakami et al. 2006; Sennhauser et al. 2009). However, FAs are hydrophobic compounds like many identified substrates of RND efflux pumps, and efflux of PAHs (Hearn et al. 2003; 2006) as well as membrane lipids such as hopanoids (Doughty et al. 2011) that partition into the bacterial inner membrane supports the possibility of replaced membrane FA efflux. In addition to tetracycline and temperature, other physico-chemical factors such as pH and Mg^{2+} are also inducers of EmhABC efflux pump (Chapter 5) supporting the perspective that efflux pumps play other physiological roles in bacteria beyond antibiotic resistance (Chapter 1; Adebusuyi and Foght 2011; Kumar and Schweizer 2011; Piddock 2006).

The presence or over-expression of RND efflux pumps increase virulence and bacterial resistance to antimicrobials but come with a fitness cost (Alonso et al. 2004; Hirakata et al. 2002). While fitness cost was thought to be a result of metabolic burden placed on the cells by the over-expression of efflux pump genes, Olivares et al. (2012) recently showed that over-expression of MexEF-OprN in *P. aeruginosa* did not cause any growth impairment in this strain but produced other physiological changes in the cells. Here, consistent with the finding of Olivares et al. (2012), it was shown that the fitness cost may be impairment of other cell functions due to membrane stress and not necessarily the result of a metabolic burden of over-expressing the efflux pump (Chapter 2; Adebusuyi and Foght 2011). Impaired growth resulting from increased incubation temperature was suppressed by the over-expression of the EmhABC efflux pump. This suggests that over-expression of efflux pumps is a response to improve the survival of bacteria in the presence of environmental stressors and may not constitute a fitness cost to the cells.

While local and global regulators may be involved in the transcriptional regulation of RND efflux pumps, only a few effector molecules for global regulators such as SoxS have been identified (Chapter1; Grkovic et al. 2002). This study hints at the likely involvement of environmental parameters as the effectors that trigger local or global regulatory responses, although this proposal is untested.

In Chapters 3 and 4, the presence of RND efflux pumps in hydrocarbon-degrading bacteria was shown for the first time to slow down, accelerate or complicate bioremediation processes (e.g., accumulation of toxic metabolite in the environment; Chapter 3). This indicates that the presence of a RND efflux pump may be an important feature to consider when selecting bacterial strains for use in bioremediation or biocatalysis of hydrocarbon compounds. This supports the idea that there are both advantages and disadvantages associated with the presence of RND efflux pumps in Gram-negative bacteria.

For human beings, the discovery of bacterial RND efflux was initially perceived as a curse because they are major determinants of bacterial virulence and antibiotic resistance (e.g., review by Piddock 2006). Increases in antimicrobial resistance complicate antibiotic treatment of bacterial diseases because certain members of most of the known classes of antibiotics are substrates of RND efflux pumps. Various efflux pump inhibitors (EPIs) such as Phenylalanyl-Arginyl-β-Naphthylamine (PAβN) have been suggested for use in combination therapy to inactivate efflux and allow the antibiotic to reach inhibitory concentrations in the cells (Kumar and Schweizer 2005; Pagés and Amaral 2009; Renau et al. 1999). PAβN and other EPIs have been shown to block the efflux of levofloxacin, a fluoroqinolone and other antimicrobials in *P. aeruginosa*, *E*. *coli* and *Salmonella enterica* (Pagés and Amaral 2009; Renau et al. 1999). The challenges associated with the use of EPIs are that resistance may be

developed towards them as well. For instance, PAβN is a substrate of the MexB efflux pump of *P. aeruginosa* (Lomovskaya et al. 2007). In addition, EPIs may be toxic to and inhibit eukaryotic transporters that are structurally and functionally similar to bacterial efflux pumps (Kumar and Schweizer 2005). Research on efflux pumps has dwelt on the negative aspects of their presence in bacteria as it affect humans, but they can be beneficial as well to humans if their function is exploited appropriately (see section 6.3).

6.2 Conclusions

The authentic role of the EmhABC efflux pump in *P. fluorescens* LP6a is adaptation to variation in environmental factors (both physical and chemical) by mitigating membrane damage (Fig. 6.1). In the case of chemical membrane stressors, the EmhABC efflux pump often takes care of both the stressor and the consequence of the stress on the membrane. Since environmental factors are cues for the induction of RND efflux pumps, new antimicrobial drugs should be designed and strains to be used for microbial remediation or biocatalysis of hydrocarbon compounds should be selected with consideration of the environmental factors affecting the bacterial species to be eradicated or used.

