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Régulation de la perméabilité membranaire chez les bactéries à Gram négatif et la relation avec la sensibilité aux antibiotiques

T H È S E

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Par M Alexander MOLITOR
UMR-MD 1
Transporteurs Membranaires,
Chimiorésistance et Drug Design

Date et lieu de naissance: 16.08.1979 à Munich, ALLEMAGNE

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Membres du Jury de la Thèse :

Prof Jean-Louis MÈGE	Président du Jury
Prof Marie Helene NICOLAS-CHANOINE	Rapporteur
Dr Jean-Philippe LAVIGNE	Rapporteur
Prof Mathias WINTERHALTER	Examinateur
Dr Jean-Marie PAGÈS	Examinateur
Dr Anne DAVIN-REGLI	Directrice de thèse

pour ma mère...

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Science is organized common sense where many
a beautiful theory was killed by an ugly fact

- *Thomas Henry Huxley*

Iucundi acti labores

- Cicero

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LIST OF ABBREVIATIONS

AAC	Aminoglycoside-N-acetyltransferases
ABC	ATP-binding cassette
ACC	Ambler class C-1
ADH	Arginine dihydrolase
AMPC	beta-lactamase type C
ANT	Aminoglycoside-O-nucleotidetransferases
APH	Aminoglycoside-O-phosphotransferases
BES	Brazil extended spectrum beta lactamase
BLI	Beta-lactamase inhibitors
CAT	Chloramphenicol-acetyltransferase
CFU	Colony forming units
CMY	Cephamycinase
CTX	Cefotaximase
DHPS	Dihydropteroate synthetase
DNA	Deoxyribonucleic acid
EDP	Energy dependant phase
ESBL	Extended spectrum beta-lactamase
FOX	Cefoxitinase
GES	Guiana extended-spectrum beta-lactamase
GYR	Gyrase
IBC	Integron associated beta lactamase class C
ICU	Intensive care units
IPTG	Isopropyl-beta-D-thiogalactopyranosid
IRT	Inhibitor resistant TEM
KPC	*Klebsiella pneumoniae* carbapenemase
LDC	Lysine decarboxylase
LPS	Lipopolysaccharide
MAR	Multiple antibiotic resistance
MATE	Multidrug and toxic compound extrusion

MDO	Membrane derived oligosaccharides
MDR	Multidrug resistance
MFS	Major facilitator superfamily
MH	Muller Hinton
MIC	Minimum inhibitory concentration
NAG	N-acetyl glucosamine
NAM	N-acetyl muramic acid
ODC	Ornithine decarboxylase
OMP	Outer membrane protein
OXA	Oxacilinase
PAP	Periplasmic adaptor proteins
PBP	Penicillin binding region
PMQR	Plasmid mediated quinolone resistance
QRDR	Quinolone resistance determinant region
QNR	Quinolone resistant determinant
RAM	Resistance antibiotic multiple
RNA	Ribonucleic acid
RND	Resistance nodulation division
ROB	Right origin binding protein
SDS	Sodium dodecylsulfate
SFO	*Serratia fonticola* beta-lactamase
SHV	Sulfhydryl reagent variable
SMR	Staphylococcal multi-resistance
SOX	Superoxide regulon
TEM	beta-lactamase named after first patient infected, Temoneira
TRNA	Transfer RNA
VIM	Verona integron-encoded metallo-beta-lactamase
WT	Wildtype

CHAPTER I:

RECAPITULATIF - SUMMARY

1.1 Récapitulatif

Enterobacter aerogenes est une Enterobactérie caractérisée par sa forte implication depuis une quinzaine d'années dans des infections nosocomiales chez des patients immuno-compromis en réanimation, comme chez les personnes âgées en institution ou hospitalisées et par son phénotype de résistance multiple aux antibiotiques. Les patients acquièrent la bactérie soit de leur voisinage, par l'intermédiaire des différentes manipulations du personnel soignant, soit de la flore entérique endogène, après sélection de souches résistantes au cours de l'antibiothérapie. Les bactéries appartenant au genre *Enterobacter* sont largement distribuées dans l'environnement au niveau des eaux et des sols. *E. aerogenes* est, par ailleurs, un commensal du tube digestif de l'homme.

Son épidémiologie en France est marquée par la dissémination d'un clone majoritaire hébergeant un plasmide porteur du gène codant pour la beta-lactamase à spectre élargi de type TEM-24 et exceptionnellement d'autres types de BLSEs. Cette BLSE est souvent associée, chez les isolats cliniques, à une céphalosporinase de haut niveau d'origine chromosomique. Ainsi doté de mécanismes enzymatiques efficaces, il est vraisemblable que l'importante dissémination du clone prévalent de *E. aerogenes* a résulté de sa bonne adaptation à la pression de sélection des antibiotiques ainsi qu'à l'environnement hospitalier.

L'imipénème est la seule beta-lactamine qui reste encore active sur ces souches, de ce fait, sa prescription a augmenté dans cette situation et l'inéluctable adaptation de la bactérie aux agents antimicrobiens a rapidement permis, dans ce contexte, l'émergence de souches devenues résistantes aux carbapénèmes chez les patients traités. Le plus souvent, on observe une résistance à l'imipénème associée à la perte d'une ou plusieurs protéines membranaires, telle la porine majoritaire Omp36. Cette résistance par imperméabilité est un mécanisme d'adaptation rapide, faisant intervenir simultanément ou indépendamment la mise en place d'un efflux actif par surexpression des composants de la pompe AcrAB-TolC.

La modulation de la perméabilité membranaire est sous la dépendance de plusieurs systèmes génétiques, éléments fonctionnant de manière combinée sur des cibles potentiellement identiques par reconnaissance de motifs similaires au niveau des promoteurs des gènes. Il en découle la mise en place d'une cascade de régulation aboutissant à l'activation ou l'inhibition de nombreux gènes et en particulier, les gènes intervenant dans l'expression des porines et des constituants des pompes d'efflux.

Récemment le régulon *marRAB* et le gène *ramA* ont été caractérisés chez *E. aerogenes.* Ces systèmes appartiennent à une même famille d'activateurs transcriptionels, fonctionnant sous la dépendance de répresseurs spécifiques, d'activateurs et d'inducteurs. L'objectif de notre travail était d'identifier un répresseur pour RamA et une fois identifié et nommé RamR, prouver qu'il était capable de moduler l'expression de RamA. Le régulon *ram* étant ainsi identifié au complet, nous avons utilisé nos connaissances au niveau génétique sur l'impact des effecteurs, activateurs et cibles de ces éléments de régulation, pour étudier les stratégies utilisées par les isolats cliniques *in vivo* comme *in vivo* dans la mise en place de la MDR. La sélection de 60 souches d'*Enterobacter* précédemment caractérisées phénotypiquement au niveau membranaire et sensibilité aux antibiotiques, nous a permis de confirmer le rôle central joué par RamA dans la régulation, ainsi qu'identifier des mutations pouvant être critiques, lors de l'adaptation à la thérapeutique antibiotique, au niveau de RamR. Ainsi, l'absence variations observées dans le régulon *marRAB* et l'expression modérée des transcrits montrée par qRT-PCR et à l'inverse la variabilité identifiée du répresseur RamR et la forte expression de RamA chez les souches résistantes, tente à prouver de manière globale, sur les souches sélectionnées, que RamA a un rôle clef dans la régulation de l'expression des porines et des pompes d'efflux chez *E. aerogenes*. Cependant le rôle précis joué par RamR ainsi que les mutations identifiées chez les souches cliniques ou de laboratoire semblent intervenir de façon secondaire, sachant que peu de variations de phénotype ont pu être observées chez les souches transformées par les différents gènes *ramR*. Il semblerait que l'activation directe de RamA par des inducteurs chimiques ou issus du stress oxydatif soit primordiale dans la régulation de la MDR chez *E. aerogenes*. La boucle d'amplification suggérée par la présence des régulateurs analogues chez *E. aerogenes* n'est que modérée, comparée à l'activation préférentielle de RamA.

L'autre partie de notre travail reposait sur l'étude de la translocation des antibiotiques au travers des porines et le rôle de ces dernières dans les caractéristiques de pénétration de différents antibiotiques au travers de la paroi bactérienne. Notre modèle d'étude fut la porine OmpF d'*Escherichia coli*, et le rôle joué au niveau interne par la boucle L3 présente dans la zone de constriction plus particulièrement son intervention dans les étapes d'accueil et de diffusion des carbapénèmes au travers du pore, sachant que ces molécules ont un rôle clef dans l'antibiothérapie. L'étude des interactions porine-carbapénème s'est faite sur la

porine sauvage OmpF et ses deux mutants, possédant une alanine neutre au lieu des aspartates chargés négativement aux positions 113 et 121. Ces porines ont été clonées dans la souches dépourvue de porines *E. coli* BL21Δ*omp*. La mesure de l'activité des trois carbapénèmes, ertapénème, méropénème et imipénème a permis de mettre en évidence des différences de translocation dans chacune des trois porines. Les résultats indiquent également l'importance de l'aspartate en position 113 dans la sélectivité de translocation des différents carbapénèmes au sein de la porine OmpF.

Ce travail montre aussi par approche multidisciplinaire que la translocation des pénicillines, ampicilline et benzylpénicilline est sous la dépendance des interactions qui se créent entre le substrat et le résidu en position 113 et limitent alors leur passage au niveau du canal porine.

Dans la dernière partie de notre travail, nous avons recherché la contribution attribuée à la porine Omp36 d'*Enterobacter aerogenes* dans la translocation de certaines béta-lactamines, par la combinaison d'observations à partir de méthodes biologiques et biophysiques. Cette porine est celle qui, *in vivo*, est majoritairement exprimée par la bactérie. La mesure (i) de l'activité de pénétration et d'action des beta-lactamines dans des bactéries exprimant exclusivement Omp36, (ii) de la conductance ionique de haute résolution dans Omp36 purifiée et reconstituée dans des membranes artificielles par la méthode "black-lipid-bilayer", ont permis une analyse poussée et informative de la fonction de Omp36 dans le passage des beta-lactamines. Les deux beta-lactamines étudiées, l'ertapénème et le céfépime, présentent des interactions avec le canal qui facilitent leur passage et en conséquence favorisent le transport.

1.2 Summary

The family of *Enterobacteriaceae* includes several pathogenic species. One of them, *Enterobacter aerogenes*, is of increasing interest due to being the cause of several nosocomial infections in the recent years, especially in intensive care units.

Nowadays various clinical strains of *Enterobacter aerogenes* present the problem of multidrug resistance (MDR). By a combination of several resistance mechanisms they gain increasing resistances to even the latest antimicrobial agents. *Enterobacter aerogenes* can acquire a large variety of enzymatic responses and genetic alterations to protect itself from harming effects.

In addition to these intracellular mechanisms there is another way to decrease antibiotic effects in the bacteria: regulation of membrane permeability. In the recent years this resistance mechanism became more and more important since it is the first way for a bacteria to acquire resistances against a broad spectrum of unrelated antibiotics and therefore allows the acquisition of other resistance mechanisms later on as well.

One of the aims of this work was to characterize and better understand the genetic regulation cascade of membrane permeability in *Enterobacter aerogenes*. We therefore focused on the two known global regulators in *E. aerogenes*: *mar* and *ram*. Of these two regulons we compared the sequences of the genes *marA* and *ramA* together with their respective assumed regulators *marR* and *ramR* of about 60 clinical isolates with two laboratory mutants that were raised under increasing antibiotic concentrations and two laboratory strains. We observed alterations in *ramR* and in the upstream region of *ramA* but no mutations in neither *marA* nor *marR*. Overexpression of *ramA* or *ramR* due to cloning with a multi-copy plasmid led to alterations in the MIC-value of several tested antibiotics, and to increased or decreased expression of porins or efflux-pump components. No differences could be reported between the different *ramR*-mutations. Transcription-measurements by qRT-PCR pointed out the estimated importance of the *ram*-regulon in the regulation cascade.

Another part of this work was to characterize the translocation of compounds through porins and the role of porins in drug uptake in general. In the second part of this work we tried to better understand the role of the *E. coli* porin OmpF, especially the constriction zone around the L3-loop, in uptake of carbapenems. The wildtype OmpF-porin and two mutations having the aspartates in the L3-loop at position 113 and 121 substituted with

alanine were transformed into the otherwise porin-deficient *E. coli* strain BL21Δ*omp*. Measurement of the rate of antibiotic action of the three tested carbapenems ertapenem, meropenem and imipenem therefore directly allowed investigation of the translocation rate of these antibiotics through the three different porins. The results clearly indicated the importance of the aspartate at position 113 in antibiotic translocation through the porin.

Other parts of this work were contributions to investigations about translocation of beta-lactam antibiotics through the *E. aerogenes* porin Omp36. By combination of measurement of the rate of antibiotic action with *E. coli* bacteria solely expressing Omp36 and high resolution ion conductance measurements with purified Omp36 reconstituted into artificial lipid membranes, the translocation of two representative beta-lactam molecules, ertapenem and cefepime, was studied. The results suggested that interactions between the porin channel and the antibiotic facilitate and therefore fasten transport.

We also participated to a multi-disciplinary three way approach to better understand antibiotic translocation through the *E. coli* OmpF porin, especially the role of the amino acid residue 113 placed inside the so-called constriction zone. Collaboration of computer modeling, artificial bilayer experiments and measurement of biological activity revealed clear differences in the translocation of the two investigated beta-lactams, ampicillin and benzylpenicillin, for the wildtype OmpF porin and the mutant D113A. The data suggests that interactions with residue D113 are rate-limiting for transport through the porin channel.

CHAPTER II:

INTRODUCTION

2.1 *Enterobacteriaceae*

The family of *Enterobacteriaceae* is a large group of nearly 200 species in more than 40 genera that are known today. All are Gram-negative and do not form spores and the majority (except *Klebsiella*, *Shigella* and *Yersinia pestis*) possesses peritrichous cilia for movement. They are viable in both aerobic and anaerobic conditions and show the best growth on MacConkey without additives where they prefer fermentation of D-glucose and other sugars to oxidation. With exception for *Shigella dysenteriae* serotype 1, which is catalase negative, they are all catalase positive, oxydase negative and all of them with the exception of *Erwinia* deoxidize nitrates to nitrites (Freney, et al., 2006). This characterization allows to separate the family of *Enterobacteriaceae* from other Gram-negative bacteria and is valid for all members with the exceptions shown in **Table 2.1**.

They include well known pathogens such as *Escherichia coli* and *Salmonella* Typhimurium. Of *Enterobacteriaceae* the genera *Klebsiella*, *Citrobacter* and *Enterobacter* can be found in several different ecosystems like soil, water, plants and in animals from insects to humans where they preferably inhabit the digestive system and play a major role in the intestinal flora (Dorland, 2009). In contrast to for example *Shigella* spp. that normally can only be found inside the intestinal tract or *Escherichia coli* which can survive just for brief periods outside the body and therefore is a good indicator for fecal contamination.

In clinic *Enterobacteriaceae* are known as being associated with many types of human infections (Brenner, et al., 2005).

Characteristics	**Exceptions**
Gram-negative bacilli	*Alterococcus agarolyticus*
Oxydase negative	*Plesiomonas shigelloides*
Catalase positive	*Shigella dysenteriae serogroup* O1
	Xenorhababus nematophila
Absence of spores	*Serratia marcescens* subsp. *sakuensis*
Nitrate reductase positive	Some *Erwinia* and *Yersinia*
Common antigen of *Enterobacteria*	*Dickeya* (*Pectobacterium*) *chrysanthemi*
Growth on "ordinary" media	Endosymbiotes

Table 2.1: General phenotypic characteristics of members of the family *Enterobacteriaceae* with their exceptions (Janda, et al., 2006)

2.1.1 *Enterobacter*

In the family of *Enterobacteriaceae*, the genus *Enterobacter* comprehends 15 species. With *Enterobacter cloacae* and *Enterobacter aerogenes* it plays an increasing part in nosocomial infections (Sanders, et al., 1997). They are nowadays the most frequent isolated species in clinical environments in France (Davin-Regli, et al., 2006).

In the pre-antibiotic era, they were not encountered in surveys of nosocomial infections. By the 1970s, it was established that *Enterobacter spp.* could be nosocomial pathogens, although they were much less commonly encountered than *Escherichia coli* and *Klebsiella* strains (McGowan, 1985). In the last years the importance of *Enterobacter* spp. grew and at the turn of the century *Enterobacteriaceae* were the third most common pathogen recovered from the respiratory tract (Sanders, et al., 1997). Nowadays bacteria of the genus *Enterobacter* represent the 5th most frequent cause of nosocomial infections and the 4th most frequent cause of nosocomial infections of the urinary tract in the United States (Jarvis,

et al., 1992) (Langley, et al., 2001) and are also often involved in urinal and respiratory tract infections in France (Ehrhardt, et al., 1993) (Mathai, et al., 2001).

It has developed several remarkable resistance mechanisms during its evolution and the different species vary greatly in their resistances and thus in the role they play during opportunistic infections of hospitalized or immunodeprived subjects (Sanders, et al., 1997). Today the patients in intensive care units are most commonly subject of infections caused by multi-resistant strains and *E. cloacae* and *E. aerogenes* together with *Escherichia coli* are the principle *Enterobacteria* to express this phenotype (Allerberger, et al., 1996) (Davin-Regli, et al., 1996) (Georghiu, et al., 1995) (Wenger, et al., 1997).

2.1.2 *Enterobacter cloacae*

The complex *Enterobacter cloacae* constitutes the majority of *Enterobacteria* and includes the species *E. asburiae*, *E. cancerogenus*, *E. cloacae*, *E. hormaechei*, *E. kobei* and *E. nimipressuralis*. All species of the complex share about 60% of DNA homologies (Hoffmann, et al., 2003).

The species *E. cloacae* also sub-divides into two subspecies: *cloacae* which is esculine-negative and *dissolvens* which is esculine-positive (Hoffmann, et al., 2005). The strains differ from the biochemical point of view by the absence of lysine decarboxylase (LDC) and the presence of an arginine dihydrolyse (ADH) and an ornithine decarboxylase (ODC). As nutrients they are able to ferment D-sorbitol, saccharose and melibiose (Davin-Regli, et al., 2006).

Two studies using 54 and 38 clinical isolates of *E. cloacae* respectively showed that most of the strains produced siderophores (aerobactine) and three isolates showed furthermore a hemolytic character (Barnes, et al., 1997) (Keller, et al., 1998). Certain pathogenic factors like a hemolytic and leucotoxic membranotropic cytotoxine have been identified recently (Paraje, et al., 2005).

E. cloacae is naturally resistant to ampicillin, cefalotin and cefoxitin due to a natural cephalosporinase (Davin-Regli, et al., 2006). Other penicillins like ureidopenicillins and carboxypenicillins are only active in half of the investigated strains (Sanders, et al., 1997). *E. cloacae* also possesses a chromosomal cephalosporinases that grants resistance to numer-

ous beta-lactams including ceftazidime when derepressed (Nauciel, et al., 1985) (Zaher, et al., 1998). Second generation cephalosporins like cefamandole and cefuroxime are also just poorly active in clinical isolates. Presence of beta-lactamase inhibitors induces enhanced expression of beta-lactamases what prohibits the use of combinations like amoxicillin with clavulanic acid (Davin-Regli, et al., 2006). De Champs and staff described in 1989 the first nosocomial infections caused by an extended-spectrum beta-lactamase (ESBL) producing *E. cloacae*. These ESBLs grant a resistance to a couple of beta-lactams, in particular to third generation cephalosporins with the exception of latamoxef and cephamycins (De Champs, et al., 1989). Several classes of ESBL have been identified in *E. cloacae* up to now, including TEM, SHV, CTX-M and also inhibitor resistant TEM (IRT) (Arpin, et al., 2002) (Szabo, et al., 2005). Cephalosporins of the fourth generation rest most active, only 0.2-9% of the studied strains were resistant against cefepime (Sanders, et al., 1997). The most effective antibiotic against *E. cloacae* still remains imipenem. However already two types of carbapenemases have been identified (Nordmann, et al., 1993) (Galani, et al., 2005) and a small rate of 0.4% of *E. cloacae* have been reported to be resistant to imipenem (Lee, et al., 2005). High percentage of resistance could also be observed for aminoglycosides like gentamicin (up to 51%), amikacin (up to 34%) and ciprofloxacin (36%) (Sanders, et al., 1997). *E. cloacae* together with *E. coli* and *Klebsiella pneumoniae* are also the *Enterobacteria* were the presence of the plasmidic protein QnrA, that grants resistance to quinolones, was initially observed (Corkill, et al., 2005) (Davin-Regli, et al., 2006).

2.1.3 *Enterobacter aerogenes*

E. aerogenes also belongs to the family of *Enterobacteriaceae* and therefore shares its principal characteristics. Phenotypically and genotypically they resemble much to their close neighbor *Klebsiella* with presence of decarboxylases for ornithine (ODC) and lysine (LDC), absence of arginine dehydrolase (ADH), that distinguishes *E. aerogenes* from *E. cloacae*, and also by absence of urease (Farmer, et al., 1985) (Sanders, et al., 1997) (Davin-Regli, et al., 2006). Contrary to *Klebsiella*, *E. aerogenes* is mobile due to a peritrichous flagella arrangement.

In the early 1990s *E. cloacae* was the most frequent isolated *Enterobacter* pathogen in hospitals. But in recent years up to today, that changed to *E. aerogenes* (Flynn, et al., 1987) (Falkliner, 1992) (Weischer, et al., 1992) (Neuwirth, et al., 1996) (De Gheldre, et al., 2001). *Enterobacter* species rarely cause disease in healthy individuals. Patients most susceptible to *Enterobacter aerogenes* infections are those who stay in the hospital, especially in the ICU, for prolonged periods. The source of infection may be endogenous (via colonization of the skin, gastrointestinal tract, or urinary tract) or exogenous (hands of personnel, endoscopes, stethoscopes,…). Nowadays *E. aerogenes* is known as a common and important nosocomial pathogen that is most often found in intensive care units. It is here known to be the origin of numerous epidemics in Europe, in particular in France, Spain and Belgium, but also in the United States and Japan (Georghiu, et al., 1995) (Arpin, et al., 1996) (Davin-Regli, et al., 1996) (Glupczynski, et al., 1998) (Jalaluddin, et al., 1998) (De Gheldre, et al., 2001) (Mammeri, et al., 2001) (Goshi, et al., 2002). In France, *E. aerogenes* is the third most common cause of respiratory infections and the fifth most common cause of urinary infections in intensive care units. It is found most often in surgical wards, but present in all other sections as well. Infections with *E. aerogenes* lead to a mortality of 32% and the usage of third generation cephalosporins leads to an increasing risk of a selection of multi-resistant clinical isolates (Chow, et al., 1991).

E. aerogenes exhibit an excellence in acquiring resistance mechanisms against antibiotics (Davin-Regli, et al., 2006). In the last decade, the increasing number of studies regarding antibiotic resistance of *E. aerogenes* demonstrate clearly the ongoing evolution of resistance mechanisms in this species (Georghiu, et al., 1995) (Grattard, et al., 1995) (Allerberger, et al., 1996) (Arpin, et al., 1996) (Davin-Regli, et al., 1996) (Glupczynski, et al., 1998) (Jalaluddin, et al., 1998) (De Gheldre, et al., 2001) (Sirot, et al., 2002). In 1991, the first strains of *E. aerogenes* bearing a plasmid with genes coding for several ESBLs were isolated in France (De Champs, et al., 1991). An investigation realized in 2002 revealed that 47.7% of 2353 isolated *E. aerogenes* strains isolated in northern France were carrier of ESBL compared to 11.4% of the 6121 isolates of *K. pneumoniae* (Albertini, et al., 2002). These results are slightly different to those of southern France, where *E. aerogenes* shows a clear predominance with 9.2% isolated strains against 6.2% *E. cloacae* and 2.4% *K. pneumoniae* (C-CLIN Sud-Est, 2002). Of these isolated *E. aerogenes* strains about 70% featured ESBLs and other resis-

tances like against aminoglycosides but remained sensible to fourth generation cephalosporins as cefepime and cefpirome (Sanders, et al., 1997).

Strains of *E. aerogenes* possess a chromosome-coded cephalosporinase that grants resistance to 1st generation cephalosporins as well as an high-level AmpC-type beta-lactamase which is activated by treatment with 3rd generation cephalosporins or by mutation of the repressor AmpR and through which the bacteria becomes resistant against all beta-lactam antibiotics except cefepime and imipenem (Steffee, et al., 1992) (Bryskier, 1999) (Davin-Regli, et al., 2006) By overexpression of a mutated AmpC beta-lactamase, some strains can even acquire resistance against cefepime (Barnaud, et al., 2004).

Diverse typological studies document that the major ESBL-type TEM-24 is responsible for several epidemics in France and Belgium in past and present years (Neuwirth, et al., 1996) (Bosi, et al., 1999) (De Champs, et al., 2000) (Galdbart, et al., 2000) (De Gheldre, et al., 2001) (Mammeri, et al., 2001) (Dumarche, et al., 2002). The plasmid carrying the TEM-24-type ESBL, which is nearly exclusively found in *E. aerogenes* (Bertrand, et al., 2003), imparts resistance against fourth generation cephalosporins (cefpirome, cefepime) and also against aminoglycosides, except gentamicin, by expression of a type AAC6' aminoglycoside acetyltransferase (Marchandin, et al., 2000). Two years ago *E. aerogenes* represented the primary hydrolytic enzymes producing *Enterobacteria* in France, even before *E. coli* (Bertrand, et al., 2003) (Dumarche, et al., 2002). Nowadays *E. coli* again has the lead, in consequence of frequent expression of CTX-M-ESBL. Among *Enterobacteriaceae* expressing ESBL, 48% are *E. coli* strains against 24% *E. aerogenes* and 15% *K. pneumoniae* (Biendo, et al., 2008). Other identified beta-lactamases include TEM-1, TEM-2, TEM-3, TEM-10, TEM-12, TEM-121 which is considered as IRT, SHV-4 and SHV-5 (Dumarche, et al., 2002). Recently TEM-20, SHV-5 and SHV-12 have been identified as well (Biendo, et al., 2008).

E. aerogenes appears to be an important host for the type TEM-24 ESBL (Arpin, et al., 2003). It can on the one hand acquire plasmids, but also transfer it to other species. Therefore type TEM-24 ESBL could already be isolated from *K. pneumoniae*, *E. coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa.* The source of these transfers was always identified as *E. aerogenes* which was actually always isolated before or with the other bacteria during clinical extractions (Marchandin, et al., 2000) (Neuwirth, et al., 2001).

Other studies revealed a derepressed chromosomal AmpC-type cephalosporinase (Preston, et al., 2000) (Lee, et al., 2003), or combinations of derepressed cephalosporinases with ESBL (Davin-Regli, et al., 1996).

In France the frequent use of fluoroquinolones led to a gained resistance against these antibiotics by target mutation in the QRDR-region of the DNA gyrase beta-subunit (Davin-Regli, et al., 2006). The plasmid-mediated quinolone resistance (PMQR) determinants has been rarely described in *E. aerogenes* for instance (Park, et al., 2007).

Beside the enzymatic inactivation of an antibiotic with ESBL or cephalosporinases or target mutations, *E. aerogenes* has another resistance mechanism: alteration of membrane permeability. One way to alter the membrane permeability and therefore the amount of materials which is transported across this barrier is to vary the quantity of porins in its external membrane. These porins are hydrophilic canals, and therefore present one way of entering the cell for hydrophilic substances like antibiotics such as beta-lactams. The evolution of membrane impermeability allows *E. aerogenes* to gain even resistances against fourth generation cephalosporins. Charrel and coworkers revealed in 1996, that about 6% of the *E. aerogenes* strains that were resistant to a broad spectrum of antibiotics showed an altered expression of porins (Charrel, et al., 1996). *E. aerogenes* also seem to lack a carbapenemase like the one described in *E. cloacae* (Nordmann, et al., 1993), nevertheless some clinical isolates show a imipenem-resistant phenotype (De Gheldre, et al., 1997). This resistance is explained by a overproduction of a beta-lactamase and the lack of a 40kDa protein of the outer membrane (Hopkins, et al., 1990) (Chow, et al., 1991) (Tzouvelakis, et al., 1992) (Tzouvelakis, et al., 1994) (Bornet, et al., 2000) (Stürenburg, et al., 2002) (Fernandez-Cuenca, et al., 2006). Recently it was also described that strains can gain membrane impermeability and thus resistance to antibiotics by alteration or mutation of the porin interior (Thiolas, et al., 2004). Examples are the accumulation of imipenem (Chevalier, et al., 2000) or the translocation of ertapenem and imipenem through *E. aerogenes* porin Omp36 (James, et al., 2009). A clinical study described that 62.9% of strains, selected during a two year period, that were resistant to an extended spectrum of cephalosporins, also were resistant or less susceptible to imipenem This resistance can occur in ESBL-producing *E. aerogenes* isolates by carbapenemase production (67% of the resistant isolates) or by the loss of porins in the

outer membrane (33% of the cases) (Biendo, et al., 2008). The metallo-beta-lactamases were identified as IMP-1 and VIM-2.

Another way to evolve antibiotic resistance via the alteration of membrane permeability is to develop active efflux. *E. aerogenes* is able to assemble a complex proteomic structure, explained later on, that enables ejection of structurally unrelated antibiotics like fluoroquinolones, tetracycline and chloramphenicol (Malléa, et al., 1998) (Pradel, et al., 2002) (Chollet, et al., 2004) (Pagès, et al., 2005) (Poole, 2005) (Thiolas, et al., 2005) (Davin-Regli, et al., 2006) (Ghisalberti, et al., 2006) (Lomovskaya, et al., 2006) (Masi, et al., 2006). An increased efflux by overexpression of this proteomic structure, called efflux pump, induced by increasing chloramphenicol concentrations was shown to enable resistance against chloramphenicol and other structurally unrelated antibiotics (Ghisalberti, et al., 2005).

Together, these two mechanisms alter the permeability of the outer membrane for antibiotics with different chemical structures and therefore allow *E. aerogenes* to develop resistances against a broad spectrum of unrelated antibiotics. Since 1998 several multi-resistant *E. aerogenes* strains were described that showed a combination of different enzymatic countermeasures in combination with decreased porin expression and increased efflux activity. The combination of these three different resistance mechanisms resulted in several failed therapeutic strategies (Malléa, et al., 1998) (Pradel, et al., 2002) (Gayet, et al., 2003).

2.2 Mechanisms of antibiotic activity

Antibiotics are biochemical substances of natural, semi-synthetic or synthetic origin. In addition to this origin-based classification they can also be divided into two broad groups according to their effect on microorganisms: those that kill bacteria (bactericidal agents) and those that only impair growth (bacteriostatic agents). Beside other specifications as for example their target specificity, all antibiotics can also be divided into four big groups or categories that are defined by their mechanisms of antibiotic activity: antibiotics that target the bacterial cell wall, antibiotics that act on the bacterial membrane, those that affect the DNA synthesis and those that inhibit bacterial protein synthesis. The first three groups are usually bactericidal in nature, the last one bacteriostatic. All four groups will be introduced and explained in the following.

2.2.1 Antibiotics inhibiting cell-wall synthesis: β-lactams

β-lactam antibiotics act on the cell wall synthesis by inhibiting the synthesis of the peptidoglycan layer which is important for the structural integrity of the cell wall. Especially in Gram-positive bacteria, but also in Gram-negative. The final transpeptidation step is facilitated by the transpeptidases, that are also known as penicillin-binding proteins (PBP).

Due to their structural similarity as analogues of D-alanyl-D-alanine, which is the terminal amino acid residue on the precursor NAM/NAG-peptide subunits of the nascent peptidoglycan layer, their binding to the active site of the PBPs is facilitated. After the β-lactam nucleus of the molecule binds irreversibly to the active site of the PBP, it prevents the final crosslinking (transpeptidation) of the nascent peptidoglycan bilayer and therefore disrupts the cell wall synthesis. This inhibition of the cell wall synthesis results in the halt of the bacterial growth (bacteriostatic effect) (Bryskier, 1999) (Tankovic, 2000). The bactericidal effect dates from the activation of autolytic cell wall hydrolases, triggered by the cross-linking of beta-lactams causing an accumulation of peptidoglycan precursors. These hydrolases digest the existing peptidoglycan without building new one and therefore cause the cellular death (Tankovic, 2000).

The β-lactams form a large group of antibiotics that all share a similar structure with the β-lactam ring as a central part of its structure (**Figure 2.1**).

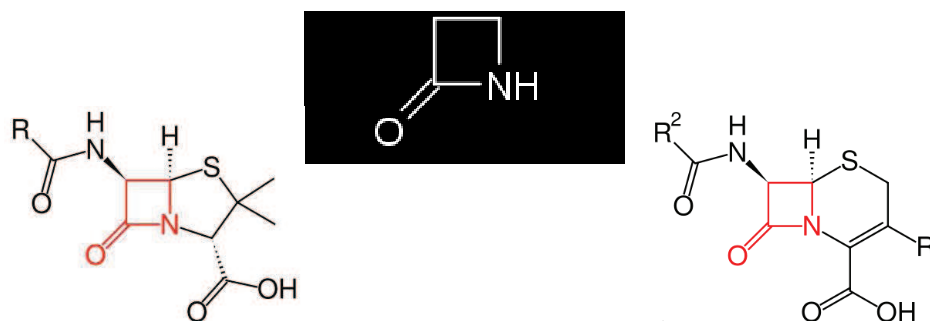


Figure 2.1: beta-lactam ring with penicillin (left) and cephalosporin (right)

http://de.wikipedia.org/wiki/%CE%92-Lactam-Antibiotika

http://en.wikipedia.org/wiki/Beta-lactam_antibiotic

The oldest representative is penicillin G and the others can be classified in four groups: penicillins, cephalosporins, carbapenems and monobactams.

2.2.2 Antibiotics acting on the bacterial membrane: polymyxins

Polymyxins are antibiotics consisting of a general structure of a cyclic polypeptide with a long hydrophobic chain. They are produced by the Gram-positive bacterium *Bacillus polymyxa* and are selectively toxic for Gram-negative bacteria. The selectivity stems from their specificity to lipopolysaccharide, a molecule that can be found in the outer membrane of many Gram-negative bacteria but does not exist in the cell-wall structure of Gram-positives.

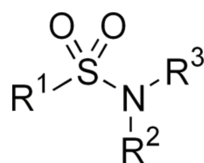
The amphipathic structure of polymyxins enables them to penetrate the bacterial cell and to insert into the cell wall. Here they cause disorganization and disruption of both the inner and outer membrane, probably by a detergent-like action, and hereby cause cell death (Bryskier, 1999).

Polymyxins remain active also in multi-resistant *Enterobacteriaceae*, but due to their heavy secondary effects like eye, ear and bladder infections and kidney and nerve damage they remain rarely used even after the global problem of increasing resistances renewed the interest in their usage. Recent studies revealed that *Enterobacteriaceae*, namely *E. aerogenes*, can also develop resistances against polymyxin, namely colistin, by alteration of membrane permeability (Thiolas, et al., 2005).

2.2.3 Antibiotics affecting DNA synthesis

2.2.3.1 Sulfonamides

Sulfonamides were one of the first antimicrobial drugs, already discovered in the 1930s in Germany in the laboratories of Bayer AG. They are synthetic antimicrobial agents that contain the sulfonamide group (**Figure 2.2**).



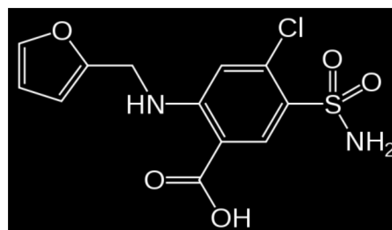


Figure 2.2: Sulfonamide functional group and furosemide as example

http://en.wikipedia.org/wiki/Sulfonamide_%28medicine%29

In bacteria, antibacterial sulfonamides act as competitive inhibitors of the enzyme dihydropteroate synthetase (DHPS), an enzyme involved in folate synthesis. Folate or folic acid, also known as vitamin B9, is necessary for the production and maintenance of new cells, for DNA synthesis and RNA synthesis. In bacteria DHPS is an enzyme producing dihydropteroate, or dihydropteroic acid, in humans it does not exist or function. This makes it a useful target for antibiotics. Nevertheless Sulfonamides are known to have the potential to cause a variety of side effects, including urinary tract disorders, hemopoietic disorders, porphyria and hypersensitivity reactions. When used in large doses, they may cause a strong allergic reaction.

2.2.3.2 Quinolones

Quinolones are a synthetic-produced type of antibiotics that possess a highly conserved structure (**Figure 2.3 and 2.4**), usually connected to piperazine. If a fluorine atom is attached to the central ring system, the antibiotic is called a fluoroquinolone. Quinolones in general are active against *Enterobacteriaceae* whereas fluoroquinolones are even more efficient and also have a broader range of bacteria.

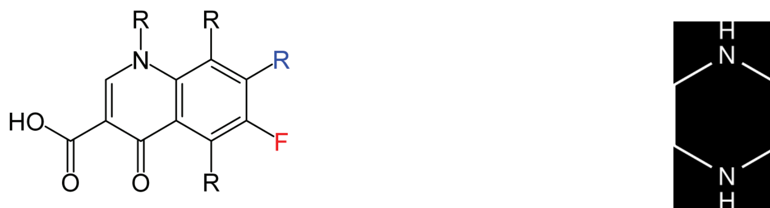


Figure 2.3: general quinolone structure (left) with blue R usually piperazine ring (right) and red F fluorine in fluoroquinolones

http://en.wikipedia.org/wiki/Quinolone

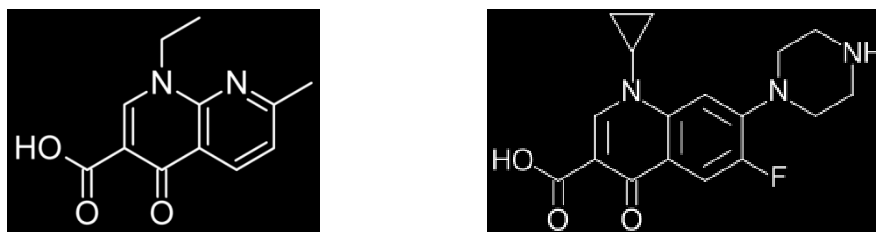


Figure 2.4: Nalidixic acid (left) and ciprofloxacin (right)

http://en.wikipedia.org/wiki/Quinolone

The more hydrophilic fluoroquinolones take use of the porins to cross the barrier of the external membrane of Gram-negative bacteria (Hirai, et al., 1986) (Chevalier, et al., 2000). Inside the cell, the preferred target for the quinolones is the DNA-gyrase. This topoisomerase, a tetrameric enzyme consistent of 4 subunits (GyrA2-GyrB2), controls the supercoiling of the DNA-strand in prokaryotic organisms such as bacteria. Quinolones now block the enzymatic cycle, that is necessary for the cleavage of the double-strand DNA and hereby inhibit DNA replication and transcription. Another target for quinolones is the DNA topoisomerase IV, that holds structural analogies with the gyrase and also is an analogous enzyme. For most of the Gram-negative bacteria, the DNA gyrase forms the target contrary to Gram-positives where it is the topoisomerase IV (Hooper, 2000) (Tankovic, 2000).

2.2.5 Antibiotics inhibiting protein synthesis

2.2.5.1 Tetracyclines

Tetracyclines are a group of broad-spectrum antibiotics composed of four hydrocarbon rings. They can easily pass through the bacterial cell wall by diffusion which is a reason for their wide antibacterial range. Inhibition of the protein synthesis occurs during the elongation phase of the translation. The tetracycline binds to the 30S subunit of the ribosome, close to site A thus blocking the fixation of new tRNA-amino acids. This results in an inhibition of the prolongation of the new amino acid-chain (Chopra, et al., 2001).

2.2.5.2 Aminoglycosides

An Aminoglycoside is a molecule composed of a sugar group and an amino group. It acts usually as a protein synthesis inhibitor but also disrupts the cell-wall. Also their exact mechanism remains yet unknown. Aminoglycosides are known to interfere with the proofreading process and therefore cause an increased rate of error during the synthesis but also inhibit ribosomal translocation when the peptidyl-tRNA moves from the A- to the P-site, hence they alternate nearly all steps during translation (Bryskier, 1999).

Despite their small size and their hydrophilic character, aminoglycosides only have a low translocation rate through porins. Therefore they have to penetrate the external and cytoplasmic membrane. This entry is divided into two steps EDP I and EDP II (Energy Dependent Phase I and II) (Tankovic, 2000). During EDP I an accumulation of aminoglycosides occurs on the outer membrane where the polycationic structure of the aminoglycosides helps to interfere with the LPS. This step already has a bacteriostatic effect. EDP II ends in a faster accumulation and the aminoglycosides create fissures in the outer membrane resulting in a faster uptake of antibiotics but also leakage of intracellular contents. This rapid action probably accounts for most of the bactericidal activity. (Lorian, 1996)

The plasmids found in *Enterobacteriaceae* carrying ESBLs and also the TEM-24-type ESBL found in *E. aerogenes* express determinants for resistances against aminoglycosides (Aminoacyl-transferases type AAC-6’) (Davin-Regli, et al., 1996) (Bosi, et al., 1999).

2.2.5.3 Macrolides, lincosamides and streptogramins

These three groups of antibiotics show a different chemical structure but share the same antibacterial spectrum and also the same mechanism. Since they are just seldom used against *Enterobacterial* infections, they will be just shortly explained.

Macrolides are usually build up of one large macrocyclic lactone ring to which one or more deoxy sugars are attached. The lactone rings are usually 14, 15 or 16 membered. Lincosamides derive from six-ring-monosaccharides, the so called pyranoses. They are based on an alkylated hydric acid at position 4 and substituted with a 6-amino-thio-octopyranosid group using an amid function (Tankovic, 2000). The streptogramins finally are a mixture of two molecule types: streptogramin A and B. they act in a synergetic kind like fashion. Their structure is pretty complex, with the type A molecule corresponding to an unsaturated macrocyclic lactone and the molecule type B corresponding to a cyclic polypeptide (Tankovic, 2000).

All three classes of antibiotics inhibit the protein synthesis: they inhibit the translation by binding to the 50S subunit of the ribosome. This reversible binding at position P blocks the transfer of tRNA-peptides from position P to A and therefore stops the elongation process during the translation from DNA to RNA (Tenson, et al., 2003).

2.2.5.4 Chloramphenicol

The last antibiotic to explain is chloramphenicol. It has a large bacteriostatic spectrum for both Gram-positive and Gram-negative bacteria (Neu, et al., 1996) with the exception of *Haemophilus influenza*, where it acts bactericidal. Due to resistances and safety-concerns, since its most serious adverse side effects are bone marrow suppression and aplastic anemia which is generally fatal, its use was reduced in the recent years. Nevertheless the global problem of advancing bacterial resistances to newer drugs has led to renewed interest in its use (Falagas, et al., 2008). Chloramphenicol acts on the 50S ribosomal subunit like the macrolides and inhibits therewith the protein synthesis (Bryskier, 1999) (Johansson, et al., 2005).

2.3 Resistance mechanisms against antibiotics

There are several different strategies and mechanisms that allow bacterial organisms to develop or improve resistances against all different kinds of antibiotics. First of all they can be distinguished by their place of action: inside the cell or at the cell surface.

Bacteria possess two different possibilities of countermeasures against an antibiotic inside the cell. They can either degrade it using enzymes (Bradford, 2001) (Paterson, 2006) or they can modify or overexpress the bacterial protein that serves as target for the antibiotic and therefore alter the affinity (Cambau, et al., 1993) (Spratt, 1994).

The most important mechanism of resistance to penicillins and cephalosporins is to hydrolyze the antibiotic with the bacterial enzyme beta-lactamase. Among them are distinguished Extended Spectrum Beta-lactamases (ESBL) and chromosomic cephalosporinases (Bradford, et al., 1997) (Paterson, 2006), which have emerged in the last decades in clinic coincidental with the development of therapeutic molecules. Methods to overcome resistance to beta-lactam antibiotics include the development of new antibiotics that are stable to attacks and the co-administration of beta-lactamase inhibitors (BLI) (Bradford, 2001) (Paterson, 2006).

Metallo-beta-lactamases grant resistance against carbapenems and were first reported in *Pseudomonas aeruginosa* (Castanheira, et al., 2004), later it was also described for Gram-negative bacteria in *Acinetobacter sp.*, *Citrobacter sp.*, *E. coli, Enterobacter sp.*, *K. pneumoniae*, *Pseudomonas sp.*, *Proteus sp.*, *Providencia sp.*, *Serratia sp*. (Castanheira, et al., 2004).

Production of antibiotic-modifying enzymes and synthesis of altered bacterial targets are the primary resistance mechanisms for the other classes of antibiotics including rifampicin, aminoglycosides, phenicols and quinolones (Cambau, et al., 1993) (Spratt, 1994). These mechanisms affect only a single class of molecules.

In contrast to this, reduced antibiotic penetration by alteration of membrane permeability is a general resistance mechanism concerning several classes of antibiotics: beta-lactams, aminoglycosides, phenicols and macrolides. The membrane permeability is crucial for the intrinsic resistance of the bacteria (Nikaido, 2003). One way to lower the uptake rate is to decrease the expression of porins or the expression of porins with modified structure (Chow, et al., 1991) (Dé, et al., 2001) (Raimondi, et al., 1995) (Simonet, et al., 2000). Another

way to lower the intracellular concentration is an energy-driven active efflux. First discovered in 1980, five different families have been identified up to today (Nishino, et al., 2001) (Piddock, 2006). Together with the porins they play a major role in both intrinsic and acquired antibiotic resistance (Blair, et al., 2009). Both mechanisms will be explained in more detail later on.

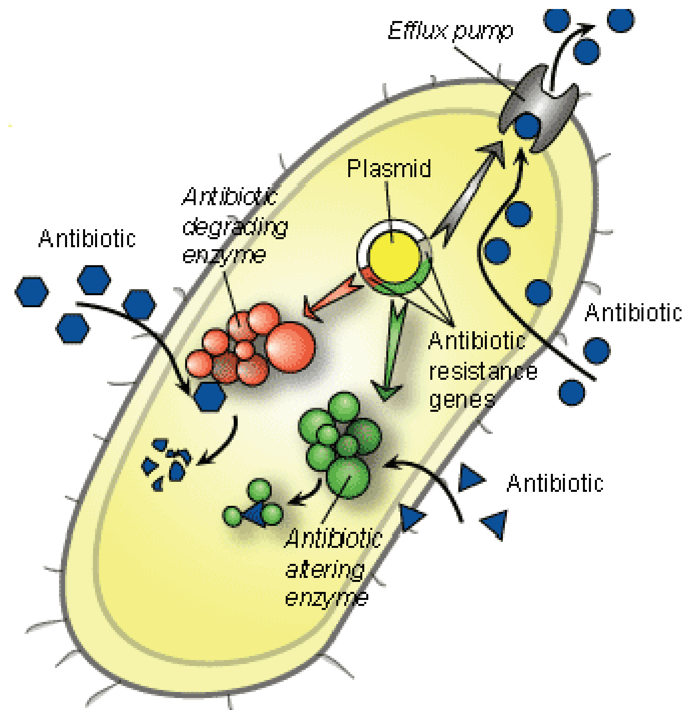


Figure 2.5: overlook of resistance mechanisms in Gram-negative bacteria (porins not shown)

http://textbookofbacteriology.net/themicrobialworld/ResistanceMechanisms.gif

Beside these two or four different mechanisms a bacterial population has also the possibility to build a so called biofilm. A biofilm allows a bacterial colony to protect itself effectively against antimicrobial agents and is made responsible for several persistent human infections (Allison, et al., 1995) (Costerton, et al., 1999) (Hall-Stoodley, et al., 2004).

Bacterial resistance often consists of the expression of one or the combination of several of these different mechanisms and many clinical isolates possess their multi-resistant character because of the conjunction of several different mechanisms. We can also distin-

guish between natural resistances that are chromosomal, hence stable, and therefore are a major characteristic of the bacterial species, or acquired ones. Acquired resistances can also be chromosomal and been activated by mutations or by integration of foreign genes or they are attained by mobile elements such as plasmids, transposons and integrons. These mobile elements allow the exchange of acquired resistances between different bacterial species.

2.3.1 Resistance by enzymatic degradation of the antibiotic

To gain resistance against a harmful chemical inside a bacterial cell, the first way is to get rid of the substance. One way is to produce enzymes capable of modifying or digest the substrate, in this case the antibiotic, which will be explained in the following.

2.3.1.1 Enzymatic reactions against beta-lactam antibiotics

Various enzymatic systems that degrade beta-lactams are known today. The four classes of different beta-lactamases are divided by their respective substrates: penicillinases (class A), metallo-beta-lactamases (class B), cephalosporinases (class C) and oxacillinases (class C) (Thomson, et al., 2000). After current calculations, the beta-lactamases are highly efficient and the enzymes produced by one bacteria are able to destroy about 100 millions of molecules per second (Walsh, 2000).

About 31% of *Enterobacter* spp. responsible for infections in intensive care units in the United States are not susceptible to third generation cephalosporins. This resistance is typically caused by the acquisition of plasmids containing ESBL genes and these plasmids often carry other resistance genes as well (Paterson, 2006). Chromosomal penicillinases and cephalosporinases can be expressed continuously like in *Klebsiella sp.* or can be inducible by the presence of beta-lactams like in *Enterobacter sp.*, *Serratia sp.*, *Citrobacter sp.* and *Pseudomonas sp.* (Sanders, et al., 1997) (Eliopoulos, 1988) (Livermore, 1997). Since the resistance is caused by an overproduction of the enzyme, it is not defeasible by beta-lactamase inhibitors like clavulanic acid, tazobactam and cloxacillin. Bacteria with this so-called ''derepressed cephalosporinase'' show a wide variation of resistance-phenotypes, but all remain susceptible to imipenem (Barnaud, et al., 2001)

Beside the chromosomal way, resistances to beta-lactams can also be acquired by plasmids. The number of described plasmid coded beta-lactamases is very important and in constant evolution (Bradford, 2001). An actual overview is shown in **Table 2.2**.

Beta-lactamase	Examples	Substrates
Broad spectrum	TEM-I, TEM-2, SHV-I OXA family	Penicillin G, aminopenicillins, carboxypenicillins, piperacillin, narrow-spectrum cephalosporins Substrates of the broad-spectrum group plus cloxacillin, methicillin and oxacillin
Extended spectrum	TEM family, SHV family CTX-M family OXA family Others (PER-1, PER-2, BES-1, GES/IBC family, SFO-1 TLA-1, VEB-1, VEB-2)	Substrates of the broad-spectrum group plus oxymino-cephalosporins and monobactam (aztreonam) Substrates of the expanded-spectrum group plus, for some enzymes, cefepime Same as for the CTX-M family Same as for TEM and SHV family
AmpC	ACC-1, ACT-1, CFE-1, CMY family, DHA-2, FOX family, LAT family, MIR-1, MOX-1, MOX-2	Substrates of the expanded-spectrum group plus cephamycins
Carbapenemase	IMP family, VIM family, GIM-1, SPM-1 (metallo-beta-enzymes) KPC-1, KPC-2, KPC-3 OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-40, OXA-48	Substrates of the expanded-spectrum group plus cephamycins and carbapenems Same as for IMP family, VIM family, GIM-1 and SPM-1 Same as for IMP family, VIM family, GIM-1 and SPM-1

Table 2.2: Selected beta-lactamases of Gram-negative bacteria
(Paterson, 2006)

ESBLs are no penicillinases in the classical meaning but still target penicillins and cephalosporins except the ones of the fourth generation. In *Enterobacteriaceae* the types TEM and SHV are most frequent (Jacoby, et al., 1991) (Davies, 1994). They emerge by point mutations of the beta-lactamases TEM-1, TEM-2 and SHV-1. The exemplary type of plasmidic beta-lactamases is TEM-1, found in *E. coli*, and responsible for its acquired resistance to penicillinases but without a really versatile activity. Beta-lactamases are generally susceptible to beta-lactam inhibitors like clavulanic acid *in vitro*, there are also TEMs that are not inactivated by inhibitors, which is an effect of particular mutations in the earlier mentioned TEMs. They were proven in clinical isolates of *E. coli* that were particularly resistant to the combination of amoxicillin and clavulanic acid.

2.3.1.2 Enzymatic inactivation of aminoglycosides, chloramphenicol and quinolones

Although aminoglycosides cannot be inactivated by hydrolysis, their binding to their ribosomal RNA target can be anticipated. This can be done by aminoglycoside-N-acetyltransferases (AAC), aminoglycoside-O-phosphotransferases (APH) and aminoglycoside-O-nucleotidetransferases (ANT) (Shaw, et al., 1993) (Wright, et al., 1998) (Wright, 1999). The disposition of the corresponding genes varies from species to species. *Serratia marcescens* and *Providencia stuartii* for example carry the AAC-coding gene on their chromosome and therefore are resistant to different degrees according to the expression of the gene (Shaw, et al., 1992) (Shaw, et al., 1993) (Franklin, et al., 2001). Nevertheless these resistance genes are often carried on plasmids, together with ESBLs, which is another reason for their large diffusion.

The enzymes are characterized by the resistance phenotype they cause against the four aminoglycosides that are most commonly used in hospital therapy: gentamicin (G), tobramycin (T), netilmicin (N) and amikacin (A). The profile of the enzyme is then symbolized with the initials of the inactivated aminoglycosides. For example the most often found enzyme in *E. aerogenes* is AAC6', that is often associated on the same plasmid as the type TEM-24 ESBL. It disables netilmicin, amikacin and tobramycin but is inactive against gentamicin and therefore is classified TNA (Marchandin, et al., 2000).

Resistance to chloramphenicols is granted by a chloramphenicol-acetyltransferase (CAT) (Murray, et al., 1997).

2.3.2 Resistance by modification of the target

To be efficient, an antibiotic needs to be able to detect its target inside the cell. If the target is modified or replaced so that the antibiotic is no longer able to bind to it, the bacterial cell gains a resistance that is normally not specific but extended to a whole family of antibiotics.