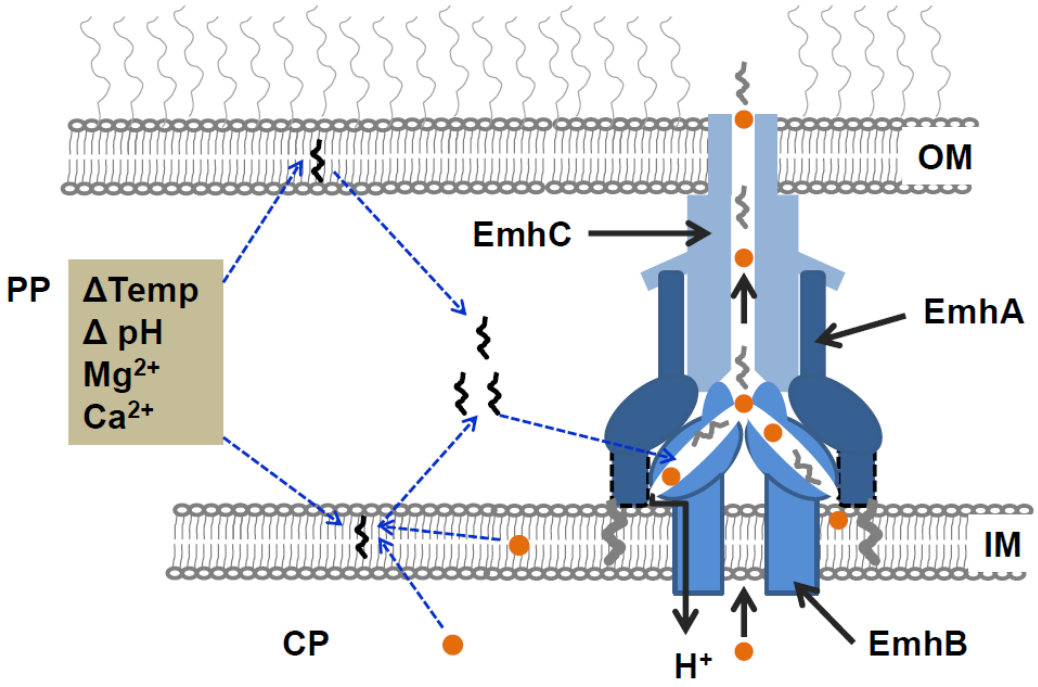

Figure 6.1 Proposed model for induction and substrate transport of *P. fluorescens* LP6a EmhABC efflux pump. Membrane modification in the presence of physico-chemical factors triggers the efflux of released and/or damaged fatty acids (zigzag line), constituents of the inner membrane (IM) and/or outer membrane (OM) and sometimes the membrane-damaging agents themselves (antimicrobial agents, polycyclic aromatic hydrocarbons, orange circle). CP, cytoplasm; PP, periplasm; H^+, proton.

6.3 Future perspective

There are potential benefits (clinical and biotechnological) that can be gained from the knowledge of RND efflux pumps. As stated in previous chapters, RND efflux pumps transport a variety of structurally diverse compounds such as antibiotics, PAHs, solvents, fatty acids, and metabolites. Discussed below is how the knowledge of efflux pump substrates and inducers can be utilized for beneficial purposes in the future.

6.3.1 The possibility to ‘tweak’ efflux pumps to modulate substrate specificity

The periplasmic (Elkins and Nikaido 2002; Mao et al. 2002), transmembrane (Middlemiss and Poole 2004) and outer membrane protein docking (Hearn et al. 2006) domains and the central cavity (Yu et al. 2003) of RND efflux pumps are responsible for substrate recognition, specificity and translocation (Tikhonova et al. 2002; Murakami et al. 2004). It is possible to tweak bacterial efflux pumps to improve affinity for substrates (Hearn et al. 2006) or to enable the efflux of a desired product. This type of efflux pump is referred to as a ‘designer’ pump (Dunlop 2011) and may be the focus of research for the next decade.