2.3.2.1 Resistance to beta-lactams by modification of the penicillin-binding-proteins (PBP)

In Gram-negative bacteria, beta-lactams find their target the PBPs, in the periplasm after having crossed the outer membrane via porins. PBPs are proteins that are responsible for the correct construction of the sub-units of the bacterial cell-wall. Alteration of the PBPs that leads to resistance against beta-lactams concerns especially Gram-positive bacteria, but has also been described in *Neisseria gonorrhoeae* (Hakenbeck, et al., 1998).

2.3.2.2 Resistance to quinolones by target-modification

Generally the DNA gyrase and the topoisomerase IV (respectively *gyrA* and *parC*) serve as targets for quinolones, two enzymes that are responsible for the correct folding of the DNA as explained before. Mutations that lead to slight structural changes of the DNA gyrase are liable that the quinolones cannot bind any longer and cause therefore resistance (Cambau, et al., 1993). The mutations are located in a 40 amino-acid long region of the sub-unit A of the gyrase. This region is known as *Quinolone Resistance Determining Region* (QRDR) and is found in several bacterial species (Hooper, 2000).

Recent findings indicate that also plasmid-mediated resistance mechanisms against quinolones exist. The plasmids encode enzymes of the pentapeptide family, QnrA, QnrB and QnrS, that protect the DNA-gyrase in Gram-negative and the topoisomerase IV in Gram-positive bacteria (Poirel, et al., 2006) from the inhibitory activity of quinolones (Tran, et al.,

2002) (Tran, et al., 2005). Hereby they facilitate the selection of chromosomal mutants in the presence of a quinolone. They are frequently described in *E. coli, E. cloacae, K. pneumonia* and *Citrobacter freundii*. They are also frequently found on plasmids that also carry other resistance mechanisms like ESBL or plasmid-mediated AmpC-type beta-lactamases (Park, et al., 2007).

2.3.2.3 Resistance by alteration of the ribosomal target

Alteration of the ribosomal target is an effective way to gain resistances against tetracyclines (Chopra, et al., 2001), macrolides, streptogramins and lincosamides (Leclerq, et al., 1991) and in a minor extend also against aminoglycosides (Wright, et al., 1998)

Gram-positive *cocci* are often resistant to macrolides, lincosamides and streptogramin caused by methylation or dimethylation of an adenine in the 23S RNA located in the ribosomal 50S subunit (Leclerq, et al., 1991).

2.3.3 Resistance by regulation of membrane permeability

Before any antibiotic can operate, it has to enter the cell. Therefore they have to cross the bacterial cell-wall that separates the bacteria from the surrounding medium. Depending on its group or phylum, bacteria can have several different kinds of cell-wall organizations of which Gram-negative and Gram-positive are the biggest and best known. Since this thesis will deal with Gram-negative species, this type of cell-wall will be explained in the following. To enter a Gram-negative bacteria the antibiotic (and other substances as well) has first to cross the outer membrane. The outer membrane composes a for antibiotics impermeable membrane, which alone explains at least partially the intrinsic higher resistance of Gram-negative bacteria compared to Gram-positives (Nikaido, 2003).

2.3.3.1 Organization of the Gram-negative cell wall

The cell wall of Gram-negative bacteria is organized in three layers. These layers are (from inside to outside): the inner membrane or cytoplasmic membrane, the periplasm with its peptidoglycan layer and the outer membrane (**Figure 1.6**).

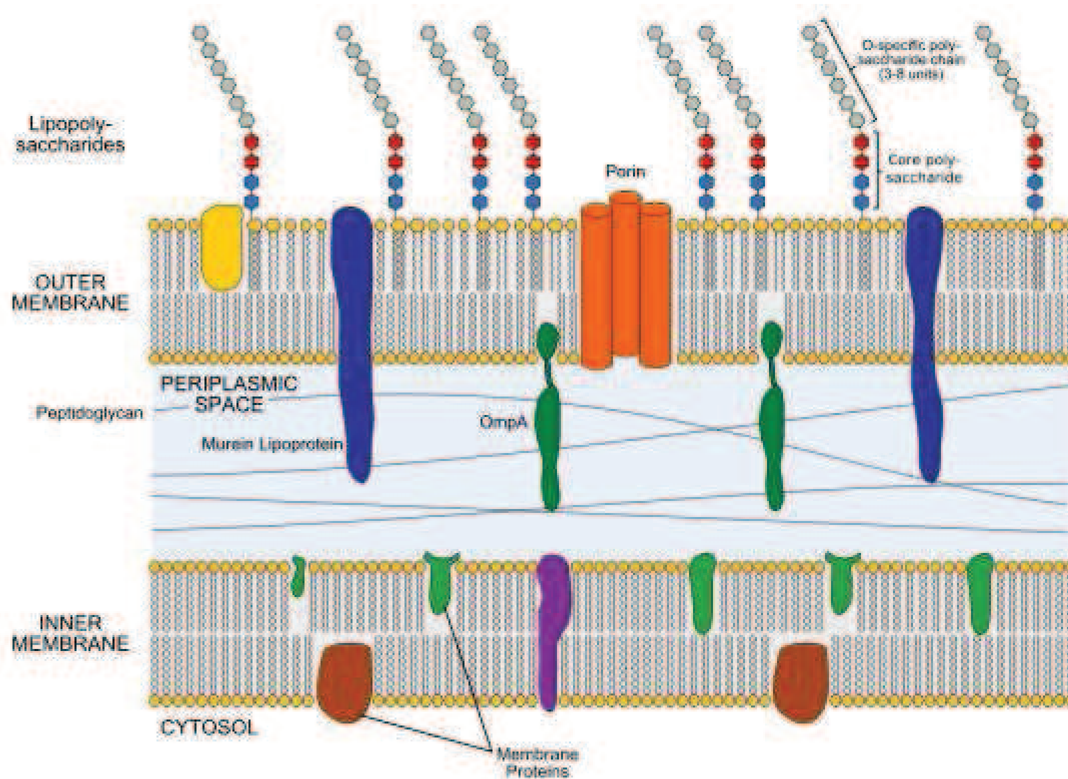


Figure 2.6: organization of the Gram-negative cell-wall

http://en.wikipedia.org/wiki/File:Gram_negative_cell_wall.svg

2.3.3.1.1 Inner membrane (cytoplasmic membrane)

The inner membrane is build up of a phospholipidic bilayer, rich of phosphatidyletha-nolamine, that also contains several cardiolipins (phosphatidylglycerols and diphosphatidyl-glycerols). It assures many different functions, performed by numerous specific proteins res-ident in it. It is for example important for the oxidative phosphorylation because several pro-teins (dehydrogenases, coenzymes, ...) are settled in the inner membrane. But also many proteins necessary for the energy or non-energy dependant nutrient transport (permeases and phosphotransferases) or for the signal transduction (two component system EnvZ-

OmpR) are settled here. Last but not least the inner membrane also plays an important role in the biosynthesis of components of the cell-wall itself (Cronan, et al., 1987).

2.3.3.1.2 Periplasm

The periplasm is the hydrophilic compartment in between the two phospholipid membranes. It contains oligosaccharides (membrane-derived-oligosaccharides: MDOs), the peptidoglycan and proteins of several different functions. These periplasmic proteins intervene in the transport of sugars, amino acids and ions. Another class of enzymes that can be found here are the beta-lactamases that were described earlier.

2.3.3.1.3 Outer membrane

The outer membrane plays an important role for the physiology of Gram-negative bacteria. In the case of *Enterobacteriaceae*, whose natural environment is the mammalian intestinal tract, the outer membrane acts as protection barrier against the detergent action of bile salts and the degradation by digestive enzymes (Nikaido, et al., 1979). At the same time it is impermeable for various types of antibiotics and also acts here as a veritable protection (Nikaido, et al., 1979).

The foundation of this impermeability builds the asymmetric structure of the outer membrane with the lipopolysaccharide (LPS) that constitutes the major part of the external side of the outer membrane. LPS is an amphipathic molecule with three regions: the lipid A, the oligosaccharide core and the O-antigen on its distal side (Gronow, et al., 2001) (**Figure 2.7**).

Lipid A is the hydrophobic part of the LPS that is integrated in the liposome bilayer of the outer membrane. It is composed of two phosphorylated N-acetyl-D-glucosamines with several saturated fatty acids connected to them (Karibian, et al., 1993). The hydrophilic part of the LPS is formed by the core oligosaccharide and the O-antigen. The core is build up of ten sugars, followed by the O-antigen made of repetitive sequences of hexoses (Gronow, et al., 2001).

The two factors that lead to the impermeability of the outer membrane are on the one hand that the fatty acids of the LPS are all saturated. This leads to a very stable conformation that is only marginal fluid even at 37°C. On the other hand the LPS is rich in negative charges that

can bind divalent cations such as Mg^{2+} and Ca^{2+}. The electrostatic interactions lead to an even higher bonding between the adjacent molecules and stabilize the membrane even more (Nikaido, 2003).

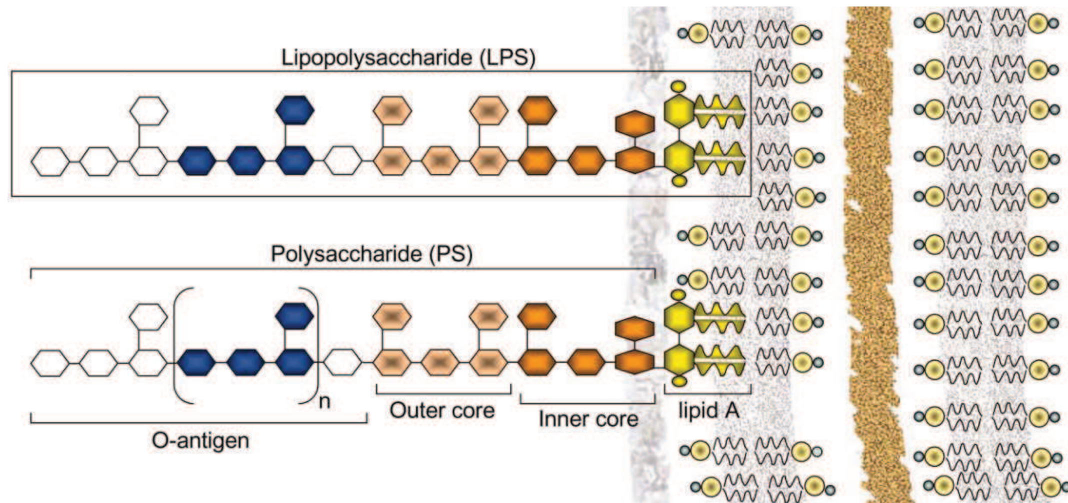


Figure 2.7: outer membrane and LPS

http://jdr.sagepub.com/content/vol84/issue7/images/large/DARVEAUX1.jpeg

This increased rigidity of the outer membrane dampens the diffusion rate of lipophilic substances like for example macrolides, hydrophobic β-lactams and so on. At the same time the LPS also forms a weak spot in the bacterial defense. Polycationic antibiotics like polymyxins can form complexes with the LPS chains instead of the stabilizing divalent cations and thus lead to an increased instability (Nikaido, 2003). Beside the impermeability the LPS also grants other advantages to Gram-negative bacteria, like increased protection against phagocytosis and the complement system of circulating antibodies in a mammalian body (Mäkelä, et al., 1980).

The outer membrane hosts also several important proteins like Braun's lipoproteins, OmpA, OmpF, OmpC and many others (Koebnik, et al., 2000). Both OmpA and Braun's lipoprotein participate in the cohesion of the outer membrane and also the integrity of the cell form.

OmpF and OmpC belong to another important group of proteins, the so-called porins. Their expression is strictly regulated and they are strongly bound to the peptidoglycan and

LPS. Since the outer membrane as a lipid bilayer forms an effective barrier for any hydrophilic substrate, the cell needs another way to let the, often hydrophilic, nutrients enter the cell. It carries therefore porins in its outer membrane that form hydrophilic channels that allow these substrates to pass across the membrane. The porins are assembled as trimers, of which each monomer forms a hydrophilic pore in the membrane that allows molecules of about 600Da to pass. However it is hard to determine what substrates and especially what antibiotics may pass the porins. In *Enterobacteriaceae*, it seems that small hydrophilic molecules (beta-lactams, tetracyclines, chloramphenicol, fluoroquinolones) are able to pass through the porins (Nikaido, 2003). Other bacteria like *P. aeruginosa* produce porins with lower permeability that are resistant to several of these antibiotics (Hancock, et al., 2002). Big lipophilic molecules (macrolides, novobiocin) on the other side cannot pass through the porins and thus have to take the slower way by diffusion through the lipid bilayer of the membrane (Nikaido, 2003). Another example for protein channels found in the outer membrane are LamB and PhoE. LamB is a so called maltoporin that is produced in presence of maltose and maltodextrin which are the preferred substrates of this channel (Gehring, et al., 1991) (Charbit, et al., 1998). PhoE on the other hand is a anion-selective phosphoporin that is expressed in presence of a phosphate (Koebnik, et al., 2000).

2.3.3.2 Characterization of resistance mechanisms due to permeability modification in Gram-negative bacteria

As previously mentioned, the first line of protection of the bacterial cell against all harming chemicals is the cell wall. The Gram-negative cell with its asymmetrical lipid bilayer composed of lipopolysaccharides (LPS) and phospholipids forms an almost impermeable barrier for hydrophilic solutes and also slows down the transport of lipophilic substances. To insure that substances like necessary nutrients can pass the envelope, the cell possesses highly selective transport channels called porins. These porins allow the crucial exchange of information and material for the survival of cells in the environment (Benz, 2004) (Hancock, 1987) (Nikaido, 1994) (Nikaido, 2003) but they also allow antibiotics like cephalosporins to cross the bacterial envelope. In recent decades bacteria gained a broad spectrum of resistances by a combination of enzymatic reactions and modifications of membrane permeabili-

ty by alterations in porin expression (Bradford, et al., 1997) (Charrel, et al., 1996) (Chow, et al., 1991) (Dé, et al., 2001) (Thiolas, et al., 2005). Alteration of membrane permeability is not only affected by porin expression but also by active efflux via the so called efflux pumps (Bornet, et al., 2000) (Malléa, et al., 1998) (Pagès, 2004). The outer membrane is thus involved in the intrinsic resistance of Gram-negative bacteria (Hancock, 1997) (Nikaido, 2003) which will be explained in the following.

2.3.3.2.1 Regulation of Influx (transport into the cell) by porins

The outer membrane of Gram-negative bacteria is best known and understood for *Escherichia coli* . It is composed of different classes of proteins such as lipoproteins like OmpA, general porins which are expressed at high level like OmpC, OmpF, PhoE, substrate specific porins like LamB and ScrY and some minor proteins (Hancock, 1987) (Koebnik, et al., 2000) (Nikaido, 2003).

Porins are pore-forming proteins that can be found in the outer membrane of Gram-negative bacteria but also with rich amount in mitochondria (Benz, et al., 1978). Due to their beta-barrel structure (**Figure 2.8**) with a lipophilic exterior they can integrate into the lipid bilayer core of the cell envelope (Koebnik, et al., 2000). Their hydrophilic interior allows small hydrophilic molecules up to a molecular mass of about 600Da to pass the channel and hereby cross the otherwise impermeable bacterial outer membrane. Matrix porins such as OmpF of *E. coli* are linked to the peptidoglycan (Benz, 2004) (Benz, et al., 1978). The general porins also allow the diffusion of a large scale of antibiotics including fluoroquinolones and beta-lactams (Benz, 2004).

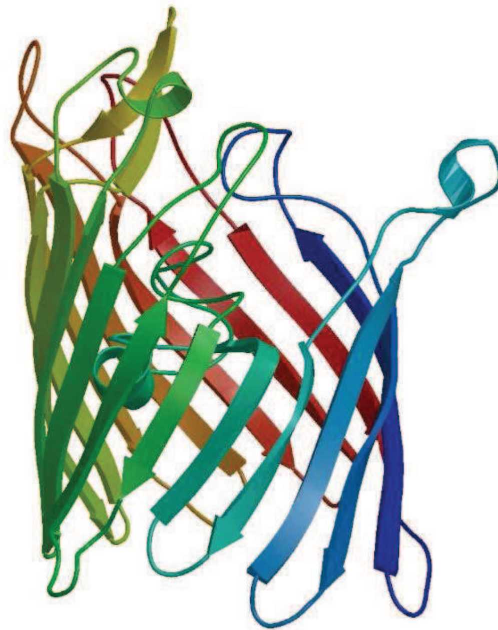


Figure 2.8: beta-barrel structure of *E. coli* porin OmpF

http://www.strubi.ox.ac.uk/strubi/gilbert/image/1OPF.jpg

Porins can be cation or anion substrate selective (Benz, et al., 1985). Four different kinds of porins are known to exist nowadays: general or non-specific porins, substrate-specific porins, gated porins and efflux porins (Hancock, et al., 2002). The expression of porins and the interaction of amino-acid residues in the water filled channel are the main constituents of membrane permeability and make the cell envelope semi-permeable (Hancock, et al., 2002) (Pagès, 2004). In the recent years crystallization and characterization of their chemico-physical properties has elucidated the relation between structure and function of the porins and hereby illustrated their role in membrane permeability (Baslé, et al., 2006) (Cowan, et al., 1992) (Schulz, 2002).

OmpF and OmpC are the most studied non-specific porins in *E. coli*. They are expressed depending on osmolarity, OmpC if the osmolarity is high, OmpF if the osmolarity is low (**Figure 2.9**). (Alphen, et al., 1977). They are known to build homotrimeric anti-parallel beta-barrels with 16 beta-strands hydrogen bonded to the nearest neighbors along the chain. The polypeptide loops line the inner barrel wall and therefore restrict the pore size. Loop 3 is the only internal loop that forms the so called constriction zone inside the channel

and therefore limits the diffusion of substrates (Baslé, et al., 2006) (Cowan, et al., 1992) (Jeanteur, et al., 1994). In *E. coli* OmpF it measures 7 x 11 Å.

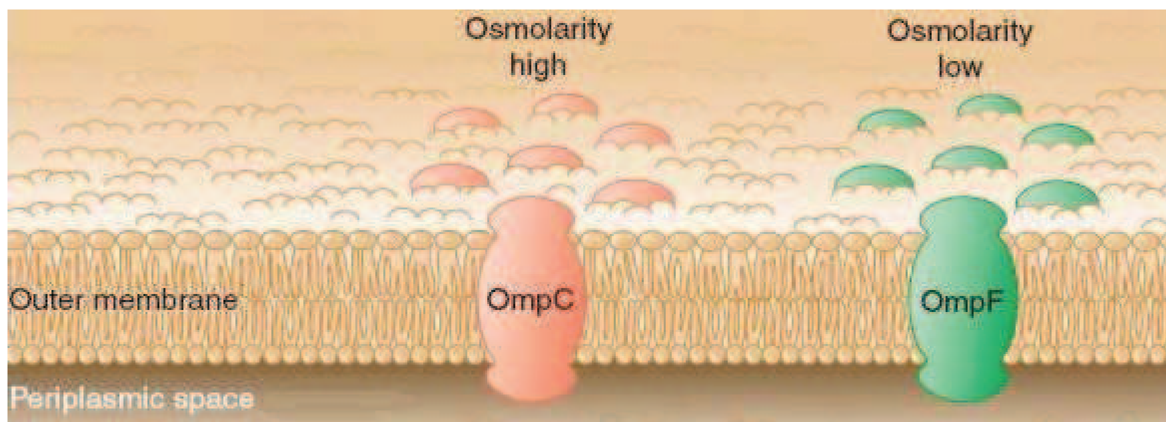


Figure 2.9: Expression of OmpF and OmpC depends on environment osmolarity

http://www.uic.edu/labs/kenneyl/index.shtml

The homologues of OmpF and OmpC are OmpK35 and OmpK36 in *Klebsiella pneumoniae* and Omp35 and Omp36 in *Enterobacter aerogenes* (Malléa, et al., 1998) (Bornet, et al., 2004). Despite *E. aerogenes* important role as one of the most frequently described Gram-negative pathogens for nosocomial respiratory tract infections and the prominent role of porins in drug susceptibility, only a few *E. aerogenes* porins are functionally characterized nowadays. Besides the already mentioned Omp35 and Omp36 these are OmpA and OmpX

The function of OmpA is not yet fully known but seems mainly to play a role as membrane anchors and is responsible for the stabilization of the bacterial envelope (Wang, 2002). Characterization of OmpA by reconstitution into lipid bilayer has given some controversial arguments whether the protein is pore-forming with two different states of conductance (Arora, et al., 2000). However, the structure of OmpA suggests that the protein is not a pore but has large water-filled cavities (Pautsch, et al., 1998) (Pautsch, et al., 2000).

OmpX is a small outer membrane protein of 18kDa that was identified to be related to virulence by promoting bacterial adhesion and entry into mammalian cells (Vogt, et al., 1999). Its structure is closely related to the eight beta-strand OmpA (Wang, 2002). In *Enterobacter aerogenes* the overexpression of OmpX is known to lower the production of OmpF

and therefore alter the level of resistance to beta-lactams (Gayet, et al., 2003) (Dupont, et al., 2004).

2.3.3.2.2 Role of porins in the resistance to antibiotics

The regulation of porin expression and the diffusion mechanisms of substances through the pore may play the key role in the resistance to antibiotics in bacteria (Benz, 2004). Three porin mechanisms have been reported in response to antibiotics: decrease of porin expression, alteration of expressed porin-type and porin mutation (Pagès, 2004) (Pagès, et al., 2008).

In *K. pneumoniae* the loss of porins associated with the production of ESBL has been described to cause the resistance to cephalosporins of the last generation and to carbapenems (Bradford, et al., 1997) (Martinez-Martinez, et al., 1999).

In the case of members of the family *Enterobacteriaceae*, resistance to new beta-lactam compounds can be frequently associated with alterations of envelope permeability (Charrel, et al., 1996). Earlier studies suggested the loss of a 40kDa outer membrane protein to be responsible for the transport of imipenem across the outer membrane of *Enterobacter aerogenes* (Chow, et al., 1991). Diminished membrane permeability due to porin alterations in conjunction with an enzymatic barrier has been proven to be a way to lower intracellular concentrations of antibiotics and therefore increase resistance (Malléa, et al., 1998).

Porin alterations, especially in its putative L3-loop region, are known to alter gravely the biophysical characteristics like single channel conductance, ion-selectivity or voltage sensitivity (Dé, et al., 2001). Structural mutation of the residue Gly112 to Asp in the conserved eyelet of the *Enterobacter aerogenes* Omp36 porin was characterized to be involved in the high resistance to beta-lactams (Thiolas, et al., 2004).

The substitution Gly119Asp in the *E. coli* porin OmpF also caused a decrease of conductance and increased cation selectivity and gating potential (Jeanteur, et al., 1994). This mutation led to a high resistance to beta-lactams associated with a decrease of cephalosporin diffusion through OmpF (Simonet, et al., 2000). Due to similarities in structure and function of OmpF to OmpK35 of *Klebsiella pneumoniae* and Omp35 of *E. aerogenes* (Bornet, et

al., 2004) we assume likewise changes for interactions between antibiotic and porin for these porins.

2.3.3.2.3 Regulation of Efflux (transport out of the cell) by efflux pumps

The complex cell envelope of Gram-negative bacteria that is almost impermeable for a large variety of compounds requests various protein channels involved in transport across the membrane. Beside the already mentioned porins which allow nutrients like sugar and salt to enter the cell (influx), so called efflux pumps contribute to decrease the intracellular concentration of toxic compounds like drugs or detergents and are consequently involved in the control of antibiotic susceptibility.

The first drug-pumping protein to be reported was the plasmid-encoded tetracycline resistance Tet protein in 1980 (Nishino, et al., 2001). Five different families of drug extrusion translocases or efflux pumps have been identified based on sequence similarity. These are the MFS (major facilitator superfamily), SMR (staphylococcal multi-resistance) family, RND (resistance nodulation division) family, ABC (ATP-binding cassette) family, and MATE (multi-drug and toxic compound extrusion) family (Nishino, et al., 2001) (Piddock, 2006) (**Figure 2.10**).

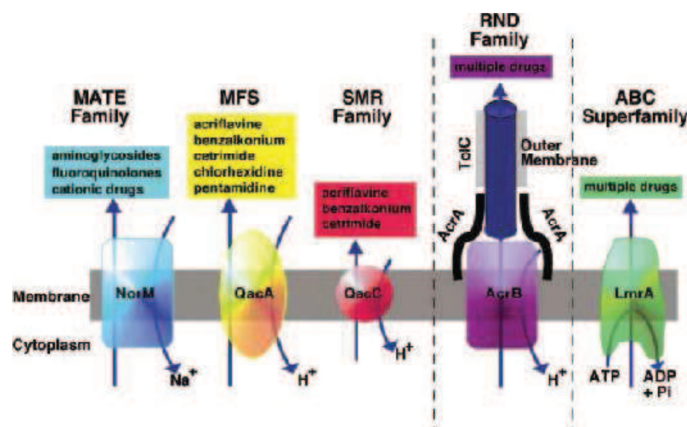


Figure 2.10: Diagrammatic comparison of the five families of efflux pumps (Piddock, 2006), courtesy of Melissa Brown)

The resistance nodulation division (RND) efflux systems have a major role in both intrinsic and acquired multi-drug resistance in Gram-negative bacteria (Blair, et al., 2009) and are the most important chromosomally encoded bacteria efflux pump in the *Enterobacteriaceae*. The resistance nodulation division is situated within the inner membrane in Gram-negative bacteria and functions in complex with two other proteins, an outer membrane channel and a periplasmic adaptor protein, to form a tripartite efflux pump spanning both inner and outer membrane that works by proton motive force (**Figure 2.11**).

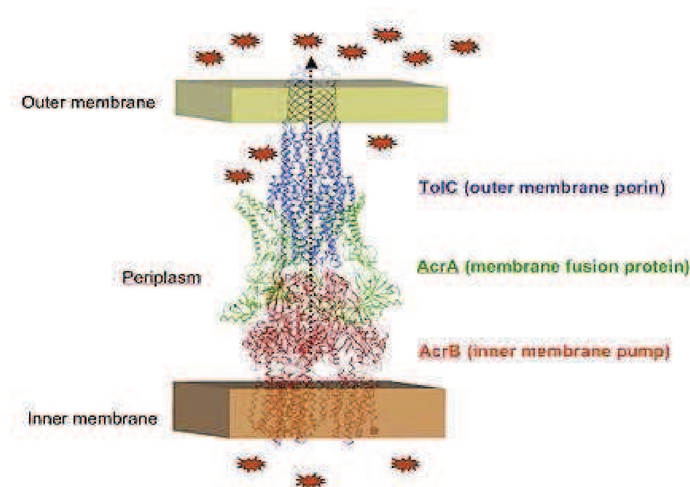


Figure 2.11: Schematic diagram of a tripartite RND system, AcrAB-TolC of *E. coli*

http://www.mpexpharma.com/images/illustration.gif

AcrAB-TolC of *E. coli* is the best described and most understood RND efflux pump and therefore will act as model in the following. The translocation site is located in the cytoplasmic part, AcrB in the *E. coli* model. The triplet of charged amino acid residues responsible was recently located in the fourth and tenth transmembrane domain (Murakami, et al., 2006) (Takatsuka, et al., 2006) (Seeger, et al., 2009). The periplasmic domain, TolC in the *E. coli* model, is partially responsible for the substrate specificity (Yu, et al., 2003) (Yu, et al., 2005). It is thought that the transporter protein AcrB captures its substrates either from within the phospholipid bilayer of the inner membrane or from the cytoplasm, and then transports them to the extracellular medium through TolC, which forms a channel in the outer membrane (Eswaran, et al., 2004) (Piddock, 2006). The periplasmic adaptor proteins (PAP), AcrA in the *E. coli* model, were formerly described as membrane fusion proteins. Their

function is yet not fully understood and PAPs of other organisms cannot complement each other's function (Tikhonova, et al., 2002), but they are supposed to mediate the cooperation between the cytoplasmic and the periplasmic domain.

2.3.3.2.4 Role of Efflux in the resistance to antibiotics

It is known that the increase of the expression of efflux pump genes is one of the first steps of a bacteria to become fully resistant, not an additive mechanism like target gene mutation or enzymatic resistance (Viveiros, et al., 2007) (Piddock, 2006). Efflux decreases the intracellular concentration of an antibiotic or other harmful drug and hereby allows a strain to survive even more if it is coincident with a drastic reduction in drug uptake due to the loss of porin in the outer membrane (Thiolas, et al., 2005).

The fact that efflux played an important part in the intrinsic resistance against antibiotics was first described in 1994 for *P. aeruginosa* (Li, et al., 1994). Intrinsic resistance to certain antibiotics or antimicrobial agents in general is conferred by basal level of efflux. In contrast resistance to harmful substrates can also be obtained by increase in expression of the efflux-pump protein. The drug substrate profile of bacteria overexpressing efflux pumps typically includes a broad range of structurally unrelated molecules, for example antibiotics like chloramphenicol, quinolones (like nalidixic acid, ciprofloxacin and norfloxacin) and tetracycline (Piddock, 2006). The drug substrate profile for the previously described RND-family AcrAB-TolC efflux pump of *E. coli* includes the antibiotics chloramphenicol, fluoroquinolones, fusidic acid, beta-lactam antibiotics, nalidixic acid, novobiocin, rifampicin and tetracycline as well as acriflavine, ethidium bromide, bile salts, short-chain fatty acids, SDS, Triton X-100 and triclosan (Okusu, et al., 1996) (White, et al., 1997) (Nikaido, 1998) (Fernandes, et al., 2003).

Like in *E. coli* the best described efflux pump of *Enterobacter aerogenes* is also AcrAB-TolC. As in *E. coli*, AcrAB-TolC in *E. aerogenes* shows a broad spectrum of transported substrates including detergents and structurally unrelated antimicrobial agents such as quinolones, tetracyclines and chloramphenicol (Poole, 2005) (Piddock, 2006) (Pradel, et al., 2002). The expression of an efflux pump in multi-drug resistant *E. aerogenes* strains has also been demonstrated to contribute to a severe decrease in intracellular concentrations of various antibiotic classes (Davin-Regli, et al., 1996) (Charrel, et al., 1996) (Malléa, et al., 1998)

(Bornet, et al., 2000) (Gayet, et al., 2003) (Thiolas, et al., 2005). A partial deletion of efflux pump genes leads to increased susceptibility to a wide range of antibiotics like chloramphenicol, norfloxacin, ciprofloxacin, tetracycline, mitomycin and acriflavine (Pradel, et al., 2002). Treatment of clinical *E. aerogenes* isolates with imipenem increased resistances against structurally unrelated antibiotics norfloxacin, chloramphenicol and tetracycline conjointly with increased expression of the efflux pump protein *acrA* (Bornet, et al., 2003). The activity of efflux pumps in various resistant clinical isolates has been characterized by using different efflux pump inhibitors that for example compete with the antibiotic efflux or that lead to the collapse of the membrane potential required for the active antibiotic transport (Pagès, et al., 2005) (Malléa, et al., 2002). Due to their poly-specificity, efflux transporters confer a general resistance phenotype that can drive to the acquisition of additional mechanisms of antibiotic resistance such as target mutation or secretion of enzymes that degrade antibiotics and also reinforce the effect of these acquired mechanisms (Piddock, 2006) (Chevalier, et al., 2008).

2.4. Genetic regulation of membrane permeability

The complex genetic regulation cascade of *Enterobacterial* pathogens is explained with the help of the review 'Membrane permeability and Regulation of Drug "Influx and Efflux" in *Enterobacteria*l pathogens' on the following pages.

Review

Membrane Permeability and Regulation of Drug "Influx and Efflux" in *Enterobacteria*l Pathogens

Davin-Regli A., Bolla JM., James CE, Lavigne JP, Chevalier C,
Garnotel E, Molitor A, Pagès JM

Current Drug Targets, 2008, 9, 750-759

CHAPTER III:

RESULTS

3.1 Article 1

Ram locus is a key regulator to trigger MDR in *Enterobacter aerogenes*

Molitor A, Farrell-Ward S, James CE, Fanning S.,

Pagès JM, Davin-Regli A

presented as manuscript

3.2 Article 2

How beta-lactam antibiotics enter bacteria: a dialogue with the porins

James CE, Mahendran KR, Molitor A, Bolla JM, Bessonov AN, Winterhalter M, Pagès JM

PLoS One. 2009, 4(5):e5453

3.3 Article 3

Towards screening for antibiotics with enhanced permeation properties through bacterial porins: proof of concept

Hajjar E, Bessonov AN, Molitor A, Kumar A, Mahendran KR, Winterhalter M, Pagès JM, Ruggerone P, Ceccarelli M

presented as manuscript

3.4 Supplementary Results

Characterization of differences in translocation of three different carbapenems through the porin OmpF and two of its structurally altered mutations by the 'killing assay' method

3.4.1. Introduction

Carbapenems, like other beta-lactam-antibiotics, need to enter the target cell to work. In Gram-negative bacteria beta-lactam-antibiotics use porins to cross the bacterial membrane. This translocation is the first step of antibiotic action. A variation of the membrane permeability, consistent of transport into the cell (influx) and out of the cell (efflux), is therefore the first way for a bacteria to regulate antibiotic action. In many multi-drug-resistant bacteria the phenotype is linked to altered membrane permeability, characterized by porin modification and efflux pump expression.

In this work, the influx of three different carbapenems, imipenem, ertapenem and meropenem (**Figure 3.1**), through the *Escherichia coli* porin OmpF is studied. To better understand the exact mechanism and drug translocation through the porin two mutations, constructed by substitution of single amino-acids located in the lumen of the porin, were also examined and compared to the wild-type porin.

The rate of translocation was determined by measuring the so called bacterial 'killing rate' of the different antibiotics on bacterial cells. Here, a certain concentration of an antibiotic is given to a culture with a calculated concentration of bacterial cells and then the number of surviving cells is measured by taking the colony forming units (CFU) in certain time intervals. To exclude the possibility of transport through other porins, a porin deficient mutant of *Escherichia coli*, *E. coli* BL21 DE3 Δomp, was used. To also rule out the action of bacterial countermeasures against the used antibiotics inside the cell, three beta-lactamase inhibitors were added to the medium.

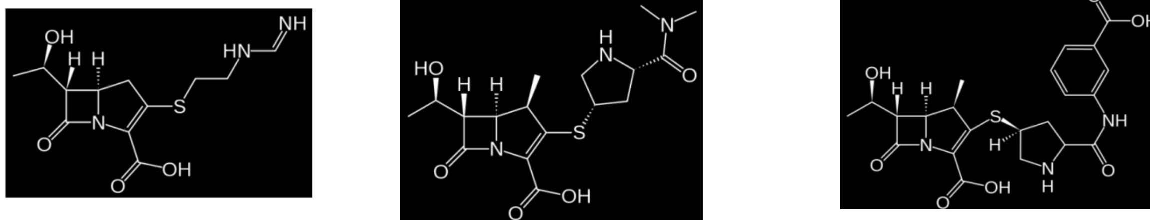


Figure 3.1: Imipenem (left), ertapenem (center), meropenem (right)

http://en.wikipedia.org/wiki/Ertapenem

http://en.wikipedia.org/wiki/Meropenem

http://en.wikipedia.org/wiki/Imipenem

3.4.2. Material and methods

3.4.2.1 Bacterial strain and culture media

The MIC experiments and the 'killing assays' were performed by using a porin null *E. coli* BL21(DE3)omp8 (Δ*lamB*, *ompF* ::*Tn5*, Δ*ompA*, Δ*ompC*) strain, referred to in the following text as BL21Δomp. Transformants of *E. coli* BL21Δomp harbored the expression vector pCold IV (4359bp) (Takara) with inserts for *ompF*-WT, *ompF*-113A and *ompF*-121A (Vidal, et al., 2005). Bacteria were grown and selected in Muller Hinton (MH) broth (Difco) and Muller Hinton (MH) agar plates (Difco) containing relevant antibiotics (kanamycin (50µg ml^{-1}) and ampicillin (100µg ml^{-1}) (Sigma)) (James, et al., 2009).

3.4.2.2 Minimum inhibitory concentration (MIC)-assay

The BL21Δ*omp* cultures harboring the empty plasmid pColdIV, pColdIV*ompF*-WT, pColdIV*ompF*-D113A or pColdIV*ompF*-D121A, were grown to OD_{600} 0.4 in MH containing appropriate antibiotics. Cultures were split into two tubes, one tube was induced with IPTG (1mM) for 1 hour. IPTG was omitted for the control. After the induction the bacteria were subcultured into MH broth with or without IPTG (0.5mM) and the beta-lactam inhibitors tazobactam, clavulanic acid and cloxacillin (4µg ml^{-1}), to quench the activity of beta-lactamase expressed by amp gene present on plasmid (James, et al., 2009), at OD_{600} 0.001

containing no antibiotics. 2-fold dilution series of each studied antibiotic were prepared and added to 1ml aliquots of bacterial suspension in MH. Afterwards the assays were incubated for 18-24 hours at 37°C. Each assay was repeated independently at least three times and classified according to the 'Antibiogram Committee of the French Society for Microbiology' (http://www.sfm.asso.fr).

3.4.2.3 Rate of Antibiotic Action Assay ('killing assay')

BL21Δomp *E. coli* cultures harboring either pColdIV*ompF*-WT, pColdIV*ompF*-D113A or pColdIV*ompF*-D121A were prepared as for the MIC-assays. In order to accurately quantify the rate of action over a number of time points, all induced and diluted cell suspensions (OD_{600} 0.01) were exposed to 2-fold the MIC of ertapenem and meropenem (0.125µg ml^{-1}) and the exact MIC of imipenem (0.125µg ml^{-1}) for the cultures producing OmpF-WT, OmpF-D113A and OmpF-D121A. At 15 or 30min time intervals 10-fold dilution series of the exposed cultures were prepared with MH and spread on MH agar containing appropriate antibiotics. Plates were incubated overnight (18h) at 37°C and colonies were counted afterwards. The number of colony forming units (cfu ml^{-1}) was calculated for each time point and plotted as percentage decrease compared to t=0. All experiments were repeated independently at least four times.

3.4.3 Results

3.4.3.1 Minimum Inhibitory Concentration (MIC)-assay

Minimum Inhibitory concentration assays for the *E. coli* BL21 Δ*omp* recombinants harboring the empty plasmid pColdIV showed values of 4µg ml^{-1} for ertapenem, 1µg ml^{-1} for meropenem and 0.5µg ml^{-1} for imipenem (**Table 3.1**). The recombinants harboring the plasmid with *ompF* and its two mutants exhibit the same antibiotic susceptibility when not induced by IPTG (data not shown). As also seen in **Table 2.1** the MIC-values for the three recombinants with *ompF*-WT, D113A and D121A lie between 0.003µg ml^{-1} and 0.006µg ml^{-1} for ertapenem and meropenem and at 0.125µg ml^{-1} for imipenem.

	Ertapenem	Meropenem	Imipenem
E. coli* BL21Δ*omp			
pColdIV	4	1	0.5
pColdIV*ompF*-WT	0.03	0.03	0.125
pColdIV*ompF*-D113A	0.06	0.03	0.125
pColdIV*ompF*-D121A	0.06	0.03	0.125

Table 3.1: MICs in μg ml^{-1} of different carbapenems for *E. coli* BL21Δomp harboring different plasmids and induced with IPTG

3.4.3.2 Rate of Antibiotic Action Assay

The following figures present the percentage of CFU decrease obtained with the three different tested porins OmpF-WT, 113A and 121A during the incubation times in presence of the tested carbapenems ertapenem (**Figure 3.2**), meropenem (**Figure 3.3**) and imipenem (**Figure 3.4**).

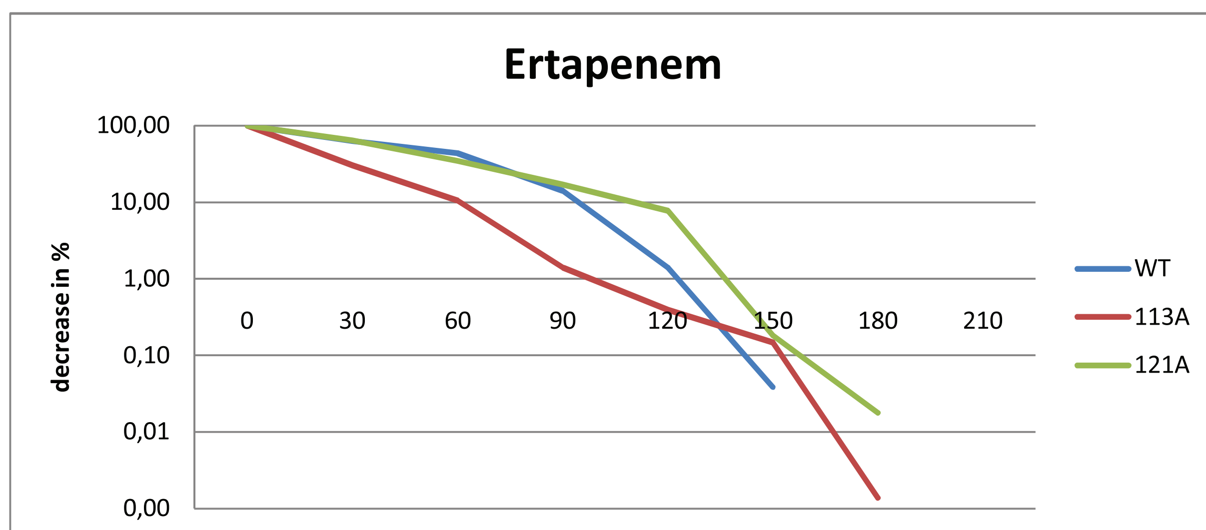


Figure 3.2: antibiotic action assay for ertapenem at concentration 0.125μg ml^{-1} (2-4-fold MIC-value) on *E. coli* BL21Δ*omp* harboring plasmids for OmpF-WT, OmpF-D113A and OmpF-D121A

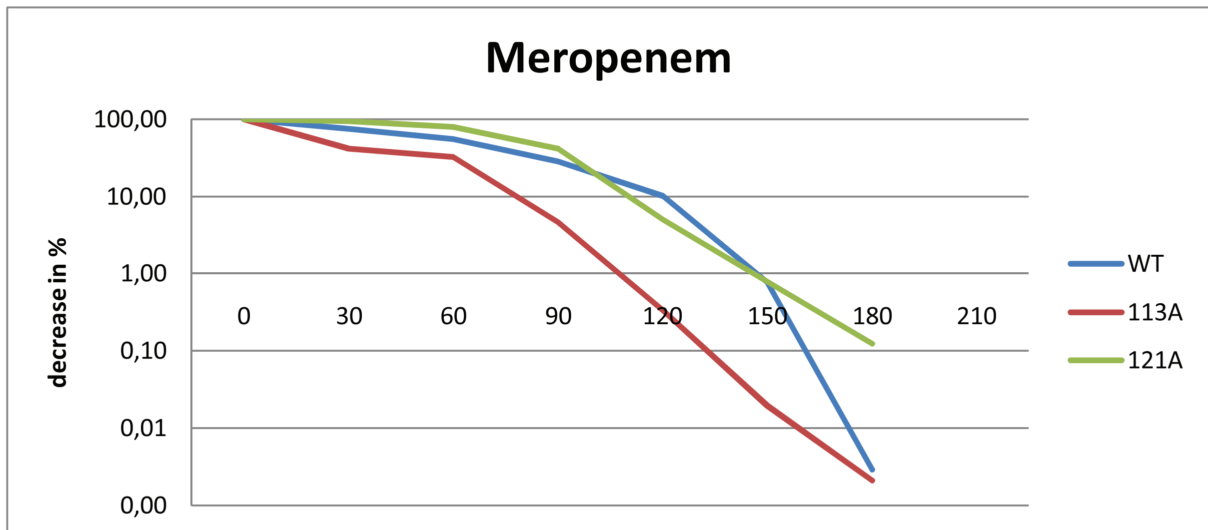


Figure 3.3: antibiotic action assay for meropenem at concentration 0.125µg ml^{-1} (4-fold MIC-value) on *E. coli* BL21 Δ*omp* harboring plasmids for OmpF-WT, OmpF-D113A and OmpF-D121A

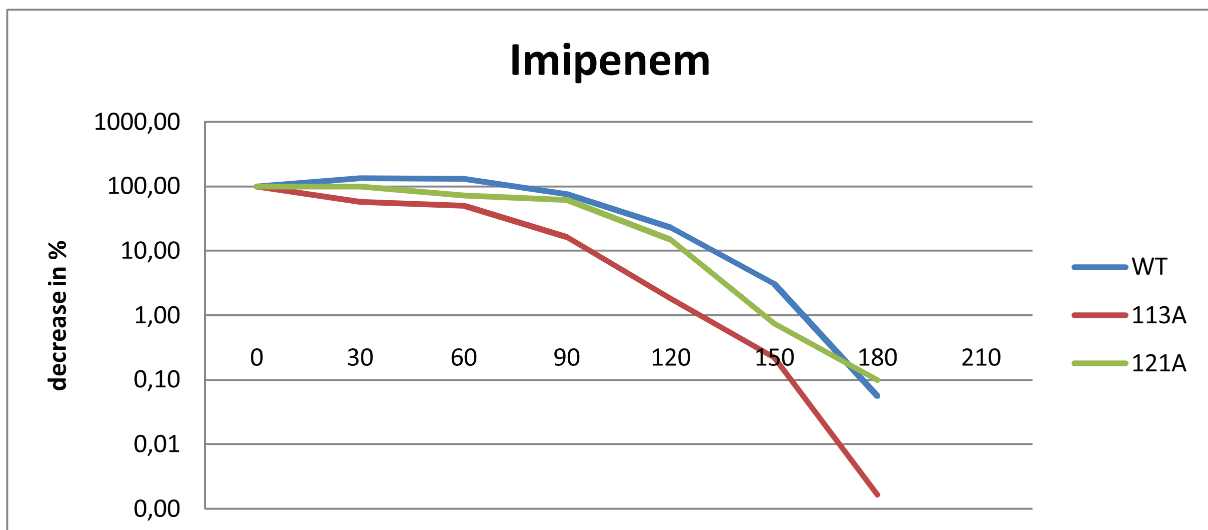


Figure 3.4: antibiotic action assay for imipenem at concentration 0.125µg ml^{-1} (1-fold MIC-value) on *E. coli* BL21 Δ*omp* harboring plasmids for OmpF-WT, OmpF-D113A and OmpF-D121A

In our approach we compared the level of susceptibility and CFU reduction by carbapenemes in bacteria producing solely OmpF-WT, 113A and 121A. With this method we focus

on the effect of the mutations of the porins in the uptake of a specific molecule. Since the background like target affinity, activity of the antibiotic on the target or action of beta-lactamases are conserved by using the same host bacterium, changes in the 'killing rate' can directly be attributed to the different porin characteristics.

3.4.4 Discussion

The results of the MIC-experiments indicate that *ompF* plays a major role in the influx of various carbapenems. The values for the three tested carbapenems vary between a 4-fold increase for imipenem, a 33-fold increase for meropenem up to a 133-fold increase for ertapenem between the porin-deficient BL21-strain bearing the empty plasmid and the recombinants expressing *ompF* or one of its mutants (**Table 3.1**).

The fact that a strain expressing porins is more susceptible to a specific antibiotic assists the previously mentioned assumption that porins play a major role in beta-lactam transport across the bacterial membrane (Pagès, et al., 2008) (James, et al., 2009).

Comparison between the values of the different carbapenems also leads to the conclusion, that differences may exist during the transport through the porin channel. A 133-fold increase of antibiotic for ertapenem between strains expressing porins and the porin deficient strain may hint to a faster translocation rate through the bacterial membrane compared to a 33-fold increase for meropenem and even more to a 4-fold increase for imipenem. Although differences in target binding between the three carbapenems play a role, we assume that these differences are also caused by the structural differences between the three carbapenems and the hereby different interactions with the charged amino acid residues inside the porin channel (Pagès, et al., 2008) (James, et al., 2009).

To define a more exact view on the involvement of structural differences of the antibiotics and the residues inside the porin-channel, the rate of antibiotic action was measured.

In presence of all three tested carbapenems, ertapenem, meropenem and imipenem, BL21Δ*omp* cultures expressing D113A as the sole porin were killed at a more efficient rate compared to those expressing the mutant 121A or the wildtype (WT) (**Figure 2.2 to 2.4**). The corresponding IC90 of D113A is reached at least about 30min before the ones obtained with D121A or WT for the three tested carbapenems.

Regarding D121A and WT only slight differences are noticeable. For meropenem IC90 is reached at 150min for both D121A and WT compared to 90-120min for 113A. WT is killed at a slightly faster rate for ertapenem (IC90 at 120min compared to IC90 at 150min for D121A), whereas D121A shows a somewhat increased killing-rate for imipenem (IC90 150min against IC90 120-150min for WT) (**Figure 2.2 to 2.4**).

These results suggest that the respective deletions of the negative charges, of COO^- side chain located at either position 113 or 121, have different effects on the killing rates for the different carbapenems tested here. The increased rate of bacteria killing when OmpF D113A is produced as sole porin compared to the wildtype or the mutant D121A, may be associated with the faster rates of influx across the mutated porin channel.

Previous studies have shown that zwitterionic compounds penetrate proteoliposomes and live cells very rapidly (Nikaido, et al., 1983) and induce increased ion current blockages through OmpF in lipid bilayer models (Danelon, et al., 2006) compared to other charged compounds. In addition, large molecules with bulky side-chains, such as azlocillin and piperacillin have shown low permeation rates (Nikaido, et al., 1983) (Danelon, et al., 2006). Single substitutions of D113A were found to dramatically increase the uptake of beta-lactam antibiotics (Simonet, et al., 2000) (Vidal, et al., 2005). The translocation of ampicillin through OmpF and the role of D113A was characterized and modeled recently (Hajjar, et al., 2010).

Another recently done study of Mahendran et al. indicated that ertapenem with its negative charges interacts more strongly with OmpF and its mutants than zwitterionic cephalosporins. The strength of these interactions (binding affinity) also varied between wildtype and mutant porins, indicating that the affinity of the antibiotics is changed by mutation.

Substitutions of D113A and D121A alter translocation pathways via an imbalance of charge distribution. In our study, the D113A mutation has been shown to increase susceptibility to ertapenem (mono anionic, positive), and both meropenem and imipenem (zwitterionic) whereas the D121A substitution shows hardly any variations at all compared to the wildtype porin. Both acid residues D113 and D121 lie on the L3-loop of the porin and are responsible for the negative side of the electrostatic field within the constriction zone of the porin that plays an important part in properly orienting molecules for passage through the porin (Cowan, et al., 1992) (Jeanteur, et al., 1994) (Baslé, et al., 2006). Previous studies have shown, that positively charged functional groups of antibiotics form strong H-bonds with the nega-

tively charged residues D113, D121 and E117 of the L3-loop of OmpF (Vidal, et al., 2005) (Danelon, et al., 2006) (Ceccarelli, et al., 2004). Many theoretical models have been developed to correlate the influence of the antibiotic interaction with the channel surface to calculate flux across the membrane (Berezhkovskii, et al., 2005) (Kasianowicz, et al., 2006) (Bauer, et al., 2006). Brezhkovski and Bezrukov 2005 presented a diffusion model proposing that (i) the antibiotic translocation is facilitated by the affinity site in the channel and (ii) there should be optimal strength of interaction between antibiotic drug and porin contributing to high translocation probability through the membrane channel.

The expression of OmpF and its two mutants D113A and D121A now provided the opportunity to test the ability of three different carbapenems to cross the bacterial outer membrane via these channels. Our MIC data explains the role of the porins in drug uptake: the bacterial cells were more susceptible to all three tested antibiotics when a porin was expressed. This indicates the important role of porins for transport across the bacterial membrane of antibiotics. The assay for antibiotic activity showed faster killing rates for ertapenem, meropenem and imipenem with the D113A mutant compared to the wildtype and D121A. No significant difference could be observed between the wildtype and D121A. We assume that the substitution of the negative charged residue at position 113 has a stronger effect for the carbapenem-porin-interactions and the carbapenem translocation through the channel than the substitution at position 121.

Special thanks to Kozhinjampara R. Mahendran. Without his provided data about the biophysical characteristics of the different OmpF porins, the discussion of the gained results would not have been possible.

CHAPTER IV:
OUTLOOK

4.1 Future investigation and characterization of the *ram*-regulon

In the future we aim to process further investigations on the role of the global regulator RamA in the control of membrane permeability and the MDR phenotype. The role of the repressor RamR is still poorly understood and the possible effect of the different mutations found in MDR clinical isolates and laboratory strains is yet to verify. Computer modeling of the structure of the protein and the changes induced by the mutations seems to be a good way to understand if conformational changes might alter the characteristics and/or functionality of the mutated proteins.

It is also necessary to clarify the binding regions of *ramR* or *ramA* to be able to understand the exact regulation and self-regulation mechanisms of the *ram*-regulon. The role of the *marbox* in regulation of *ram* is yet unclear, studies have to be made about the binding properties of *ramA* and homologous transcription activators *mar*/*sox*/*rob*. Another aim is to sequence the promoter regions of *ramR* to identify possible variations here that could alter transcription and expression.

An interesting possibility might be the production of knockout mutants without *ramR* and/or *ramA*. MIC results of normally antibiotic susceptible strains producing no more *ramR* might give new insights into the role of *ramR* in development of the MDR phenotype. The effect of a knockout of *ramA* in strains normally showing MDR might also alter their phenotype. Validation of gene expression by qRT-PCR and testing with antibodies against porins and efflux pump components with these knockout strains will give even more insights in the role of *ramA* and *ramR*.

4.2 Purification of *E. aerogenes* porins Omp35 and Omp37 for biophysical experiments

To better understand the role of *Enterobacter aerogenes* porins in antibiotic susceptibility, a multi-disciplinary approach might be helpful. Combination of structural analysis, black lipid bilayer measurements and biological assays will give new insights in drug transport through porins and interactions between antibiotic and the porin channel. We already started with studies on Omp36 as presented in the results. Furthermore we purified Omp37 (data not shown), the porin is now ready for biophysical measurements in artificial bilayers

or liposomes. Purification of Omp35 was not successful yet. The crucial step seems to be the release of the porin out of the separated outer membrane. Several detergents need to be tested in the future.