Designer efflux pumps can be achieved by site-directed mutagenesis of amino acid residues located in critical domains of a RND efflux pump to increase or decrease affinity for a particular substrate (Hearn et al. 2006; Middlemiss and Poole 2004; Murakami et al. 2006; Sennhauser et al. 2009). It is also possible to design chimeric pumps with RND efflux components from different organisms (Tikhonova et al. 2002). This may be useful for industrial fermentation processes in which certain bacterial species are safer to work with. The RND efflux pump components of another strain with specificity for a targeted compound can be combined with the outer membrane protein and periplasmic adaptor protein of the preferred bacterial species. Alternatively, the complete RND efflux pump system from a different bacterial strain with the required

specificity can be introduced into a production strain. The ability to engineer efflux pumps opens new opportunities to improve microbial production of platform chemicals, secondary metabolites and biofuels (Dunlop et al. 2011) and also to improve or speed up biocatalysis and bioremediation of hydrocarbon compounds.

6.3.2 Efflux pumps and biofuels

With the current push for green energy (Dunlop 2011), one of the areas of RND efflux pump research for beneficial purposes is the production of biofuels. Recently efflux pumps have been engineered to efflux certain biofuels (Dunlop 2011) with more ongoing work to expand the possibility of using this strategy to generate more fuels synthesized by microbes. In the production of second-generation biofuels such as 2-butanol, terpenoids and higher lipids from lignocellulosic feedstocks, toxicity of feedstock components such as organic acids and phenolic compounds and end-products (Fischer et al. 2008; Sakai et al. 2007) is one of the major limitations to commercial production of these fuels by microbes. To achieve high biofuel productivity, microbial producing strains must be able to tolerate toxic compounds during fermentation. Recently, Dunlop et al. (2011) heterologously expressed HAE1 efflux pumps in an *E. coli* host and found that some of these efflux pumps improved tolerance to feedstock constituents and products, and thereby increased production of biofuels such as longer chain alcohols, alkanes, alkenes and cyclic hydrocarbons (Dunlop 2011; Dunlop et al. 2011).

6.3.3 Development of antimicrobial drugs and other mechanisms of treating bacterial infections

Membrane stress has been highlighted in this study as the main trigger for induction and function of the EmhABC efflux pump (Chapter 1; Adebusuyi and Foght 2011) and likely other bacterial efflux pumps as well (Fraud et al. 2008). This suggests that the antimicrobial efficacy of a compound may be improved if it has little to no effect on the membrane of pathogenic bacteria. In the future, drugs may be designed to have no effect on bacterial membrane. This may be a good start in reducing the occurrence of antimicrobial resistance due to efflux and/or membrane modification. Other ways to treat bacterial infections without the use of drugs may be by modulating the environment of bacterial cells.

6.3.4 Efflux pumps in anaerobes

Anaerobes are a major group of bacteria involved in *in situ* remediation of hydrocarbon pollutants in oil spills and oil sand tailings ponds (Foght 2008). Genome sequences of some of the anaerobic species such as *Desulfatibacillum alkenivorans* isolated from polluted environments revealed the presence of RND efflux pumps in these species (Callaghan et al. 2012). These anaerobes are exposed to various toxic chemicals in their environment but there have been no studies to determine how they survive high concentrations of toxic chemicals. Future studies to determine the role of RND efflux pumps in anaerobes isolated from polluted sites may be worthwhile as these efflux pumps may be

engineered into industrial strains to improve tolerance to toxic compounds, thereby increasing product yield.

6.4 Literature cited

Adebusuyi AA, Foght JM (2011) An alternative physiological role for the EmhABC efflux pump in *Pseudomonas fluorescens* cLP6a. BMC Microbiol 11:252

Alonso A, Rojo F, Martinez JL (1999) Environmental and clinical isolates of *Pseudomonas aeruginosa* show pathogenic and biodegradative properties irrespective of their origin. Environ Microbiol 1:421-430

Callaghan AV, Morris BEL, Pereira IAC, McInerney MJ, Austin RN, Groves JT, Kukor JJ, Suflita JM, Young LY, Zylstra GJ, Wawrik B (2012) The genome sequence of *Desulfatibacillum alkenivorans* AK-01: a blueprint for anaerobic alkane oxidation. Environ Microbiol 14:101-113

Doughty DM, Coleman ML, Hunter RC, Sessions AL, Summons RE, Newman DK (2011) The RND-family transporter, HpnN, is required for hopanoids localization to the outer membrane of *Rhodopseudomonas palustris* TIE-1. PNAS 108:E1045-E1051

Dunlop MJ (2011) Engineering microbes for tolerance to next-generation biofuels. Biotechnol Biofuels 4:32

Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Syst Biol 7:487

Elkins CA, Nikaido H (2002) Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops. J Bacteriol. 184:6490-6498