4.3 Biophysical characterization of OmpF mutants D113A and D121A

After the biological assays to determine interactions between the OmpF porins WT, 113A and 121A now a biophysical characterization has to occur. We already purified OmpF and its two mutants (**Figure 4.1**) and the first measurements were done as explained in the results.

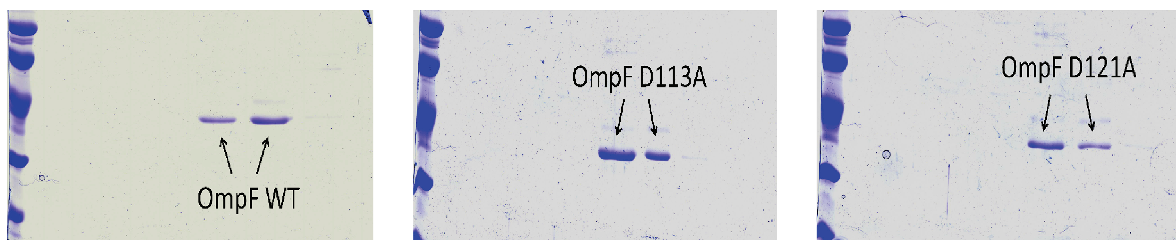


Figure 4.1: Purified OmpF WT, D113A, D121A

The next step will be to test several other antibiotics and their interaction with the porin channel to gain new insights in translocation and the exposed status of the constriction zone with the L3-loop.

CHAPTER V:

DISCUSSION -CONCLUSION DE THESE

5.1 Discussion

In this thesis we studied the outer membrane permeability and its role in antibiotic resistance of Gram-negative bacteria in general. We adjusted a special focus on the regulation mechanisms at the genetic level in *Enterobacter aerogenes* that lead to modification of membrane permeability and also on the function of porins of *E. aerogenes* or *E. coli* in antibiotic uptake.

In *Enterobacter aerogenes* several regulatory proteins are known to be implicated in development of MDR. Control of efflux pumps and porin expression, which together alter the permeability of the outer membrane, is carried out on several levels. A special role is fulfilled by the so-called global regulators which coordinate the expression of several genes. The global regulator *ramA* plays a special role in the regulation cascade of *Klebsiella*, *Salmonella* and *Enterobacter* since it is not only a self-governing activator of the MDR cascade but also a transcriptional activator of the global regulator *marA*.

A putative repressor of *ramA*, named *ramR*, has been identified and sequenced in *E. aerogenes*. Sequence analysis revealed several mutations for *ramR* in various clinical isolates and laboratory strains showing the MDR-phenotype compared to the laboratory reference strains that show normal antibiotic susceptibility. The found alterations are not located in the DNA-binding region, but some are located at the C-Terminus of the protein. This region is supposed to play a role in the dimerization of the protein. Incapability to dimerize would leave the protein unable to bind to DNA and therefore inactive. Other found substitutions are known to be able alter the structure of the protein. Modifications in activity due to structural changes can't be excluded.

Investigation of *ramA* revealed that a putative self-regulatory protein binding cassette called *marbox* is present in all strains. A deletion between the *marbox* and the start codon of *ramA* is just present in the majority of the multidrug resistant strains. No alterations in *marA* or *marR* were found at all, despite numerous descriptions of *marR/O* mutations characterization in *E. coli* MDR strains. The role of the *marbox* in regulation of *ram* is yet unclear, studies have to be made about the binding properties of *ramA* and homologous transcription activators *mar*/*sox*/*rob*. A deletion around the binding site however could alter the binding properties, inactivate self-regulation, hereby lead to an increased expression of *ramA* and thus activate MDR.

Validation of expression of different regulators, porins and efflux pump components revealed a several times higher expression for *ramA* in about 90% of the MDR strains. Expression of *ramR* was also increased, but not as much as *ramA*. The expression level of *marA* and *marR* just showed marginal variations. All multidrug-resistant strains showed increased expression of efflux pumps and decreased expression of porins. The results confirm the role of RamA as a super regulator in multidrug-resistance. Increased expression of *ramA* leads to increased expression of efflux pump components and decrease of porins. Marginal variations in the expression level of MarA indicate that RamA plays a major role and is able to directly modify efflux pump and porin content in the membrane. An interaction between the two regulating systems can't be excluded, but RamA does not seem to need increased expression of MarA to regulate membrane permeability on its own.

Cloning of *ramA* on a multicopy plasmid resulted in an increasing resistances to antibiotics, reduced expression of porins and increased expression of efflux pump components. *ramR* had the contrary effect. No differences were remarked between wildtype or mutated *ramR*. The effect of overexpressed *ramA* confirms former results about RamA as global regulator of membrane permeability and MDR. Increased expression of *ramA* leads to an increase of efflux, decrease of influx and hereby to a cutback of the intracellular concentration of harmful substances inside the cell. Results of experiments with overexpressed *ramR* enforce the theory of *ramR* as repressor of *ramA*. High amount of *ramR* due to a high-copy plasmid inside an otherwise antibiotic-susceptible cell had no remarkable effect. In a bacteria that gained resistance to imipenem by alteration of its membrane profile, *ramR* reconstituted the antibiotic susceptible phenotype. In bacteria that achieved multidrug-resistance by diverse mechanisms like degrading enzymes or target mutations in addition to altered membrane permeability, *ramR* just had an effect on porin or efflux pump content in the membrane, but not on antibiotic susceptibility in general. We therefore speculate that *ramR* can repress *ramA* and hereby regulates porins and efflux pumps, but has no or less effect concerning other resistance mechanisms.

The first step of antibiotic interaction with Gram-negative bacteria is to cross the outer membrane, which forms a protective barrier against hostile environments. The exact mechanism of uptake across this lipid bilayer is yet poorly understood. One major part is estimated to be fulfilled by porins, major outer membrane proteins (OMP) that form water

filled channels allowing diffusion along the membrane. The involvement of porins in the membrane permeability of *Enterobacter aerogenes* and *Escherichia coli* with focus on their contribution to antibiotic susceptibility has been investigated in this study.

The translocation of three different carbapenems through the *E. coli* porin OmpF was characterized. Especially the L3-loop in the constriction zone of the porin was investigated. Negative charged amino-acid residues here are supposed to play an important role in translocation of positive charged antibiotics. Wildtype OmpF and two mutations with the negative charged aspartates at positions 113 and 121 replaced by the neutral alanine were transformed into an otherwise porin-deficient *E. coli* strain and antibiotic activity was measured. Due to the use of beta-lactamase inhibitors and the lack of other porins, changes in antibiotic activity could be originated to the three different transformed OmpF porins. The results indicated the importance of porins in the translocation of antibiotics through the bacterial membrane. Porin deficient *E. coli* strains showed a several times higher minimum inhibitory concentration for all three tested antibiotics. This strongly suggests that carbapenems use the OmpF channel to cross the outer membrane. The alterations at position 113 resulted in a faster killing rate for all three tested antibiotics, whereas the substitution at position 121 hardly showed any differences at all compared to the wildtype-porin. We assume that the substitution of the negative charge at position 113 has a stronger effect for the drug-porin-interactions. Substitution of the negative residue lets translocate the antibiotic faster through the porin channel.

A multi-disciplinary approach combining computer modeling, artificial bilayer experiments and biological assays supports this theory. The interactions with aspartate at position 113 was identified as rate-limiting for the translocation of beta-lactam ampicillin. Mutation of D113 to alanine opens an hydrophobic pocket and therefore allows faster translocation through the channel.

Due to the detailed knowledge about its crystal structure, most studies of antibiotic-porin interactions so far have focused on OmpF from *E. coli* which is a major porin type expressed in vitro along with homologues Omp35 in *Enterobacter* and OmpK35 in *Klebsiella*. However *in vivo* temperature and salt concentrations favor the expression of OmpC-type porins including *Klebsiella pneumoniae* OmpK36 and *Enterobacter aerogenes* Omp36. Consequently these are the dominant porins in the patient body and represent the key strategic

pathways for several antibiotics to penetrate the bacterial cell during patient therapy. We contributed to a study combining high resolution ion conductance measurements with biological susceptibility assays to explore beta-lactam translocation through the *Enterobacter aerogenes* porin Omp36. Using the results of translocation of two representative beta-lactam molecules through the porin, we hypothesize that there is a strong interaction involving hydrophobic and hydrogen bonds between antibiotics and specific amino acid residues which constitute the affinity site within Omp36. The tested ertapenem has a negative net charge and two carboxylic groups are able to form hydrogen bonds with residues inside the porin channel. For the zwitterionic cefepime a lower channel affinity was measured. These results are in agreement with previous molecular modeling studies of cefepime in the constriction zone of OmpF. Biological assays agree with the electrophysiological data, showing stronger activity of ertapenem than cefepime in bacterial cells expressing solely Omp36 as porin.

5.2 Conclusion de thèse

Dans ce travail, nous avons étudié deux aspects du rôle de la perméabilité membranaire dans la résistance aux antibiotiques chez les bactéries à Gram-négatif: (i) la régulation génétique modulant la synthèse des protéines de membrane et ses effecteurs, de manière à compléter les informations acquises ces dernières années et les hypothèses sur les différents intervenants de la cascade de régulation et (ii) les interactions porine-antibiotique lors du passage de celui-ci à l'intérieur du canal. L'étude a été menée sur les porines OmpF de *Escherichia coli* et Omp36 de *E. aerogenes* responsables du passage transmembranaire des antibiotiques.

Chez *Enterobacter aerogenes*, les protéines régulatrices décrites également chez d'autres Entérobactéries comme *Salmonella* sp. ou *Klebsiella pneumoniae* ont été caractérisées et il a été montré qu'elles jouaient un rôle dans le développement de la multi-résistance aux antibiotiques (MDR). L'expression des porines et des pompes d'efflux modulent la perméabilité membranaire et sont régulées à plusieurs niveaux. Une position particulière est tenue par les régulateurs dits globaux, capables de contrôler et coordonner l'expression de plusieurs gènes. Ce type de régulateur est représenté par *ramA,* identifié chez diverses Entérobactéries à l'exception de *E. coli* et intervenant dans la résistance comme dans la virulence chez *Klebsiella*, *Salmonella* et *Enterobacter*. RamA occupe une place privilégiée dans la mesure où il est à la fois un activateur autonome de la cascade régulatrice de la MDR, mais aussi un activateur secondaire d'un autre régulateur MarA, permettant la possible mise en place d'une boucle d'amplification de la MDR.

Les activateurs et répresseur de MarA sont connus et ont été décrits dans plusieurs travaux, cependant peu d'informations relatives à la régulation de RamA sont définies à ce jour. Néanmoins, le répresseur présumé de *ramA*, nommé *ramR*, a été identifié et séquencé dans ce travail, chez *E. aerogenes*. L'étude de la séquence de RamR des 60 souches sélectionnées et présentant un phénotype MDR a permis d'identifier plusieurs mutations en comparaison à la séquence de la protéine des souches sensibles aux antibiotiques. Les substitutions trouvées ne sont pas situées dans la région nécessaire à la fixation de la protéine à l'ADN, mais se trouveraient dans la région responsable de la dimérization de la protéine, qui appartient à la famille des régulateurs TetR dont les membres fonctionnent en dimères. Il en résulterait une incapacité de la protéine mutée à se fixer à l'ADN. Les autres substitutions

identifiées pourraient modifier la structure de la protéine, avec comme conséquence une modification dans l'activité.

L'investigation de la région promotrice en amont de *ramA* a révélé la présence d'un domaine de reconnaissance des protéines de régulation de la famille Arc/XylS, nommé *marbox*. Une délétion entre la *marbox* et le codon start de *ramA* est présent dans la majorité des souches MDR. Aucune variation dans *marA* ou *marR* n'a été trouvée, en dépit des descriptions nombreuses de mutations au niveau de *marR/O* chez des isolats de *E. coli* MDR. Le rôle exact de la *marbox* dans la reconnaissance de *ramA* à ses cibles est encore flou et à démontrer. De nouvelles études sont nécessaires pour clarifier la fixation de *ramA* et des autres régulateurs homologues comme *mar/sox/rob* au niveau des promoteurs des différentes cibles. Une délétion autour du site de fixation pourrait moduler les propriétés de fixation de *ramA* à ses cibles malgré une expression significativement augmentée en réponse aux antibiotiques.

La validation de l'expression de quelques régulateurs, des gènes des porines et des protéines des pompes d'efflux a montré, par PCR en temps réel, une expression de *ramA* augmentée significativement dans 90% des souches MDR testées. L'expression de *ramR* était également augmentée, mais pas au même niveau que *ramA*. L'expression de *marA* et *marR* est augmentée de façon marginale chez ces mêmes souches. Tous les souches présentaient une expression accrue des transcrits *tolC* et *acrB* et à l'inverse une diminution pour *omp35*. Les résultats confirment le rôle de RamA comme une super-régulateur de la multi-résistance aux antibiotiques par modification de la perméabilité membranaire chez *Enterobacter aerogenes*. La variation d'expression de *marA* reste marginale chez *Enterobacter* et ne semble donc pas générer une boucle d'amplification de la MDR. Une interaction entre les deux régulateurs ne peut pourtant pas être exclue étant donné la présence des *marbox* sur chaque promoteur, mais il semble que *ramA* n'ait pas besoin de *marA* pour être pleinement opérationnel chez les souches MDR.

Le clonage de *ramA* dans un plasmide multi-copie a eu pour conséquence une modification des la perméabilité membranaire et une MDR. Le gène *ramR* cloné a généré l'effet contraire et confirme ainsi qu'il est le gène codant pour répresseur de RamA. Aucune différence n'a pu être notée entre le répresseur RamR type sauvage et les produits des gènes où des mutations ont été identifiées. Les différentes protéines MarR surexprimées génèrent

chez des souches initialement MDR, un retour vers la sensibilité. Chez les souches exprimant des mécanismes de résistance divers, en particulier enzymatiques, la restauration de la sensibilité est très partielle, mais une modification d'expression des porines et des pompes d'efflux est systématiquement observée. RamR est capable de restaurer le niveau initial d'expression des protéines d'enveloppe impliquées dans la résistance par inhibition de RamA.

La première démarche de l'interaction d'un antibiotique avec une bactérie Gram-négative est la traversée de la membrane externe, formant une barrière protectrice contre l'environnement extérieur. Le mécanisme exact de l'admission à travers la membrane bactérienne, de l'antibiotique est jusqu'à présent mal compris. Le rôle des porines, protéines formant des canaux hydriques permettant la diffusion des substances hydrophiles, est primordial. Le rôle des porines de *E. aerogenes* et de *E. coli* et leur contribution dans le passage des antibiotiques a été également examiné dans ce travail.

Tout d'abord, la translocation de trois carbapénèmes au sein de la porine OmpF de *E. coli* a été étudiée. Le rôle sélectif dans le passage interne, de la boucle L3 présente dans la zone de constriction de la porine, a été examiné. La porine OmpF sauvage et deux mutantes substituées par des aspartates chargés négativement en position 113 et 121 à la place d'une alanine ont été clonées dans une souche déficiente en porines. Les 3 antibiotiques subissent bien une translocation dans les 3 porines. Les altérations en position 113 ont eu pour conséquence l'augmentation de la vitesse de bactéricidie associée à une diminution des CMIs à ces antibiotiques chez les souches transformées. On suppose que la substitution 113 génère de plus fortes interactions facilitant le passage de l'antibiotique au travers de la porine et ainsi une entrée accélérée. Une approche multidisciplinaire entre bioinformatique, modélisation, membranes artificielles et microbiologie confirme cette observation. Dans la deuxième partie de ce travail sur le rôle des porines dans la diffusion des antibiotiques, l'interaction entre l'aspartate en position 113 a été également confirmée comme favorisante dans la vitesse de translocation des pénicillines type ampicilline au sein de la porine OmpF. La substitution D-113-A ouvrirait une poche hydrophobe au niveau de la zone de constriction et accélèrerait ainsi le transport par le canal étroit.

A partir de nos connaissances sur l'impact de la structure de la porine OmpF de *Escherichia coli* dans le passage des β-lactamines, des expériences similaires d'interaction

entre porine et antibiotiques ont été menées sur la porine Omp35 de *Enterobacter aerogenes* et OmpK35 de *Klebsiella pneumoniae*. Cependant, *in vivo* les conditions physiologiques favorisent l'expression de la porine de type OmpC de *E. coli* ou de ses homologues Omp36 de *E. aerogenes* et OmpK36 de *Klebsiella pneumoniae*. L'étude a donc été menée par la suite, sur la porine Omp36 de *Enterobacter aerogenes*. La combinaison des mesures de conductance dans des membranes artificielles en haute résolution avec des essais biologiques mesurant l'activité des antibiotiques nous ont apporté une nouvelle compréhension sur le transport des beta-lactamines via Omp36 de *E. aerogenes*. Nous avons relevé des interactions hydrophobes et des liaisons hydrogène fortes entre l'antibiotique et les résidus des acides aminés au niveau du site d'affinité dans Omp36. L'ertapénème utilisé dans ces tests possède une charge nette négative et deux groupes carboxyliques qui peuvent former des connections hydrogènes avec les résidus à l'intérieur du canal. Pour le céfépime, molécule zwitterionique une affinité inférieure au niveau du canal a été relevée. Les résultats coïncident avec des études antérieures sur le passage de céfépime dans OmpF de *E. coli*. Les essais biologiques coïncident avec les données électrophysiologiques, montrant que l'activité de l'ertapénème est plus forte que celle de l'imipénème chez les bactéries exprimant seulement Omp36 comme porine.

CHAPTER VI:

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FINAL

Membrane Permeability and Regulation of Drug "Influx and Efflux" in Enterobacterial Pathogens

Anne Davin-Regli, Jean-Michel Bolla, Chloë E. James, Jean-Philippe Lavigne, Jacqueline Chevalier, Eric Garnotel, Alexander Molitor and Jean-Marie Pagès*

UMR-MD-1, Transporteurs Membranaires, Chimioresistance et Drug-Design, Facultés de Médecine et de Pharmacie, Université de la Méditerranée, Marseille, France

Abstract: In *Enterobacteriaceae*, membrane permeability is a « key » in the level of susceptibility to antibiotics. Modification of the bacterial envelope by decreasing the porin production or increasing the expression of efflux pump systems has been reported. These phenomena are frequently associated with other resistance mechanisms such as alteration of antibiotics or modification of the drug targets, in various clinical isolates showing a MultiDrugResistant phenotype (MDR). In *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Salmonella enterica* several genes and external factors are involved in the emergence of MDR isolates. These bacterial isolates exhibit a noticeable reduction of functional porins per cell due to a decrease, a complete shutdown of synthesis, or the expression of an altered porin and a high expression of efflux systems (*e.g.* overexpression of the pump). The combined action of these mechanisms during an infection confers a significant decrease in bacterial sensitivity to antibiotherapy ensuring dissemination and colonization of the patient and favours the acquisition of additional mechanisms of resistance. *MarA* and *ramA* are involved in a complex regulation cascade controlling membrane permeability and actively participate in the triggering of the MDR phenotype. Mutations in regulator genes have been shown to induce the overproduction of efflux and the down-regulation of porin synthesis. In addition, various compounds such as salicylate, imipenem or chloramphenicol are able to activate the MDR response. This phenomenon has been observed both *in vitro* during culture of bacteria in the presence of drugs and *in vivo* during antibiotic treatment of infected patients. These effectors activate the expression of specific global regulators, *marA*, *ramA*, or target other genes located downstream in the regulation cascade.

Key Words: Gram negative bacteria, *Enterobacteriaceae*, membrane permeability, antibiotic resistance, efflux pump, porins.

1. INTRODUCTION

Bacteria have become able to survive the action of antibiotics by employing several resistance mechanisms. Among them, the balance of the membrane permeability is a key process since the uptake of antibacterial compounds is the first event governing the efficacy of antibacterial activity. Some bacterial species are innately resistant to whole classes of antimicrobial agents due to attributes, such as their intrinsic envelope structure that limits antibiotic diffusion or to the presence of a low level of efflux systems that decrease the intracellular concentration of specific antibiotics [1, 2]. For example, *E. coli*, *K. pneumoniae* and *E. aerogenes* species are resistant to the macrolide family of antibiotics [3].

Of continuous increasing concern are those, initially susceptible, bacterial strains that become resistant in response to the selective pressure of antibiotic treatment, which may wipe out the competing flora that shares the same ecological niche. Consequently, the resistant strains are able to colonise and disseminate. Several mechanisms of antimicrobial resistance are readily spread *via* mobile genetic elements (plasmids, transposons, …) throughout a variety of bacterial genera. Today, the great threat is associated with the Multi-Drug Resistance (MDR) phenotype that renders all common antibiotic classes useless.

For classification purposes three simple technical definitions can be applied to the various stages of action of different resistance mechanisms 1) The Mechanical Barrier (acting to limit the required intracellular dose of an antibiotic), 2) The Enzymatic Barrier (producing detoxifying enzymes that degrade or modify the antibiotic), 3) The Target Protection Barrier (mutation or expression of a molecule that impairs target recognition and thus antimicrobial activity).

The mechanical barrier (see Table **1**) involves two independent processes affecting the internal drug concentration:

- Membrane permeability may be modified by a decrease of porin content or an alteration of the lipopolysaccharide structure. These two responses limit the antibiotic access to the target to levels below the required effective dose
- Alternatively or conjointly, expression of efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site greatly reduces the drug efficiency

2. REGULATION OF MEMBRANE PERMEABILITY IN *ENTEROBACTERIACEAE*

Numerous regulatory proteins have been implicated in the development of MDR and both structural and genetic

*Address correspondence to this author at the UMR-MD-1, Enveloppe Bactérienne, Perméabilité et Antibiotiques, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille cedex 05, France; Tel: (33) 4 91 32 45 87; Fax: (33) 4 91 32 46 06; E-mail: Jean-Marie.PAGES @univmed.fr

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Table 1. Major Resistance Mechanisms Associated with the Bacterial Membrane

Mechanism	Mechanical Barrier	
Type of Activity	**Influx, Passive Uptake**	**Efflux, Active Expulsion**
Bacterial component regulated in response to treatment	porin, LPS	efflux pump
Susceptible antibiotic	ß-lactam, quinolone, …etc	ß-lactam, quinolone, aminoglycoside, …etc

investigations endeavor to understand and decipher their mechanisms of action. *Enterobacteriaceae* have evolved different molecular resistance strategies in response to a variety of toxic substances and environmental stresses by way of membrane permeability modulation (Fig. **1**). Control of efflux pump and porin gene expression is carried out on several levels:

1. Positive regulation by general or specific transcriptional activators which coordinate the expression of several genes.
2. Negative regulation by repressors of porin or efflux pumps components.
3. Response to chemical or pharmaceutical factors that trigger one or more complex inter-linking regulatory cascades.

2.1. Positive Regulation by Global Transcriptional Activators

The chromosomal transcriptional regulators of bacterial influx and efflux genes described in *Enterobacteriaceae*, in particular AraC-XylS, MarR, MarA and TetR, belong to one family of regulatory proteins [4-7]. All possess α-helix-turn-α-helix (HTH) DNA-binding domains and the most described is the global activator MarA.

2.1.1. The Mar Locus

The *mar* (multiple antibiotic resistance) locus in *E. coli*, is a continual operon of 1335 bp with two divergent transcriptional units *marC* and *marRAB* separated by the operator *marO*. The *marC* gene encodes a putative integral protein of the inner membrane with a function not thought to be involved in MDR [8-10]. *marR* encodes the 144 amino acid repressor that acts to prevent expression of the regulation cascade under non-stressful conditions. *marA* encodes a transcriptional activator (125-129 amino acids depending on bacterial species) that acts at the origin of a genetic cascade triggering numerous mechanisms of MDR. *marB* encodes a small protein of the inner membrane with unknown function [11, 12]. The *marRAB* operon exhibits a genetic organization preserved among the *Enterobacteriaceae* [13] that has also been characterized in *Salmonella enterica*, *Enterobacter aerogenes* and *Yersinia pestis* [14-16]. The *marRAB* operon is constitutively repressed by MarR. Analysis of *E. coli marO* has indicated the presence of two sites that consist of repeated reversed sequences comprising palindromic domains [11]. Repression of the system requires binding of MarR at Site II. In addition, this repressor interferes with RNA polymerase interaction with the flanking -10 and -35 boxes whilst bound to site I. The expression (or de-repression) of *marRAB* is the consequence either of (i) mutations in the MarR binding sites, (ii) modification of MarR at the protein level preventing its repressor function, or (iii) the direct action of inductors of the system.

The resolution of the crystal structure of MarR has confirmed that it acts as a dimer, as many other similar bacterial regulators [7, 17]. Three regions of the MarR repressor are important for its activity: two putative helix-turn-helix DNA-

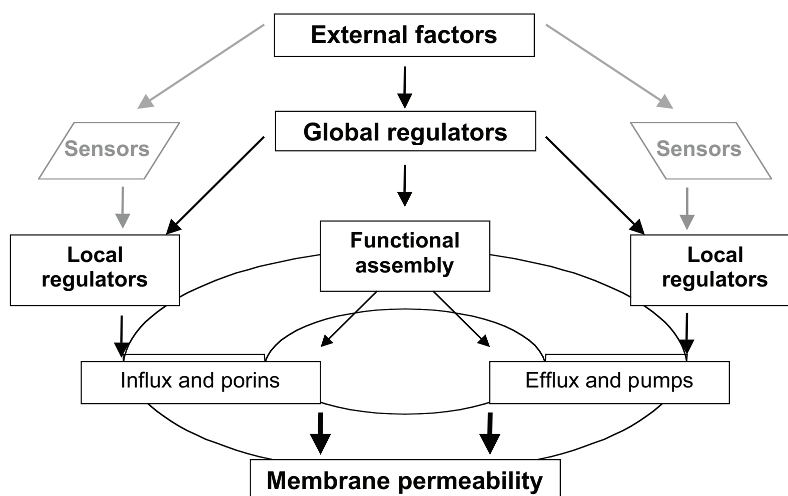

Fig. (1). Regulation of membrane permeability.

binding domains and the first 31 amino acids, which are involved in the dimerization process [17]. Numerous mutations have been described that are scattered throughout the MarR sequence and have not particularly helped define the minimal sequence necessary for function and specificity [18]. The first 8 amino acids of the C-terminal helix-turn-helix (residues 92 to 104) belong to a conserved domain, which plays a critical role in repressor function and constitutes the DNA binding site. Interestingly, major inter-specific substitutions have been found at residues 102 and 103. Furthermore, some of these inter-specific amino-acid variations are located at mutation sites described in MarR in clinical MDR *E. coli* strains (positions 3, 53, 96, 103 and 137) [9, 19-21]. The association of two or more mutations can correlate with *marA* overexpression in multidrug-resistant strains. The N terminus of the repressor gene is required for negative control, and deletion of the first 19 amino acids of MarR has been found to eliminate repressor activity [22, 23].

MarA is an important regulator in *E. coli* implicated in adaptation to the environment and protection against external aggressions, by inducing the direct or indirect action of more than 60 genes [24, 25]. It contains a DNA-binding region involved in the activity of MDR. The MarA binding site, (the marbox), is located within the promoter *marO*, implying that MarA triggers its own autoactivation [26]. Analysis of the promoter region also shows the presence of a binding site for the Fis protein, just upstream of the marbox, which plays an additional role in the activation of the operon [27]. The expression of *marA* triggers a cascade that leads to the MDR phenotype by simultaneous reduction of influx and increase in efflux of antibiotics. MarA induces a decrease in OmpF, a major outer membrane porin involved in the entry of hydrophilic antibiotics in *E coli*. This negative regulation is performed by the transcription of small non-translated antisense RNAs such as *micF* located upstream of the gene coding the OmpC porin [28]. *micF* hybridizes with *ompF* mRNA and thus prevents the translation. The *micF* promoter harbours a marbox-type consensus sequence. In addition, MarA activates the expression of OmpX involved in the control of porin expression (see below) [29, 30]. Expression of these regulators acts synergistically to reduce porin expression and limit antibiotic influx. A second arm of the MarA cascade acts to upregulate expression of AcrAB, two structural components of the major enterobacterial efflux pump. The overall effect of the *marA* system is to reduce intracellular and periplasmic concentrations of antibiotics that enter the bacterial cell through porins and are substrates of active efflux *via* AcrAB-TolC (Fig. **2**).

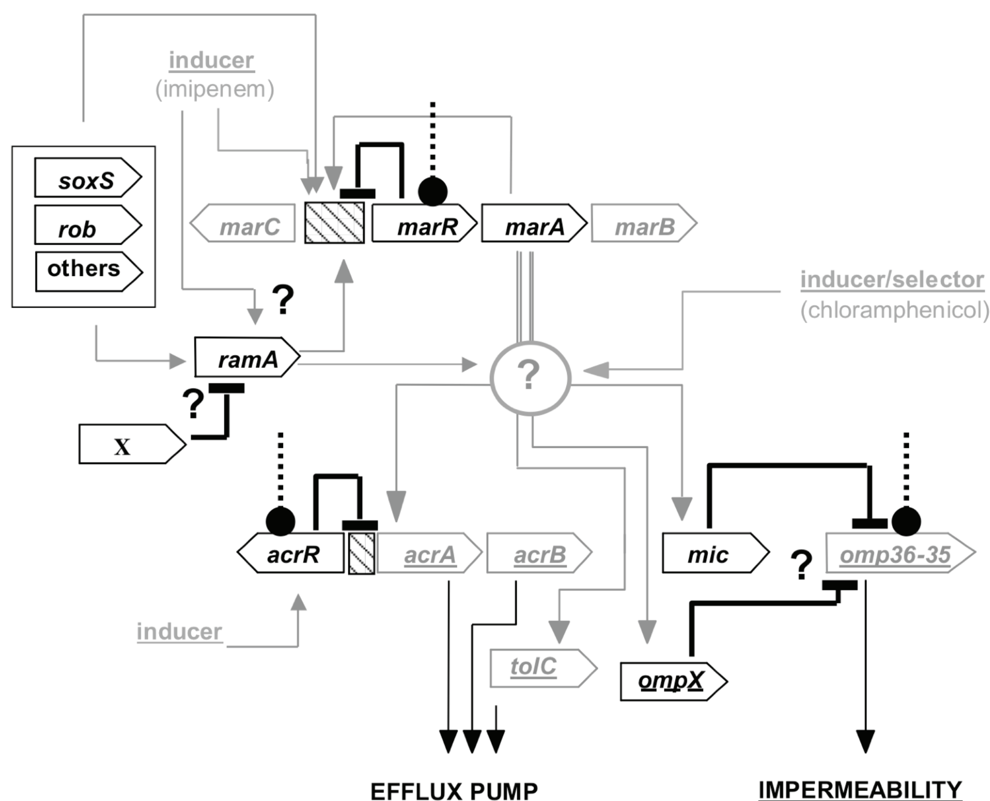

Fig. (2). Regulation cascade of membrane permeability, the *E. aerogenes* case.

The various involved genes (coding for regulators in black and structural proteins in grey, respectively) are indicated and their effects are illustrated by different arrows/lines. Negative controls are presented in black and thick lines, activation by grey and thin arrowed lanes; broken lines with bullets indicate the presence of mutations. The hatched squares represent the promoter region. X, putative regulator detected in *E. aerogenes* although the required detailed information is yet to be obtained. ?, detailled informations are missing (AcrAB-TolC efflux and porin (Omp36, 35) genes are presented (grey and underlined)).

2.1.2. The Oxidative Stress Regulon SoxRS

SoxS is the effector of the *soxRS* global superoxide response regulon. SoxS exhibits about 50% homology with MarA and also belongs to the AraC family of transcriptional activators [31]. It is activated by superoxide-generating agents *via* conversion of SoxR, a divergently transcribed local transcriptional activator, into an active form [32, 33]. In the presence of oxidising agents (*e.g.* H_2O_2, NO, Paraquat), SoxR shifts from the reduced to the oxidized state and triggers the transcription of the gene *soxS* [31]. SoxS is involved in activation of the MDR phenotype in *E. coli* and *Salmonella enterica* Serovar Typhimurium and can induce the transcription of *micF* and *acrAB*. The marboxes are also target sequences for the binding of SoxS and the phenotype induced by SoxS is similar to that induced by MarA [34]. SoxS is also able to activate MarA expression and together they activate many of the same genes [27, 35]. Mutations in the *soxR* activator have been identified in clinical isolates of *E. coli* and *S. enterica* from patients undergoing quinolone treatment and shown to confer an increased MDR phenotype [36, 37].

2.1.3. The Rob Regulon

Rob is also a member of the AraC/XylS family and it regulates genes involved in resistance to antibiotics, organic solvents and heavy metals [38, 39]. Over-expression of Rob in *E. coli* produces both increased organic solvent tolerance and low-level resistance to multiple antimicrobial agents, due to increased expression of AcrAB [40]. In contrast to MarA and SoxS, Rob is constitutively expressed, and only its suppression confers a MDR phenotype [40]. There is considerable overlapping of the genes targeted by Rob and those under the control of Mar and Sox but Rob has a limited effect on the level of expression [41]. The precise role of this regulator in resistance to antibiotics has not yet been deciphered. Its constitutive expression in the cell should saturate target genes with MarA and SoxS and should induce a continuous MDR phenotype. In fact, Rob activates the target genes only after the binding of inducers such as medium-chain fatty acids and bile salts, with its C-terminal end. It was recently shown that SoxS and MarA down-regulated *rob* transcripts *in vivo* and *in vitro* [35, 42].

2.1.4. The Super Regulator RamA

RamA is a 113 amino acid regulatory protein belonging to the AraC-XylS transcriptional activator family, first described in a *Klebsiella pneumoniae* MDR mutant [43]. It shares 45% identity with MarA with high conservation of the two DNA binding motifs essential to the regulatory function. Thus, it can be expected that RamA and MarA might recognize an overlapping set of operator sequences. The *ramA* locus cloned in *E. coli* elicits a high-level resistance to diverse antibiotics (chloramphenicol, tetracycline, tigecycline, fluoroquinolones, trimethoprim), a decreased expression of OmpF and an active efflux [44, 45]. RamA has been identified in *Salmonella enterica* serovar Paratyphi B and Typhimurium, and in *E. aerogenes* and *cloacae* [3, 45, 46]. In *E. aerogenes*, this regulator is involved in the modification of outer membrane permeability and in the active extrusion of intracellular antibiotics [3]. RamA has also been demonstrated to bind to the *mar* operator in *E. coli* and enhance its transcription, suggesting an interaction between these two systems [3, 46]. The putative marbox sequence within the *ram* promoter is well conserved according to the consensus [32]. This suggests that MarA could regulate the transcription of *ramA*, however constitutive expression of RamA results in a MDR phenotype even in the absence of the *mar* locus. RamA is a transcriptional activator of the Mar regulon and is also a self-governing activator of the MDR cascade. A putative *ramA* repressor has recently been identified in *E. aerogenes* that also belongs to the TetR family of transcriptional repressors (A. Molitor, unpublished results). Surprisingly, no *ramA* locus has been identified in the *E. coli* genome. RamA plays a role in the oxidative stress response in partnership with *soxRS*, but seems more important than *marA* and *soxS* in the development of MDR in *Salmonella* spp. [47, 48].

2.1.5. Other Regulators

Another global regulator of outer membrane permeability, H-NS (histone-like structuring nucleoid protein), was initially described as a transcription factor and plays a role in the structure and functioning of chromosomal DNA. H-NS controls about 5% of *E. coli* genes, most of which are involved in virulence or adaptability to stressful environmental conditions [49-51]. This protein regulates the expression of porins and several efflux pumps in *E. coli* and *E. aerogenes* in response to osmotic stress [51-54]. There is evidence that this H-NS also controls expression of OmpX [30].

A recent study demonstrated that AcrAB is also positively regulated by SdiA, a protein which regulates cell division genes in a manner dependant upon quorum sensing [55]. FIS is a nucleoid-associated global regulatory protein that modifies *acrAB* transcriptional activity in response to various growth conditions and can also bind to a site within *marO* upstream the marbox. This regulator is proposed to limit the overall level of negative superhelicity and stabilize the local DNA architecture of certain promoters, providing an additional two-fold stimulation to MarA-, SoxS-, and Rob-mediated activation of transcription [27]. In *Proteus vulgaris* and in *Providencia stuartii*, PqrA and AarP respectively are MarA-like elements responsible for a MDR phenotype [56, 57]. A new putative MarA-like activator, TetD, has also been identified in *E. coli* that directly activates the same subset of genes as MarA/SoxS/Rob and confers resistance to redox-cycling compounds and antibiotics [58]. All these activators share similar characteristics and approximately 50% homology, which is higher in the two HTH domains. Table **2** summarizes the principal regulators involved in the control of MDR, and the species in which they have been identified.

The relevance of global regulators in clinical resistance is difficult to appreciate and its prevalence is for the moment only partially studied. Indeed, even if the reduction of susceptibility to antibiotics of a mutant is demonstrated, levels of resistance measured in the laboratory, despite suppression of *marRAB*, often remain quite low compared to the MICs observed in the clinical isolates [18]. This can be partly explained by the fact that clinical isolates often exhibit multiple resistance mechanisms that act in synergy. An isolate resistant to the quinolones is obtained more easily if bacterial growth is carried out in the presence of inductors of the *mar* system (tetracycline or chloramphenicol) [59]. 40% of a

Table 2. Transcriptional Regulators Controlling Expression of Porins and Efflux Pumps in *Enterobacteriaceae*

Protein	Regulator Family	Function	Species
MarA	AraC	Global activator	*E. coli, S. flexneri, S.* Typhimurium, *S. enterica, E. cloacae, E. gergoviae, Y. pestis*
SoxS	AraC	Global activator of the oxydative stress response	*E. coli, P. aeruginosa, S. typhimurium*
Rob	AraC	Global activator	*E. coli, E. cloacae , S. typhimurium, Y. pestis*
RamA	AraC	Regulator of MDR	*K. pneumoniae, S. enterica* serovar Typhimurium, *S. enterica* serovar paratyphi
PqrA	AraC	Regulator of MDR	*P. vulgaris*
AarP	AraC	Regulator of MDR	*P. stuartii*
MarR	MarR	Repressor of MarA	*E. coli, S. flexneri, S. typhimurium, S. enterica, E. cloacae, E. gergoviae*
SoxR	MarR	Regulator of the oxydative stress response	*E. coli, S. enterica*
AcrR	TetR	Repressor of AcrAB	*E. coli, E. aerogenes, H. influenzae, S. enterica*
EmrR	MarR	Repressor of EmrAB	*E. coli, P. aeruginosa*

fluoroquinolone-resistant *E. coli* population were found to constitutively express either *marA* or *SoxS.* Furthermore, spontaneous mutants presenting high degrees of fluoroquinolone resistance were described in clinical isolates that had developed mutations in the repressor MarR [9, 23, 60].

2.2. Negative Regulation by Repressors of Porin or Efflux Pump Synthesis

2.2.1. OmpX, a Repressor of Porin Synthesis

OmpX is a small outer membrane protein (18 kDa) forming an eight stranded anti-parallel β-barrel and has been identified in various enterobacterial species [24, 61, 62]. Over-expression of OmpX has been associated with a decrease in the expression of Omp36, a major *E. aerogenes* porin, and a decreased susceptibility to β-lactams [63]. This observation was reported in clinical isolates of *E. aerogenes* exhibiting a MDR phenotype [29, 64]. Recent studies indicate that OmpX expression is influenced by a number of environmental factors and by the global *mar* regulator [63, 65, 66]. Microarray analysis has indicated that *ompX* transcription is activated by salicylate *via* MarA (1.8 – 1.5-fold) and by paraquat *via* SoxS (1.7 - 1.2-fold) [67]. A noticeable increase in *ompX* expression has been observed within 60 minutes of exposure to compounds such as salicylate, quinolones, novobiocin, dipyridyl and high ionic strength, with a simultaneous decrease in porin synthesis [30]. This rapid response highlights the efficiency of this cascade, which enables an outer membrane protein equilibrium that is highly adaptable to antibiotic therapy. Comparison of *ompX* and *ompF* expression in response to environmental stresses in *marA-, hns-* and *ompX-* mutants has clearly demonstrated the involvement of *marA* and *hns* in OmpX control. This outer membrane protein has also been hypothesized to play a central role in an alternate *mar*-independent regulatory cascade to modulate outer membrane permeability [30]. Recently, a post-translational hypothesis, involving the role of Deg protease, has been proposed to explain the strong decay of porin content in a resistant selected strain that conjointly overproduced efflux pumps and OmpX [68].

2.2.2. Small Non-Coding RNAs

Small anti-sense RNAs correspond to an emerging class of regulators of bacterial gene expression and have often been found to control the expression of outer membrane proteins. *E. coli* is known to express at least eight OMP-regulating sRNAs (*invR, micA, micC, micF, omrAB, rseX* and *rybB*) [69]. Post transcriptional regulation by *micC* and *micF* reduces OmpC and OmpF synthesis respectively in response to stress conditions. *In vitro* experiments have shown that *micF* interacts with *ompF* mRNA to prevent an active translation initiation complex [69]. This mechanism performs an essential negative osmoregulation of OmpF and is associated with OmpR repression of *ompF* under high osmotic stress [69]. The exact physiological role of *micC* remains to be confirmed, but it is thought to negatively regulate *ompC* expression by the same mechanism as *micF*. Northern analysis suggests that, in general, *micC* and *micF* are differentially expressed depending on osmotic conditions [69]. This parallels the well documented shift of porin expression from OmpF to OmpC under high osmotic pressure.

2.2.3. Repressors of Efflux Pump Genes

Many operons that encode efflux pump components contain a physically linked regulatory gene that plays a monospecific role in modulating expression of that pump. In *E. coli*, *acrR* encodes a TetR type repressor, which is known to repress both its own and *acrAB* transcription [70]. Mutations in *acrR* have been shown to de-repress *acrB*. Such mutations have been found in clinical isolates of *E. coli*, *S. enterica* and *E. aerogenes* [71-73]. Spontaneous laboratory mutants of *E. coli* generated by selection were shown to harbor IS*186* in

acrR or IS*2* up-stream of *acrEF*. An IS in *acrS* (the putative repressor of *acrEF*) of *S. enterica* DT204 was also detected. These mutations lead to the over-expression of *acrAB* and partially contributed to increased resistance to several unrelated antibiotics [74].

2.3. Chemical Effectors or Inductors

Many toxic compounds that are substrates for efflux pumps or porins are able to induce a MDR phenotype *via* induction of global activators. Modification of porin or efflux pump expression has been shown to occur in response to aggressive conditions due to toxic substrates acting as chemical effectors or inducers of regulation cascades. Enteric bacteria such as *E. coli* inhabit the intestinal tract, where they are exposed to bile salts and fatty acids that are able to induce the expression of the AcrAB-TolC pump [75]. The effectiveness of this regulation is such that bile salts simultaneously induce efflux pump expression and are then extruded as substrates of this pump. The same observation has been reported with imipenem and porin expression [76]. When the *mar* system was first described, chloramphenicol, tetracycline and salicylate were reported to induce the transcription of the *marRAB* operon [9]. Some inducers have been shown to bind directly to the repressor MarR following entry into the bacterial cell. Indeed, salicylate and (to a lesser extent) tetracycline exhibit strong affinity for MarR, preventing binding to *marO* or shifting the repressor out of the operator site [22]. Similarly deoxycholate interacts with MarR to prevent DNA binding in *S. enterica* Typhimurium [77]. The transcriptional activators involved in MDR respond to a variety of chemically unrelated compounds including antibiotics (tetracycline, tigecycline, chloramphenicol), biocides (triclosan, household disinfectants), the uncoupling agent carbonyl cyanide m-chlorophenylhydrazone, cyclohexane, salicylate, acetylsalicylate (aspirin), acetaminophen, sodium benzoate, plumbagin, menadione, paraquat, dinitrophenol, and more generally phenolic rings [78]. Removal of such toxic substances and subculture in favorable conditions often results in the reversion of MDR to the sensitive phenotype as repression of global activators is restored. The exact mechanism of induction by each of these compounds is yet to be deciphered and further investigation of these processes may aid in the understanding of how MDR develops.

3. ANTIBIOTIC RESISTANCE AND MEMBRANE PERMEABILITY IN CLINICAL *ENTEROBACTERIACEAE*

3.1. Membrane Permeability and Gene Mutation in Clinical Isolates

It has been reported that during antibiotic therapy several Gram-negative *Enterobacteriaceae* have developed a protective response to impair the activity of clinically used antimicrobial drugs. This response may include a down regulation of porins and/or the overexpression of efflux pumps. In certain cases, an alternate modification of membrane structure has taken place involving the alteration of LPS and a concomitant change in the level of various OMPs.

The pioneer studies from S. Levy's lab reported, for the first time, the role of mutations located in the *mar* locus that are associated with the emergence of the MDR phenotype in Gram-negative bacterial clinical isolates [60, 79, 80]. The position and role of numerous *mar* mutations have been demonstrated in several *E. coli* clinical isolates exhibiting varying levels of decreased susceptibility towards several unrelated antibiotic molecules. These mutations which trigger the *mar* regulon cascade, provide an opportunity for these isolates to mutate at other loci, *e.g. gyr* or *par*, and emerge as high-level quinolone-resistant strains [60]. Moreover, it has recently been demonstrated that the expression of the efflux pump is an important prerequisite for the selection of fluoroquinolone resistant mutants that exhibit mutated DNA gyrase targets. This process has been reported in various Gram-negative bacteria such as *Salmonella* spp. or *Campylobacter* spp., two major food-borne pathogens involved in severe human diseases [47, 81]. The poly-specificity of the efflux pump transporters confers a general resistance mechanism that can reinforce the effect, and/or favour the acquisition of other resistance mechanisms such as target mutations or drug modifications [2, 82, 83].

There are several other reports documenting the modification of membrane permeability, porin deficiency, LPS alteration or efflux activation in various clinical isolates during antibiotherapy. Two clinical strains of *E. aerogenes* presenting high-level resistance to ß-lactams have been shown to exhibit a MDR phenotype with resistance to ß-lactams, quinolones (nalidixic acid, norfloxacin, ciprofloxacin), tetracycline and aminoglycosides (kanamycin, amikacin, gentamycin) [64]. One of these strains had a complex phenotype with respect to chloramphenicol resistance: it presented alteration of porin synthesis, overproduction of OmpX (see also 1.2.1), modification of the LPS (regarding the O-polysaccharide moiety), and presence of a PAßN-sensitive efflux mechanism. Similarities can be drawn between the multitude of resistance strategies employed in this isolate and the *E. coli* stress response that includes a pleiotropic change of membrane structure, permeability and expression of active efflux [24, 49, 64]. A parallel study analyzed a collection of twenty resistant *E. aerogenes* isolates [84]. Among them, three strains possessed an interesting phenotype and molecular characteristics (Table **3**). It is interesting to note that no *marR/marA* gene mutations were detected in any of these MDR *E. aerogenes* strains (A. Molitor, unpublished results). This suggests that *mar* mutation is not directly involved in the expression of MDR in these clinical isolates as reported for *K. pneumoniae* and *Salmonella* resistant strains [44]. Moreover, a mutation in the *acrR* locus has been identified favoring the overproduction of AcrAB efflux components in the *E. aerogenes* MDR isolate 27 [72].

Schneiders *et al.* have examined the role of *marA* and *ramA* in *K. pneumoniae* clinical MDR isolates [44]. Besides mutations in the local regulator *acrR* in some isolates, the authors reported a noticeable increase of *ramA* transcription while the expression of both *marA* and *soxS* were not increased. This *ramA* overexpression suggests both influx and efflux could be modified in these isolates. A study concerning a collection of clinical *K. pneumoniae* strains, isolated from different patients treated in a University Hospital in Istanbul (Turkey) indicated that 39% of studied strains exhibited a PAßN-sensitive efflux pump and that the AcrA efflux component was overexpressed in most of these isolates [85]. Interestingly, the osmotic control of the expres-

sion of OmpK35 (belonging to the OmpF porin group with large channel size) and OmpK36 (belonging to the OmpC porin group with small channel size) was abolished in the majority of these isolates suggesting an alteration in the balance of OmpK35/OmpK36 ratio. This is interesting with regards to the differential ß-lactam susceptibility afforded by these porins in *K. pneumoniae*. The level of susceptibility in bacteria expressing OmpK35 is 4-8 times higher than that conferred by OmpK36 for several ß-lactams, *e.g.* cefepime, cefotetan, cefotaxime, cefpirome [86]. Thus in these isolates, the porin dependent uptake is altered simultaneously with the overexpression of the AcrAB efflux pump. It is important to mention the various studies on *K. pneumoniae* isolates that demonstrate the interplay between porin expression including the synthesis of a selective porin, OmpK36 versus OmpK35 [86], and efflux pump activity in the emergence of MDR [87]. This tight coupling has also been evidenced using microarray analysis focused on a susceptible clinical strain and its resistant derivative isolated from the same patient [88].

Table 3. Characteristics of MDR *E. aerogenes* Isolates

Characteristics	*E. aerogenes* Isolates		
	3	5	27
ß-lactams	R	R	R
Quinolones	R	R	R
Tetracycline	I	S	R
Chloramphenicol	R	R	R
Porin detected	*	-	-
Alteration of norfloxacin accumulation	++	+/-	++
Alteration of chloramphenicol accumulation	++	nd	++
Mutation in regulator genes			acrR

From [72, 84].
R, resistant ; S, susceptible ; I intermediate.
*, mutation in the L3 loop (located in the constriction area of the channel governing the diffusion rate) ; -, no porin produced
++, high decrease of intracellular antibiotic ; +/-, low decrease of intracellular antibiotic ; nd, not determined.

Similar observations have been reported with *Salmonella enterica* during studies of human and veterinary strains [89, 90]. Table **4** summarizes the data available so far regarding mutations in the regulatory genes, *e.g. marR*, *marA*, … of *Enterobacteriaceae* clinical strains and their relation to the expression of MDR.

A major problem with these clinical data is the limited availability of key information regarding the patient situation (*e.g.* bacterial flora, level of bacterial susceptibility,…) prior to starting antibiotherapy. Treatment regiments applied during patient therapy that lead to the selection of resistant isolates are only partially documented. Future studies would benefit from full documentation of the original colonizing strain, any pre-treatment of the patient, complete description of the treatment given (dose, time, single or multi-therapy, …) and the medical history of the patient (other therapies, possible risks, …). Consequently, it is very hard to glean a clear-cut conclusion about the emergence of resistance mechanisms and their dissemination in a clinical setting. A few attractive and ambitious projects have begun to investi-

Table 4. Antibiotics, Resistance Mechanisms and Regulators Involved in Some Clinical MDR Isolates

Drug	Resistance Mechanism		Modification in Regulator Gene	
	Influx (Porin Alteration)	Efflux (Overproduction)	General	Local
ß-lactams	§, *	+	*marR** *marA** *ram A*° *soxRS** °	*acrR**, porin regulator
quinolones	only fluoroquinolone	+	*marR** *marA** *ram A*° *soxRS** °	*acrR**, porin regulator
cyclines	-	+	*marR** *marA** *ram A*° *soxRS**	*acrR**
chloramphenicol	-	+	*marR** *marA** *ram A*° *soxRS**	*acrR**

°, overexpression; * mutation; § decreased or selective expression.

gate the direct evolution of bacteria during antibiotherapy in order to associate the bacterial physiological change with the antibiotic used.

3.2. Evolution of Clinical Isolates Under Antibiotherapy Stress

It is important to stress that the true clinical role of *marA* and *ramA* in the emergence and dissemination of MDR strains may be under-estimated due to the limited number of complete clinical investigations to date. For the most part, only partial and case by case studies have been carried out in this regard. Few studies have investigated in depth the relationships between employed antibiotherapy, the evolution of antibiotic resistance and the role of genetic regulators. A large majority of results concerning the genetic control of membrane permeability come from *in vitro* studies (see chapter I).

Linde *et al.* (2000) characterized clinical *E. coli* strains isolated from the same patient who had received ciprofloxacin therapy [23]. These strains exhibited the same PFGE pattern but different antibiotic susceptibility profiles. One isolate, EP2, presented increased resistance to ciprofloxacin, chloramphenicol, cefazolin and cefuroxime compared to another. The analyses of *mar* and *acr* operons indicated that the EP2 isolate possessed a C-terminal deletion in the *marR* gene and exhibited a conjoint increase of *marA* and *acrA* transcription. Binding of functional MarR represses the *mar* regulon and this deletion was shown to disrupt such negative regulation resulting in increased MarA expression and induction of the MDR phenotype identified in this clinical isolate. The authors suggest that the corresponding mutation had been selected under ciprofloxacin treatment. A similar evolutionary process has been reported in the case of *E. cloacae* infectious disease [91]. A patient developing bacteremia due to a susceptible *E. cloacae* strain was treated for a period of 3 weeks with dual-therapy antibiotics, imipenem+amikacin. Two weeks after stopping antibiotherapy, a resistant isolate was collected. Characterization of the two strains (susceptible and resistant) collected during patient treatment indicated that the transcription levels of the major *E. cloacae* porins were severely altered and expression of the efflux gene *acrB* was increased in the resistant isolate [91]. In addition, the efflux pump inhibitor (PAßN) was able to restore the activity of antibiotics which are described as pump substrates indicating the presence of an active efflux pump. This report clearly demonstrated the intricacies of porin and efflux regulation, emphasizing a common balance of the two aspects of membrane permeability. The *mar* or *ram* genes seem to play a key role in this *in vivo* bacterial response to antibiotic pressure although no mutation or modification of effector levels (repressor or activator) have been clearly identified at this moment.