Fischer CR, Klein-Marcuschamer D, Stephanopoulos G (2008) Selection and optimization of microbial hosts for biofuels production. Met Engineer 10:295-304

Foght JM (2008) Anaerobic biodegradation of aromatic hydrocarbons: pathways and prospects. J Mol Microbiol Biotechnol 15:93-120

Foght JM, Westlake DWS (1996) Transposon and spontaneous deletion mutants of plasmid-borne genes encoding polycyclic aromatic hydrocarbon degradation by a strain of *Pseudomonas fluorescens*. Biodegradation 7:353-366

Fraud S, Campigotto AJ, Chen Z, Poole K (2008) MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*: involvement in chlorhexidine resistance and induction by membrane-damaging agents dependent upon the AlgU stress response factor. Antimicrob Agents Chemother 52:4478-4482

Grkovic S, Brown MH, Skurray RA (2002) Regulation of bacterial drug export systems. Microbiol Mol Biol Rev 66:671-701

Hearn EM, Gray MR, Foght JM (2006) Mutations in the central cavity and periplasmic domain affect efflux activity of the resistance-nodulation-division pump EmhB from *Pseudomonas fluorescens* cLP6a. J Bacteriol 188**:**115-123

Hirakata Y, Srikumar R, Poole K, Gotoh N, Suematsu T, Kohno S, Hancock RE, Speert DP (2002) Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. J Exp Med 196:109-118

Kumar A, Schweizer HP (2005) Bacterial resistance to antibiotics: Active efflux and reduced uptake. Adv Drug Del Revs 57:1486-1513

Kumar A, Schweizer HP (2011) Evidence of MexT-independent overexpression of MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa* in presence of metabolic stress. PLoS ONE 6(10):e26520 doi:10.1371/journal.pone.0026520

Lomovskaya O, Zgurskaya HI, Totrov M, Watkins WJ (2007) Waltzing transporters and ‘the dance macabre’ between humans and bacteria. Nat Rev Drug Discov 6:56-65

Mao Q, Warren MS, Black DS, Satou T, Murata T, Nishino T, Gotoh N, Lomovskaya O (2002) On the mechanism of substrate specificity by resistance nodulation division (RND)-type multidrug resistance pumps: the large periplasmic loops of MexD from *Pseudomonas aeruginosa* are involved in substrate recognition. Mol Microbiol 46:889-901

Middlemiss JK, Poole K (2004) Differential impact of MexB mutations on substrate selectivity of the MexAB-OprM multidrug efflux pump of *Pseudomonas aeruginosa*. J Bacteriol 186:1258-1269

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

Murakami S, Tamura N, Saito A, Hirata T, Yamaguchi A (2004) Extramembrane central pore of multidrug exporter AcrB in *Escherichia coli* plays an important role in drug transport. J Biol Chem 279:3743-3748

Olivares J, Alverez-Ortega C, Linares JF, Rojo F, Kohler T, Martinez JL (2012) Overproduction of the multidrug efflux pump MexEF-OprN does not impair *Pseudomonas aeruginosa* fitness in competition tests, but produces specific changes in bacterial regulatory networks. Environ Microbiol doi:10.1111/j.1462-2920.2012.02727.x

Pages J-M, Amaral L (2009) Mechanisms of drug efflux and strategies to combat them: challenging the efflux pump of Gram-negative bacteria. Biochim Biophy Acta 1794:826-833

Piddock LJV (2006) Multidrug-resistance efflux pumps – not just for resistance. Nature Rev Microbiol 4:629-636

Renau TE, Leger R, Flamme EM, Sangalang J, She MW, Yen R, Gannon CL, Griffith D, Chamberland S, Lomovskaya O, Hecker SJ, Lee VJ, Nakayama TO (1999) Inhibitors of efflux pumps in *Pseudomonas aeruginosa* potentiate the activity of the fluoroquinolone antibacterial levofloxacin. J Med Chem 42:4928-4931

Sakai S, Tsuchida Y, Okino S, Ichihashi O, Kawaguchi H, Watanabe T, Inui M, Yukawa H (2007) Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested *Corynebacterium glutamicum* R. Appl Environ Microbiol 73:2349-2353

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

Tikhonova EB, Wang Q, Zgurskaya HI (2002) Chimeric analysis of the multicomponent multidrug efflux transporters from Gram-negative bacteria. J Bacteriol 184:6499-6507