During the antibiotherapy of diverse patients infected by *E. aerogenes*, the use of imipenem therapy has been shown to be involved in the selection and emergence of particular isolates that present special drug resistance behaviour [92]. An *a posteriori* study analyzed the membrane characteristics and the antibiotic susceptibilities of the respective isolates. Under imipenem treatment, the sequentially collected *E. aerogenes* isolates rapidly began to exhibit a decrease of major porin (Omp36 and Omp35) syntheses associated with ß-lactam resistance. Simultaneously, expression of the AcrAB-TolC efflux system emerged, supporting a low level resistance to other antibiotic families. This co-ordinated adaptation was directly involved in the development of a MDR phenotype concerning the main clinical antibiotic families (ß-lactams, quinolones, cyclines, aminoglycosides). This result is of considerable interest since the single use of imipenem was shown to trigger and favour the emergence of MDR strains in various patients [76, 92]. An *in vitro* comparison assay performed using a sensitive *E. aerogenes* strain indicated that exposure to imipenem was able to select imipenem resistant variants. Several steps of bacterial adaptation could be distinguished during this experiment: a two-step differential expression of *marA* and the AcrAB efflux pump was observed as a first event, followed by complete repression of porin synthesis in a second phase, achieving the MDR phenotype [73]. Concerning *E. aerogenes*, a sequential membrane modification has been also reported during a successive antibiotherapy regimen. Five clonally related isolates were collected and four presented specific resistant phenotypes during successive imipenem and colistin treatment [53]. During the first stage of imipenem therapy, two isolates were found to display a complete lack of porins associated with an increase of OmpX expression and a decrease of imipenem susceptibility. The original porin profile was subsequently recovered when imipenem therapy was stopped and OmpX expression conjointly decreased. In a second step, during a following colistin treatment, the structure of LPS was modified in the collected isolates without noticeable effect on outer membrane protein expression but conferring resistance to polymyxin. This result emphasizes the flexibility of membrane adaptability in response to single therapy and the role of permeability balance in antibiotic resistance [93].

It is interesting to briefly mention that a similar phenomenon has been implicated in *P. aeruginosa*, another major Gram-negative bacterial pathogen. Exposure to imipenem treatment has been shown to result in restricted ß-lactam entry in combination with high-level expression of AmpC ß-lactamase conferring clinically significant levels of resistance to the antibiotic. In clinical practice, down-regulation of OprD2 expression is not a rare event *e.g.* in one study 38% of patients undergoing imipenem therapy for pneumonia caused by *P. aeruginosa* possessed OprD2-deficient isolates before the end of treatment [94]. In addition, several *P. aeruginosa* isolates displaying porin deficiencies associated with MexAB-OprM and MexXY-OprM overproduction have been described [95]. Multiple carbapenem resistance mechanisms have been demonstrated to coexist in a single *P. aeruginosa* isolate, which might explain the high-level carbapenem resistance. Moreover, these multiresistant isolates have been shown to maintain their level of virulence [96].

These studies are of importance since they demonstrate an adverse role of antibiotics, if they are misused / overused, as a potent selecting agent for strains expressing MDR capability *via* genetic cascades or mutations.

4. CONCLUSIONS

The bacterial control of membrane permeability is a complex “Pandora’s box” involving various known regulator belonging to the AraC/XylS family (*marA*, …) and the TCS

family (*EnvZ-ompR*, …), small antisense RNAs (*micF*, *micC*, ...), histone-like nucleoid structuring proteins (H-NS, …), and several external factors (chemicals, signals, ...). All of these components may target specific overlapping effectors and trigger different intricate and redundant regulation cascades (*mar, ram, sox*, ...). Furthermore, some additional factors involved in cell division, fitness, colonization and virulence may play a role in the various regulatory processes occurring during the expression of membrane channel proteins.

At this time, it is difficult to clearly portray the role of specific effectors in the regulation cascades which balance membrane permeability. Due to ethical rules, clinical constraints, time restrictions and hospital organization it is not easy to develop a complete and multifactorial investigation that considers patient history and the precise treatment details (drugs, time, dose, ...). It is not only antibiotic treatment regimens that should be documented but also the use of other drugs such as salicylate which is an excellent *in vitro* activator of the *mar* cascade. In addition, documented information of other colonizing strains would be prudent as they represent a reservoir for resistance genes and mobile genetic elements for a long-term maintenance of resistance mechanisms that may continuously resurge at various intervals and in various hospital wards.

Despite these problems, several investigations have identified some clues/keys in this biological machinery that can further our understanding of the regulatory mechanisms leading to MDR. With the support of various national or international agencies other studies regarding the crucial impact of antibiotic resistance mechanisms on human health are emerging. Current evidence, collected from several sources, suggest that the *mar* cascade appears to play a central role in governing influx and efflux. Branching off from this central core system several important regulators act to induce or enhance the efficacy of this regulation cascade of MDR response. A special role has been implicated for *ramA* in *Klebsiella spp*, *Enterobacter spp.* and *Salmonella* spp. This positive regulator can activate *marA* or directly bypass the *mar* operon. At the proximal sector, local regulators organize an amplification loop and optimize the control of components such as *micF* or OmpX which balance porin expression.

The redundancy of regulators, the overlap in control pathways and the vast array of external inducers actively contribute to the selection, dissemination and preservation of enterobacterial MDR strains. This sophisticated "pandora's box" urgently requires a complete and detailed molecular dissection of the various interactions in order to understand and circumvent the efficient bacterial adaptative shield that faces to our antibiotic arsenal.

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Ram locus is a key regulator to trigger MDR in *Enterobacter aerogenes*

Molitor A[a], Farrell-Ward S[b], James CE[c], Fanning S.[b], Pages JM[a],

Davin-Regli A[a*]

[a] Alexander Molitor, Prof Jean-Marie Pagès, Dr Anne-Davin Regli*

UMR-MD1, Faculté de Médecine, Université de la Méditerranée, IFR88, Marseille, France.

* Correspondence: anne-veronique.regli@univmed.fr

[b] Sinnead Farrell-Ward, Prof Seamus Fanning

School of Agriculture, Food Science & Veterinary Medicine, Ho85, Centre For Food Safety Belfield, UCD Veterinary Sciences Centre, Belfield, Dublin 4

[c] Dr Chloë E James

Abstract

Enterobacter aerogenes has emerged among *Enterobacteriaceae* associated hospital infections during the last twenty years due to its faculty of adaptation to antibiotic stresses. Clinical isolates of *E. aerogenes* showing the MDR phenotype possess less porines and more efflux pumps than strains with normal antibiotic susceptibility. Here were focused on the genetic regulation of this alteration of membrane permeability, especially on the global regulators *mar* and *ram*. Sequencing revealed the existence of the putative repressor *ramR* in *Enterobacter aerogenes*. Mutations were found in the majority of the tested MDR clinical isolates for *ramR* and a region upstream of *ramA*, but no mutations at all in *marR* or *marA*. Validation of expression and cloning experiments amplified the theory of the importance of *ram* in modification of membrane permeability. Found mutations in *ramR* and the self-regulating region of *ramA* could alter binding properties of the repressor *ramR.* This could lead to increased activity of *ramA* and hereby cause the MDR phenotype in *E. aerogenes*.

This study was the first to prove that overexpressed *ramR* can directly re-increase membrane permeability of MDR strains by augmentation of porin content and lowered efflux pump expression.

Introduction

The global emergence of Multidrug-resistant (MDR) Gram-negative bacilli is a linked global chronic problem. It corresponds to the association of dispersion of successful clones of MDR bacteria and local genetic adaptation of bacteria under the pressure of excessive antibiotic use [Viveiros M, 2007]. Moreover, association of different non specific resistance mechanisms as modification of membrane permeability, facilitates cross-resistance to unrelated molecules and allows development of specific resistance mechanisms such as target mutations or activation of specific enzymes [Lavigne JP, 2004; Ricci V 2006]. Today, high-level drug resistance is attributed to low membrane permeability and active efflux of drugs, combined with target mutations and hydrolytic enzymes [Lomovskaya O, 2006]. The development of antibiotic resistance is an ongoing evolutional process throughout bacteria. Resistance is not any more limited to a single class of antibacterial agents but may affect many unrelated compounds. Nowadays infections with multi-drug resistant (MDR) bacterial strains have become a major concern in hospitals worldwide. The first line of defense of bacteria against harmful substances is the bacterial membrane. The majority of antibiotics needs porins to pass through the outer membrane of Gram-negative bacteria to act on internal bacterial targets. The transport of harmful substances out of the cell is done by active expulsion by bacterial pumps in the membrane. The interaction of this 'influx' into the cell and the 'efflux' out of the cell alters membrane permeability for antibiotics and forms the major mechanism for MDR.

Among *Enterobacteriaceae* associated hospital infections, *Enterobacter aerogenes* has emerged during the last twenty years due to its efficient adaptive response to environmental stresses [Vonberg RP, 2007]. Infections caused by this organism are often not detected at an early stage and are both difficult to control and to treat and this common hospital pathogen is involved in nosocomial respiratory tract and urinary infectious diseases [Galdbart J.O., 2000]. The existence of a prevalent resistant clone of *E. aerogenes* has been reported in France,

Belgium, and Spain [Arpin C 2003; Bosi C, 1999; De Gheldre Y, 2001]. Nosocomial outbreaks due to multi-resistant *E. aerogenes* is an emerging concern in ICUs [Bosi C, 1999; Galdbart J.O., 2000]. This behaviour has been associated with a decreased susceptibility to the most recently developed cephalosporins, including cefepime and cefpirome (last cephalosporin generation), and to carbapenems [Bornet C, 2003]. An increasing number of clinical *E. aerogenes* strains exhibits a plasmid encoding extended-spectrum ß-lactamase associated with an aminoglycoside enzymatic resistance, in addition to an overexpressed chromosomal cephalosporinase. The clinical isolates also present an acquired resistance, *via* target mutation, to other antibiotic classes such as quinolones [Arpin C, 2005]. The regulation of envelope permeability including the synthesis of porins, the modification of lipopolysaccharide and the expression of efflux pumps has been reported [Thiolas A, 2005]. It has been demonstrated that during imipenem treatment, clinical isolates have developed imipenem resistance *via* a decrease of porin synthesis conjointly to a production of efflux system [Bornet C, 2000; Thiolas A, 2005]. Despite this described resistance to imipenem, resistance rate of *Enterobacter aerogenes* remains relatively stable since 2000 [Biendo M; 2009]. AcrAB-TolC, identified in *E. aerogenes* clinical isolates, expels a variety of compounds including detergents and structurally unrelated antimicrobial agents such as quinolones, tetracyclines and chloramphenicol [Gayet S, 2003; Ghisalberti D, 2005; Masi M, 2006; Pradel 2002]. The combined action of these mechanisms during an infection confers a significant decrease in bacterial sensitivity to antibiotherapy ensuring dissemination and colonization of the patient and favors the acquisition of additional mechanisms of resistance [Malléa M, 1998; Ruiz N, 2006].

In *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae* and *Salmonella enterica* several genes and external factors are involved in the emergence of MDR isolates [Bailey A. M., 2006; Davin-Regli, 2006; Schneiders, 2003; Hasdemir UO, 2004]. This tremendous

capacity to rapidly develop antibiotic resistance has been associated to the regulation cascade involving the *mar* regulon and *ramA,* the regulator gene that controls the expression of membrane transporters [Alekshun MN, 1997; George AM, 1996]. MarA and RamA are involved in a complex regulation cascade controlling membrane permeability and actively participate in the triggering of the MDR phenotype [Barbosa, T. 2000]. Mutations in regulator genes have been shown to induce the overproduction of efflux and the downregulation of porin synthesis [van der Straaten S. T., 2004; Webber M. A., 2006]. In addition, various compounds such as salicylate, imipenem or chloramphenicol for *marA* and chlorpromazine or paraquat for *ramA* are able to activate the MDR response [Alekshun MN, 1997, Bailey AM, 2008, Bornet, 2003, Keeney, D., 2008]. This phenomenon has been observed both *in vitro* during culture of bacteria in the presence of drugs and *in vivo* during antibiotic treatment of infected patients.

The *marRAB* operon (consisting of *marC, marO, marR, marA and marB*) is constitutively repressed by MarR. Analysis in *E. coli* of the operator *marO* indicated the presence of two sites that consist of repeated reversed sequences comprising palindromic motifs [Alekshun MN, 2000; McDermott PF, 2008; Sulavik MC, 1994; White DG, 1997]. The expression (or de-repression) of *marRAB* is the consequence of either (i) mutations in the MarR binding sites, (ii) modification of MarR at the protein level preventing its repressor function, or (iii) the direct action of inductors of the system. The resolution of the crystal structure of MarR has confirmed that it acts as a dimer, as many other similar bacterial regulators [Alekshun MN, 2001]. Three regions of the MarR repressor are important for its activity: two putative helix-turn-helix DNA-binding domains and the first 31 amino acids, which are involved in the dimerization process [Martin RG, 1995]. Numerous mutations have been described in several *E. coli* clinical isolates, that are scattered throughout the MarR sequence and have not particularly helped define the minimal sequence necessary for function and specificity

[Alekshun MN, 2000; Ariza RR, 1994; Linde HJ, 2000; Maneewannakul K, 1996; Seoane A, 1995]. The association of two or more mutations can correlate with *marA* overexpression in multidrug-resistant strains [Tibbetts RJ, 2005; Yaron S, 2003; Oethinger M; 1998].

RamA is regulatory protein belonging to the AraC-XylS transcriptional activator family, described in *Klebsiella pneumonia, Salmonella* sp. and *E. aerogenes* and *cloacae* MDR mutants [George AM, 1995; Martin RG, 2001; Yassien MA, 2002; Zheng J, 2009]. Sharing 45% identity with MarA with high conservation of the two DNA binding motifs essential to the regulatory function, it can be expected that RamA and MarA might recognize an overlapping set of operator sequences [Chollet R, 2004; Zheng J, 2009]. The *ramA* locus elicited a high-level resistance to diverse antibiotics (chloramphenicol, tetracycline, tigecycline, fluoroquinolones, trimethoprim . . .) due to overexpression of an active efflux and a decreased expression of OmpF [Källman O, 2008; Keeney D, 2007; Nikaido E, 2008; Ruzin A, 2005; Ruzin A, 2008]. In *E. aerogenes*, this regulator is involved in the modification of outer membrane permeability and in the active extrusion of intracellular antibiotics [Chollet R, 2004]. RamA plays a role in the oxidative stress response in partnership with *S*oxS, but seems more important than MarA and SoxS in the development of MDR in *Salmonella* spp. [Feuerriegel S, 2008; Webber MA, 2001; van der Straaten ST, 2004].

The putative *marbox* sequence, a binding region for regulators of the AraC-XylS family (like MarA, RamA, Sox and Rob), within the *ram* promoter is well conserved according to the consensus, suggesting that RamA is a transcriptional activator of the Mar regulon and is also a self-governing activator of the MDR cascade [Davin-Regli A, 2008]. A special role has been implicated for RamA in *Klebsiella*, *Enterobacter* and *Salmonella* spp. Here it was proven to be alone responsible for the MDR phenotype caused by membrane impermeability [O'Regan E 2008; Ricci 2009; Schneiders T, 2003 Kehrenberg, C., A 2009, van der Straaten, S. T., 2004].

This positive regulator can activate *marA* or directly bypass the *mar* operon [Davin-Régli A, 2006; Martin RG, 2001]. The gene *ramR*, coding for the repressor of RamA, has recently been identified in *Salmonella enterica* serovar Typhimurium and a-two nucleotides deletion in the putative binding site upstream of RamA was confirmed to play a role in the MDR phenotype [Abouzeed YM, 2008; Bailey AM, 2010]. It belongs to the TetR family of transcriptional repressors and probably acts as a dimer on the operator region [Ramos JL, 2005; Routh MD, 2009]. It is important to stress that the true clinical role of MarA and RamA in the emergence and dissemination of MDR strains may be under-estimated due to the limited number of complete clinical investigations to date. For the most part, only partial and case by case studies have been carried out in this regard [Chevalier J,. 2008].

In this study, we characterized the RamR repressor sequence and function in *E. aerogenes*. We compared its amino acid sequence in 47 characterized MDR clinical strains, 10 strains selected in vitro on antibiotics and 2 references strains. Modifications in the repressor sequence were identified and studied for their corresponding associated MDR phenotype in the isolates. The gene quantitative expression of the different regulators and their targets pointed out the key role of RamA in *E. aerogenes* MDR regulation. After overexpression of *ramA* or *ramR* in different *E. aerogenes* strains, measurements of the minimum inhibitory concentration (MICs) of several structurally unrelated antibiotics and immunoblots against porins and efflux pump components we were able to detect changes in physiology and proteomics. These results strengthened the theory of the important role of both RamR and RamA in regulation of membrane permeability.

Material and Methods

Bacterial strains

All strains studied are listed in **Table 1**.

All strains were cultivated at 37°C in Luria-Bertini (LB) Medium.

Sequencing of *marA*, *marR*, *ramA* and *ramR*

DNA of the analyzed strains was first isolated with the Wizard Genomic DNA Purification Kit (Promega). Purified DNA was then used for PCR and later on sequencing to check for presence of mutations in *marA*, *marR*, *ramA*, *ramR* and their surrounding regions. The sequences of the used primers are shown in **Table 2a**. The Taq-polymerase from Invitrogen was used with a final concentration of 0.4µM of each primer, 0.2mM of each dNTP, 1.5mM of $MgCl_2$, 1x Taq buffer and 3 units of Taq-polymerase. After 5min of denaturation at 94°C, amplification was performed over 33 cycles with steps of 30sec at 94°C, 1min at 64°C and 1min at 72°C, the final extension was held at 72°C for 7min. The PCR products were sent for nucleotide sequencing to Cogenics Online (https://www.cogenics online.com/COL/uwa.maya.engine.MayaEngine?siteid=col&mapid=home). Mutations in the amplified region were identified using BLASTN (http://www.ncbi.nlm.nih.gov /blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn) and by multiple sequence alignments using CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw2/ index.html).

Antibiotic selections

LB-Agar-plates with increasing concentrations of chloramphenicol, rifampicin and fosfomycin were prepared. The used range for chloramphenicol and fosfomycin was 2-64µg ml^{-1}, for Rifampicin 1-16µg ml^{-1} according to the French institute for microbiology (http://www.sfm.asso.fr), using 2-fold dilution series. For every antibiotic, 12 colonies of the

laboratory strain ATCC13048 were picked and grown under increasing antibiotic concentrations 24h at 37°C.

Both genes *ramA* and *ramR* of the surviving strains of the highest concentration of Chloramphenicol (64µg ml^{-1}), Fosfomycin (64µg ml^{-1}) and Rifampicin (16µg ml^{-1}) were sequenced (GenomicExpress) and compared for possible mutations due to antibiotic treatment.

Quantitative Realtime-PCR

For each of the analyzed genes, two or three primer-pairs were designed. Each primer-pair was tested at an annealing temperature of 60°C via standard PCR to determine the best working primer pairs shown in **Table 2b**. The DNA used for the standard PCR was isolated with Wizard Genomic DNA Purification Kit (Promega).

RNA for the quantitative Realtime-PCR was extracted with the RiboPure™-Yeast kit (Ambion), quantification was done using a NanoDrop spectrophotometer. Contaminating genomic DNA was eliminated by two DNase I treatments according to the manufacturer's instructions (Ambion), and its absence was confirmed by including a reverse transcriptase-minus control on each RNA sample. An Eppendorf epMotion 5070 robot was used to set up the plates and the qRT-PCR was done in an Eppendorf Mastercycler ep *realplex* Thermal Cycler.

QuantiTect SYBR Green RT-PCR (Qiagen) was used with a final concentration of 0.5µM of each primer and 500ng of the template RNA. After 30min at 50°C for the reverse transcription, the HotStarTaq DNA Polymerase was activated by a heating step at 95°C for 15min. The 3-step cycles of 15s at 94°C for Denaturation, 30s at 60°C for Annealing and 30s at 72°C for Extension were repeated 35-45 times. Each quantitative realtime PCR was repeated three times.

Cloning and expression of *ramA* and *ramR*

The *ramA*, including its putative *marbox*, and *ramR* genes were amplified from *E. aerogenes* strains ATCC 13048 (*ramA*, *ramR*), EA27 (*ramA27*, *ramR27*) and CM64 (*ramR64*) by using PCR and restriction sites were added (underlined in the primer sequence) to each end using the primers listed in **Table 2c**. PrimeStar™HS DNA Polymerase (Takara) was used to amplify products by PCR according to the manufacturer instructions. Purified PCR-products were digested using *XhoI* and *SacI* (*ramR*) or *BamHI* and *EcoRI* (*ramA*) (New England Biolabs) and cloned into the expression vector pDrive (3851bp) (Qiagen), using T4 ligase (NEB) to create pDrive*ramA-ATCC13048*, pDrive*ramA-EA27*, pDrive*ramR-ATCC13048*, pDrive*ramR-EA27* and pDrive*ramR-CM64* and then transformed into electro-competent *E. coli* strains. Plasmid constructs were purified, confirmed by sequencing (GenomeExpress), using the primer pair T7 (Eurogentec) and SP6 (Eurogentec), then transformed into *E. aerogenes* strains ATCC13048, EA289 and CM64 as shown in **Table 3**. Transformants carrying the pDriveIV plasmid were grown in the presence of antibiotics: *E. coli* transformants under ampicillin (Sigma) at 100µg ml^{-1} and *E. aerogenes* transformants additionally under kanamycin (Sigma) at 50µg ml^{-1}.

Minimal inhibitory concentration (MIC) determination by E-Test stripes

ATCC13048, EA289, CM64 and IPM240 cultures bearing plasmids were grown to OD_{600} 0.4 in LB containing appropriate antibiotics and then induced with IPTG (1mM) for 1h at 37°C. Bacteria were then subcultured to OD_{623} 0.35 (10 x 10^8 bacteria), diluted to 10 x 10^6 and 2.5ml were spread on LB Agar-plates containing 0.5mM IPTG. After drying for several minutes, E-Test stripes were placed on the plates and bacteria grew in presence of the tested antibiotics (ciprofloxacin, norfloxacin, nalidixic acid, tetracycline, chloramphenicol, imipenem, cefoxitin, cefuroxime, cefepime, ceftazidime) over night at 37°C. Assays were independently repeated 3 times.

SDS-Page and Western Blotting

Bacterial protein extracts were analyzed on SDS-Page gels containing 10% acrylamide. Samples were denaturated in Laemmli loading dye containing 2% SDS and the porin-samples were heated 3x to 95°C. Protein size was estimated by comparison with pre-stained low-range molecular weight marker (BioRad). Proteins were stained using Coomassie Brilliant Blue R-250 as precedent described (Mallea et al. 1998).

For immunodetection, proteins were electrotransferred onto nitrocellulose membranes (Schleicher & Schull, Keene, NH, USA) in transfer buffer (20mM Tris, 150mM glycine, 20% isopropanol, 0,05% SDS). Membranes were blocked using 4% milk in Tris-buffered sodium (TBS: 50mM Tris-HCl, 150mM NaCl, pH8). Polyclonal antibodies, directed against denaturated proteins OmpF, OmpC, AcrA, AcrB and TolC were used for detection. Detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove PA, USA) using BCIP and NBT (sigma) according to the manufacturer instructions.

Results

Variability in regulators

MarA and MarR

Sequencing of all 59 investigated strains shown in **Table 1** (2 laboratory reference strains, laboratory mutant raised under chloramphenicol, laboratory strains raised under imipenem and 46 clinical isolates) revealed no differences between MarA and MarR (results not shown).

RamA

All 59 strains shown in **Table 1** were sequenced. Comparison of RamA revealed no differences between tested strains and references. However a region recently identified as *marbox* (Chollet et al. 2004) was found upstream of *ramA* in every sequenced strain. A considerable amount (44 out of 47 tested clinical isolates, 93.6%) of sequenced MDR clinical isolates show a deletion between the *marbox* and the translational start of *ramA*. (**Figure 1**).

RamR

All 59 strains shown in **Table 1** were sequenced. RamR sequences of MDR clinical isolates show several amino-acid changes compared to laboratory strains (**Figure 2**).

46 of 47 (97.8%) of the sequenced clinical isolates show substitutions at positions 72 (aspartate instead of alanine), 44 of 47 (93.6%) at position 100 (serine instead of proline) and 46 of 47 (97.8%) at position 121 (serine instead of isoleucine). 93.6% (44 of 47) of these strains also show an altered C-Terminus, which is in addition four amino-acids shorter than the original of the ATCC13048 strain.

Although these mutations are not located in the putative DNA-binding area which is located close to the N-terminal ending , they could alter the three dimensional structure of the protein, since the substituted side chains possess different charges and hindrance as the original ones as discussed later.

The under increasing chloramphenicol-concentrations raised laboratory strain CM64 shows a significant deletion of amino-acids at position 154 (leucine) and 155 (phenylalanine). No other sequenced strain shows this deletion.

Effect of mutant selective antibiotics on regulating *ramA* and *ramR*

We repeated the assay of letting the laboratory strain ATCC13048 grow under increasing chloramphenicol concentrations [Ghisalberti D, 2005], in order to control if the characteristic deletion at position 154/155 in *ramR* of the strain CM64 (**Figure 2**), that was trained by presence of chloramphenicol, was reproducible. For further verification and to see if new mutations would occur we also chose rifampicin and fosfomycin for the same assay. All three used antibiotics (chloramphenicol, rifampicin and fosfomycin) are known for their capacity to select mutants on a high frequency.

After the treatment with increasing concentrations of antibiotics, the genes *ramA* and *ramR* of the surviving strains were sequenced. Of the 12 tested strains for fosfomycin and for rifampicin all strains survived the treatment with the highest concentration of the antibiotic which was 16µg ml^{-1} for rifampicin and 64µg ml^{-1} for fosfomycin. Twelve out of twelve tested strains survived stepwise treatment with high concentrations of rifampicin (up to 16µg ml^{-1}) and fosfomycin (up to 64µg ml^{-1}) and eight out of twelve strains exposure to high level of chloramphenicol (up to 64µg ml^{-1}). Likewise, the fact that the only noticeable mutation was found in *ramR* might assist the theory that it is the repressor of *ramA*. Neither the 12 colonies raised under fosfomycin, nor the 12 colonies under rifampicin showed any mutations in *ramA* or *ramR* (data not shown). Of the 12 strains raised under increasing chloramphenicol-

concentrations, 8 strains were still viable under the highest concentration of chloramphenicol of 64µg ml^{-1}. The strain CM64-10, one of the eight surviving colonies raised on a plate containing 64µg ml^{-1} chloramphenicol, shows a severe mutation in *ramR*, introducing a stop-codon at position 27 and hence altering the structure and probably functionality gravely (**Figure 3**).

Characterization and validation of expression of regulators of the MDR phenotype by qRT-PCR

Figure 4 and **Table 4** show the expression level of the examined genes in the clinical isolates EA27, EA117, EA3, EA5, GIM55621, GIM59704, MOK75586, PAP12515, RAB76089G, the under increasing chloramphenicol concentrations raised laboratory mutant CM64 and the under increasing imipenem concentrations raised laboratory strains IPM20 and IPM240. The strains were selected by their genotypic and phenotypic characteristics. The studied genes were *ramA* and *ramR* for the *ram*-regulon, *marA* and *marR* for the *mar*-regulon, *soxR* for the oxidative stress regulon SoxRS, *acrA* as representative of the efflux pump and *omp35* as porin. The expression level was determined by calculation of the threshold cycle in qRT-PCRs, the laboratory strain ATCC13048 was used as reference. An increase over 2-fold of the amount of the reference strain is defined as a remarkable increase, a decrease of less than 2-fold of the reference as a remarkable decrease.

The clinical isolate RAB76089G and the two laboratory strains that were raised under increasing imipenem concentrations, IPM20 and IPM240, exhibit the same amount of expression as the reference strain: the expression of all seven evaluated genes (with the exception of *marR* of RAB76089G) compared to the reference ATCC13048 lies between -2 and 2 (**Table 4**).

All other clinical isolates (EA27, EA117, EA3, EA5, GIM55621, GIM59704, MOK75586 and PAP12515) and the laboratory mutant CM64 show a strong increase of the

expression of *ramA*: between an over 14-fold increase for PAP12515 to an over 140-fold increase for the laboratory mutant CM64. The putative repressor or regulator of the ram-regulon, *ramR*, is also expressed on a higher level compared to the reference, but not as strong as *ramA*. The values lie between over 2-fold for GIM59704 and over 10-fold for CM64. Exception is EA5 that shows an over 3-fold decreased expression for *ramR*.

The three clinical isolates EA117, EA3 and EA5 show a significantly higher expression for the two genes of the *mar*-regulon, *marA* and *marR*, and for *soxR* (between over 20-fold to over 160-fold) whereas the remaining studied strains show only marginal variations or stay on the level of the reference strain.

The majority of the principally multi-drug-resistant clinical isolates expresses more *acrA* (up to a 3-fold increase) and less *omp35* (down to a 9-fold decrease).

<u>Cloning and expression of RamA and RamR</u>

The used inserts of ATCC13048 (*ramA*, *ramR*), EA27 (*ramA27*, *ramR27*) and CM64 (*ramR64*) were used for the following reasons: *ramA* and *ramR* from ATCC13048 were taken as reference for non-MDR strains, *ramA27* including the upstream region with the putative *marbox* as representative of MDR clinical isolates, since over 90% (**Figure 1**) of the tested strains show the same deletion in the upstream region between gene and *marbox*. *ramR27* was used since it also represents over 90% of the MDR clinical isolates (**Figure 2**). *ramA64* does not show any differences to *ramA* (data not shown) and therefore was not used as insert. *ramR64* is the only *ramR* showing the deletion at position 154/155 and therefore was used to characterize potential effects of this mutation.

The strain ATCC13048 was used as representative of non-MDR strains. EA289, a kanamycin sensitive variant of EA27 (Pradel et al., 2002) as representative of over 90% of MDR clinical isolates. CM64 as the only laboratory mutant showing the specific deletion at positions 154/155. IPM240 was used since it is a laboratory strain showing the same genotype

for *ramA* and *ramR* (**Figure 1 and 2**) as non-MDR strains, but was trained by increasing imipenem concentrations to show a MDR phenotype (**Table 1**).

Overexpression of *ramA* in susceptible laboratory strains results in increased resistance to several antibiotics

Both the laboratory strain ATCC13048 and the laboratory strain IPM240 that was raised under increasing imipenem concentrations, show a significant change in their antibiotic resistance when *ramA* is overexpressed. **Table 5a** shows that ATCC13048 strains overexpressing *ramA* have a 6-fold higher MIC to nalidixic acid, tetracycline, chloramphenicol and a 4-fold higher value for cefepime. The strain IPM240 shows a 3-fold higher MIC value for tetracycline bearing the *ramA*-plasmid. Overexpression of *ramA* had only slight effects in already MDR-strains EA289 and CM64 (data not shown).

Overexpression of the putative repressor-gene *ramR* has an effect on a laboratory strain that was raised under increasing concentration of imipenem

IPM strain shows reduction of viability in presence of antibiotic. Overexpressed *ramR* results in a nearly 3-fold decrease in the minimum inhibitory concentration of imipenem from 32µg ml^{-1} to 12µg ml^{-1} bearing *ramR* of ATCC13048 or EA27 and to 24µg ml^{-1} when overexpressing *ramR* of the strain CM64 (**Table 5b**). Only minor effects were observed after overexpression of *ramR* in EA289 and CM64. No effect at all was noticeable by overexpression of *ramR* in ATCC13048 (data not shown).

Overexpression of *ramA* and *ramR* show influence on the expression of efflux-pumps and outer membrane porins

Western Blots done with antibodies against porins (α-Omp35, α-Omp36) and parts of efflux pumps (α-AcrA, α-AcrB, α-TolC) show a significant change in expression levels in several strains if *ramA* or *ramR* are overexpressed due to a plasmid carrying them.

Figure 5 displays the effect of overexpressed *ramA* or *ramR* in the clinical isolate EA289: the plasmids harboring *ramA* of ATCC13048 and EA27 clearly effect a decrease of porins whereas *ramR* of ATCC13048, EA27 and CM64 have the adverse impact and leads to a slight increase of porins in the cell wall. Overexpressed *ramA* in ATCC13048, CM64 and IPM240 had the same effect (data not shown). Overexpression of all three tested *ramR* in ATCC13048 showed no alteration of the porin content at all (data not shown).

The effect on the expression of efflux pumps (represented by the efflux components AcrA, AcrB and TolC), is shown in **Figure 6**. Both *ramA* of ATCC13048 and EA27 have an increasing effect on the expression of AcrA, AcrB and TolC in the strains EA289, CM64 and IPM240, shown with examples in the strains EA289 for AcrA (**Figure 6a**), CM64 for AcrB (**Figure 6b**) and IPM240 for TolC (**Figure 6c**). *ramR* of ATCC13048, EA27 and CM64 has the contrary effect and decreases the amount of efflux pump components in the outer membrane of the three tested transformants (**Figures 6a, b, c**). There is no effect on the strain ATCC13048 by neither *ramA* nor *ramR* on expression of efflux pump components (data not shown).

Discussion

Numerous regulatory proteins have been implicated in the development of MDR and both structural and genetic investigations endeavor to understand and decipher their mechanisms of action. *Enterobacteriaceae* have evolved different molecular resistance strategies in response to a variety of toxic compounds and environmental stresses by way of the membrane permeability modulation (Davin-Regli, et al., 2008). Membrane permeability is regulated by the expression of porins and efflux pumps and the control of their expression is carried out on several levels: positive regulators, negative regulators and response to chemical or pharmaceutical factors.

One positive regulator of growing interest in *Enterobacter aerogenes* is the global transcriptional activator *ramA* that is known to be involved in the modification of outer membrane permeability and in active extrusion of intracellular antibiotics. RamA is a 113 amino acid regulatory protein of the AraC-XylS transcriptional activator family. It shares high similarity with MarA, a known global transcriptional activator in *E. coli* where no *ramA* locus has been identified so far. Due to the high similarity it can be expected that *ramA* and *marA* might recognize an overlapping set of operator sequences. However, overexpressed RamA elicits a high-level resistance to diverse antibiotics like chloramphenicol, tetracycline, tigecycline, fluoroquinolones and trimethoprim, reduced expression of OmpF and an active efflux (Schneiders, et al., 2003) (Keeney, et al., 2007). In *E. aerogenes* RamA is a transcriptional activator of the *mar*-regulon and also a self-governing activator of the MDR cascade (Chollet, et al., 2004).

In this work a putative repressor of *ramA*, named *ramR*, has been identified and sequenced in *E. aerogenes*. The sequence analysis revealed that the putative RamR belongs to the TetR family of transcriptional repressors. Comparison of the sequence of various multidrug resistant clinical isolates and laboratory mutants with two phenotypically normal laboratory strains revealed several mutations in *ramR* and a deletion of several amino acids in

the upstream region of *ramA* but no alterations in *marA* or *marR* at all. The substitutions in *ramR* were not located in the region that is known for DNA-binding of TetR family repressors but in the C-terminus that is known for dimerization of the protein (Ramos 2005, Routh 2009). Due to structural changes these alterations could alter the functionality and perhaps lead to an inactivation of the mutated repressor. The deletion upstream of *ramA* was located between the gene and the putative *marbox*, responsible for self-regulation of the protein. Modifications here could alter protein-DNA binding hence self-regulation of *ramA*. Precedent it has been demonstrated that sequence alterations in *ramR* or in the *ramA* upstream region led to an AcrAB upregulation in *Salmonella* (Kehremberg 2009, Zheng 2009, Ricci 2009). Both modifications could therefore be responsible for increased expression of *ramA* and hereby cause the MDR phenotype.

A characteristic deletion at position 154/155 in *ramR* was just found in a laboratory strain that was treated with chloramphenicol (CM64). To test if the finding of this mutation was reproducible, *E. aerogenes* laboratory strains were conditioned with increasing concentrations of antibiotics known to select mutants on a high frequency (chloramphenicol, rifampicin and fosfomycin). Sequencing resulted in the finding of a severe mutation in *ramR* in one of the strains that was exposed to chloramphenicol. First of all this experiment showed the well established adaption mechanism of *E. aerogenes* bacteria to antibiotic treatment. Mutations in *ramA* that would turn it defective as a global activator would have severe effects on the bacteria, probably turning it nonviable. A mutation in the repressor causing increased expression of the global activator might bring benefits with it for survival in an otherwise hostile environment.

Validation of the expression of several known regulators, porin and efflux pump and comparison between various clinical isolates and laboratory mutants with the *E. aerogenes* laboratory reference strain ATCC13048 by qRT-PCR revealed a higher expression of *ramA* for 8 out of 9 investigated clinical isolates (about 90%). The expression of *ramR* was also

increased, but not as much as *ramA*. The other validated global regulator *mar*, with *marA* and *marR*, just showed marginal variations. Expression patterns of *marA* and *marR* indicated that a balance between expression of activators and repressors exist. An increased expression of the repressor *ramR*, that does not affect expression of *ramA* indicated that the repressor is less functional or active, probably as a result of found mutations. The strains that were trained with presence of imipenem (IPM2 and IPM240) showed no variations in expression of any tested gene compared to the reference ATCC13048. An explication might be the lack of imipenem pressure during growth for the qRT-PCR experiments. After growing without presence of antibiotics, the strains rapidly re-regulated membrane permeability and therefore showed the same expression patterns as the non-MDR reference strain ATCC13048. In contrast to the laboratory mutant CM64, raised under increasing chloramphenicol concentrations and showing a non-reversible deletion in *ramR*, that evidenced a comparable expression pattern with even higher expression of *ramA* as the clinical isolates. As estimated the clinical isolates showing a multidrug resistant phenotype also exhibited a decreased expression of porins (*omp35*) and an increased expression of efflux pumps, shown by the efflux-pump component *acrA*. These results depicted a coherence between increased expression of *ramA* with increased expression of efflux pumps and decreased expression of porins that leads to the MDR phenotype in the clinical isolates. The fact that some MDR clinical isolates showed increased expression rates of *omp35*, can be explained by the post-transcriptional control of outer membrane porin genes. An increased expression of *omp35* does not obligatory lead to increased porin content in the outer membrane, since it stays also under control of *micF*, a small regulatory RNA. This conjunction pointed out the enhanced importance of the global regulator *ram* for the MDR-phenotype in *E. aerogenes*. However, a recent paper of Martin *et al*. 2008 compared activation of a set of promoters containing *marboxes*, depending of MarA or SoxS concentration. Authors observed that the half maximal activation of promoters by MarA was highly concentration variable, correlation between

experiments measuring optimal activator concentration, *in vivo* and *in vitro* was poor, and the promoter activation profile depends specifically on the activator. So the target genes activation depends on the concentration and the nature of a given activator of the *mar* regulon.

Cloning experiments with multi-copy plasmids harboring wildtype *ramA* or the mutated *ramR* of a clinical isolate EA27 or the laboratory strain CM64 showed an effect on antibiotic susceptibility, and the expression of porins and efflux pumps. When *ramA* was overexpressed in the laboratory strains IPM240 or ATCC13048, bacteria showed an increased resistance against several antibiotics. An overexpression of *ramR* in the laboratory strain IPM240 resulted in an increased susceptibility to imipenem. Overexpressed *ramA* also effected a decrease of porins combined with increase of efflux pumps in several tested strains, whereas overexpression of *ramR* had the opposite effect. In *Salmonella* Typhimurium, several studies confirmed the role of RamA in fluoroquinolones resistance due to AcrAB overexpression (Zheng, 2009; Beyley, 2010; Feuerrigel, 2008). These results confirmed the assumption of *ramA* as a global regulator activating the MDR phenotype by modification of the membrane permeability and *ramR* acting as its repressor. Nevertheless no differences could be remarked for the different mutations of *ramR*. This leaves it difficult to interpret their exact role for functional changes in *ramR*. However, Bailey et al. 2010 observed that inactivation of RamR seems to have a moderate effect on efflux pumps components expression compared to an overexpression of RamA directly. Moreover, it has been demonstrated for MarR inactivation, that several mutations are necessary for a significative inhibition of its repressor activity [Alekshun *et al* 2000; Ariza *et al* 1994; Linde *et al*, 2000; Maneewannakul *et al.* 1996] Genes regulated by RamA seemed to be dependant of its concentration as observed by qRT-PCR experiments and western blotting results.

All in all we can conclude, that the MDR phenotype in *Enterobacter aerogenes* is caused by several factors that work hand in hand. The combination of an enzymatic barrier, caused by several antibiotic-degrading enzymes, a target-protection barrier caused by

mutations in the targets of antibiotics and the physical barrier by alteration of the outer membrane profile work together to protect the bacteria from harmful substances. Alteration of just one of these barriers will not turn off MDR. This was visible by the fact, that overexpressed *ramA* or *ramR* altered the membrane profile of the tested MDR strains by increased or decreased expression of porins or efflux pumps as shown with the immunoblots, but did not completely alter the physiology, shown with MIC results. The strain EA289 remained resistant to the majority of the tested antibiotics because the enzymatic and the target protection barrier remained untouched by the overexpression of *ramA* or *ramR*. Why CM64 retained its MDR phenotype is not completely explainable with the gained data. Since the selection with chloramphenicol showed that it selects for mutants with a high frequency, we cannot eliminate the possibility that the strain CM64 possesses mutations in other regions than the ones we sequenced in this study. These mutations could also have an effect on the multi-resistant phenotype. The fact that *ramR* did not have any effect on the already antibiotic susceptible strain ATCC13048 can be explained by the circumstance, that *ramR* was already expressed and functional here. An overexpression of *ramA* on the other hand imbalanced the level between activator *ramA* and repressor *ramR*, altered the membrane permeability and thus caused increased resistance to several antibiotics as shown with the MIC results.

In summary, this study could prove the importance of the global regulator *ram* in the regulation of membrane permeability. The found mutations in *ramR* seem to alter the structure of the protein and hereby leave it less functional compared to the wild-type one. Nevertheless the activity of the global regulator also depends on its expression level. Overexpression of a less functional repressor still was able to modify the content of porins and efflux pumps in the outer membrane. In the end this is the first study to prove the exact coherence between expression of the *ram*-regulon in *E. aerogenes* and its influence on membrane permeability. Overexpression of *ramA* lead to decreased porin and increased efflux

pump components expression, thus letting less substances entering the cell. Overexpression of *ramR* had the opposite effect due to higher porin expression and less efflux pumps.

Tables and figures

Target region	Primer	Sequence	Ann Temp
For sequencing			
marR	M5	CCT-GTT-TAT-TAC-GCT-CGG-CGT	64°C
	M4IIB	CCA-GCG-ACA-ATG-GAG-ATT-CCA-GG	64°C
marA	M7	TAT-GAT-TGA-AAT-CAA-ACG-GCG	64°C
	M12	CAG-ACG-AAG-TGG-TCA-TGC-TTG	64°C
ramR	RamR1	TGA-AAG-GGT-AGG-TTA-GCG	64°C
	RamR2	CAG-ACG-AAA-GCC-CCA-TCC	64°C
ramA	Rafud	GGT-ACC-GTT-TAG-TCA-CGC-GAA-TG	64°C
	RamARevI	CCG-CTT-GGA-AAC-GCT-GTT-ATC	64°C

Table 2a: Primers for sequencing

Name		Sequence		Annealing Temp
omp35	5'	GGCGGCGACGGCTGGAACTACAC	3'	65°C
	5'	GCAAAGCTCAGGCCGTCCACCAGA	3'	
omp36	5'	AGACCTACAACGCAACTCGCACTG	3'	60°C
	5'	AACGGCCCATGTCTTTACCTTTAG	3'	
acrA	5'	ATCTACCGCGCTGGCTACCGTTCA	3'	61°C
	5'	TCGCCTTACCGTTTTCTTGCTTCA	3'	
ramA	5'	ACATTCGCGAGCGTAAACTGC	3'	58°C
	5'	CCCGGCGGCTGATTGAAGGTA	3'	
ramR	5'	AGATGCGCTTAGTACAGACAATG	3'	53°C
	5'	AACCGCTTTCACCGACAG	3'	
marA	5'	AAGTTTCGGAGCGCTCAGGTTAC	3'	58°C
	5'	AGTTTTTGCGCAATCTCCGTCAG	3'	
marR	5'	CCAATTTCGGGTGCTCTGTTCC	3'	57°C
	5'	TGCCGCGCTTGTCATTGG	3'	
soxR	5'	GCTGCTGTCGCCTGGGGAAGTG	3'	62°C
	5'	GTCGCCAGCGGAATACCAATACG	3'	

Table 2b: primers for quantitative realtime PCR

Target region	Primer	Sequence	Ann Temp
For cloning			
ramR	RamR(Fw)XhoI	GTT-ACG-CTC-GAG-GTG-GCT-CGT-CCT-AAG-AGT	64°C
	RamR(Rev)SacI	GTT-ACG-GAG-CTC-TTA-CGA-CTC-ATT-CTC-ATG	64°C
	RamR(Rev)SacI EA27	GTT-ACG-GAG-CTC-TTA-CGA-CTC-ACT-CTC-ATG	64°C
ramA	RamA(Fw)BamHI	CCT-GTA-GGA-TCC-CAT-TTA-ACG-CCT-GGT-GGC-GC	64°C
	RamA(Rev)EcoRI-2	GTT-ACG-GAA-TTC-TCA-GTG-CGC-GCG-GCT-GTG	64°C
For control			
	T7	TAA-TAC-GAC-TCA-CTA-TAG-GG	64°C
	SP6	ATT-TAG-GTG-ACA-CTA-TAG	64°C

Table 2c: Primers for cloning

Strain	Plasmid with insert	Strain	Plasmid with insert
ATCC 13048		**CM64**	
	pDrive - *ramA* pDrive - *ramA27*		pDrive - *ramA* pDrive - *ramA27*
	pDrive - *ramR* pDrive - *ramR27* pDrive - *ramR64*		pDrive - *ramR* pDrive - *ramR27* pDrive - *ramR64*
EA289		**IPM240**	
	pDrive - *ramA* pDrive - *ramA27*		pDrive - *ramA* pDrive - *ramA27*
	pDrive - *ramR* pDrive - *ramR27* pDrive - *ramR64*		pDrive - *ramR* pDrive - *ramR27* pDrive - *ramR64*

Table 3: Combinations of strains and plasmids used for cloning experiments

strains	genes						
	ramA	*ramR*	*marA*	*marR*	*soxR*	*acrA*	*omp35*
EA27	28.42	4.66	-1.24	1.07	-2.88	1.92	-2.75
CM64	147.80	10.90	2.98	3.39	-2.10	3.19	-4.59
IPM20	1.01	1.12	1.05	1.28	-1.00	1.06	1.13
IPM240	1.09	1.09	-1.15	-1.10	-1.38	-1.26	1.69
EA117	40.61	5.42	39.26	27.29	91.09	2.18	3.63
EA3	29.78	6.14	22.73	15.41	51.94	1.91	2.96
EA5	68.76	-3.52	54.93	52.85	169.59	3.80	9.02
GIM55621	18.07	3.03	5.01	2.84	9.25	1.64	1.03
GIM59704	19.33	2.26	-1.32	-1.37	-2.73	2.37	-2.14
MOK75586	17.62	2.28	1.02	1.02	-4.25	3.02	-2.10
PAP12515	14.37	2.49	-3.26	-2.19	-4.83	1.41	-2.31
RAB76089G	-1.81	-1.80	-1.16	-2.88	-1.42	-1.02	-1.19

Table 4: DDCt qRT-PCRs

Strains	MIC (µg ml^{-1})									
	CI	**NX**	**NA**	**TC**	**CL**	**IP**	**FX**	**XM**	**PM**	**TZ**
ATCC13048										
pDrive	0.25	1	4	4	8	2	---	8	0.25	1.5
p*ramA*	0.5	4	24	24	48	2	---	16	1	3
IPM240										
pDrive	0.19	2	6	16	48	>32	---	---	3	12
p*ramA*	0.25	3	12	48	48	>32	---	---	3	8

Table 5a: Combinations of strains and plasmids with corresponding MICs in µg ml^{-1}. CI Ciprofloxacin, NX Norfloxacin, NA Nalidixic acid, TC Tetracycline, CL Chloramphenicol, IP Imipenem, FX Cefoxitin, XM Cefuroxime, PM Cefepime, TZ Ceftazidime. --- means no inhibition could be remarked

Strains	MIC (µg ml^{-1})									
	CI	NX	NA	TC	CL	IP	FX	XM	PM	TZ
IPM240										
pDrive	0.19	2	6	16	48	>32	---	---	3	12
p*ramR* ATCC	0.125	1	4	24	12	12	---	---	3	16
p*ramR* EA27	0.125	1	8	12	16	12	---	---	2	12
p*ramR* CM64	0.125	0.5	4	6	4	12	---	---	3	24

Table 5b: Combinations of strains and plasmids with corresponding MICs in µg ml^{-1}. CI Ciprofloxacin, NX Norfloxacin, NA Nalidixic acid, TC Tetracycline, CL Chloramphenicol, IP Imipenem, FX Cefoxitin, XM Cefuroxime, PM Cefepime, TZ Ceftazidime. --- means no inhibition could be remarked

Strain	Sequence	AA N°
ATCC13048	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
ATCC15038	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
IPM	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
CM64	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
EA103280	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
EA112978	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
NEA7	CATTTAACGCCTGGTGGCGC----------------------G A-----ATG------	60
NEA19	CATTTAACGCCTGGTGGCGC----------------------GGAGAGA-----ATG------	65
GIM59704	CATTTAACGCCTGGTGGCGC----------------------G A-----ATG------	60
MOK75586	CATTTAACGCCTGGTGGCGC----------------------G A-----ATG------	60
PAP12586	CATTTAACGCCTGGTGGCGC----------------------G A-----ATG------	60
RAB73482	CATTTAACGCCTGGTGGCGC----------------------G A-----ATG------	60
EA27	CATTTAACGCCTGGTGGCGC----------------------G A-----ATG------	60

*****

marbox deletion

Figure 1: Sequence upstream of *ramA* (represented by startcodon ATG) with putative *marbox* and deletion found in several clinical isolates. IPM represents the 9 strains IPM1-IPM240. EA103280 and EA112978 are non-MDR clinical isolates. Strains GIM59704, MOK75586, PAP12586, RAB73482 and EA27 represent 91.5% (43 of 47) of all tested MDR clinical isolates.

Strain	Sequence	N° AA
ATCC13048	VARPKSEDKKQALLEAATAAFAQSGIAASTSAIARSAGVAEGTLFRYFATKDELLNELYL	**60**
	AIKMRLVQTMIAGLNPDEKRPKENARNIWNSYIDWGMRNPMEYRAIRRMALSERITDETR	**120**
	IQVKESFPELNEMCQLSVKAVFLSDAYRAFGDALFLSLAETTIEFASHDPQRAREIIALG	**180**
	FEAMWNALHENES	**193**
CM64	VARPKSEDKKQALLEAATAAFAQSGIAASTSAIARSAGVAEGTLFRYFATKDELLNELYL	**60**
	AIKMRLVQTMIAGLNPDEKRPKENARNIWNSYIDWGMRNPMEYRAIRRMALSERITDETR	**120**
	IQVKESFPELNEMCQLSVKAVFLSDAYRAFGDA--LSLAETTIEFASHDPQRAREIIALG	**178**
	FEAMWNALHENES	**191**
CM64new-10	VARPKSEDKKQALLEAATAAFAQSGL	**26**

Figure 3: mutations in RamR after treatment with chloramphenicol; ATCC13048 shows amino acid sequence of RamR in laboratory reference strain; CM64 shows sequence of ATCC13048 after stepwise treatment with chloramphenicol (Ghisalberti D, 2005); CM64new-10 shows sequence found in new attempt after stepwise treatment with chloramphenicol

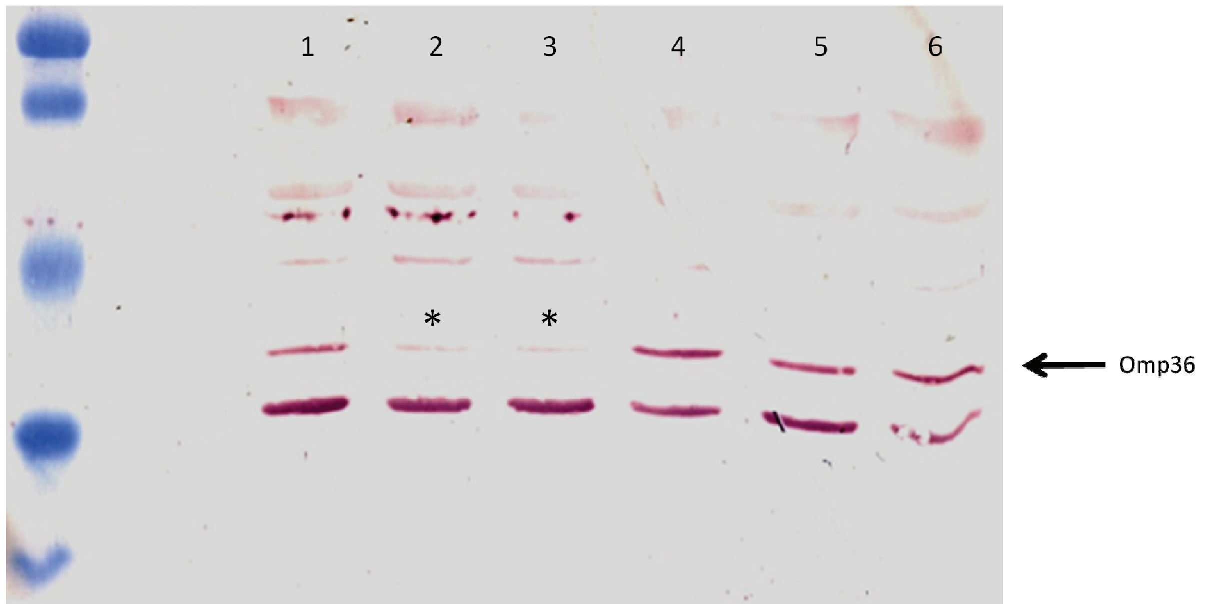

Figure 5: expression of Omp36 in *Enterobacter aerogenes* strain EA289 harboring different plasmids. Lines 1-6: **1** empty plasmid pDriveIV, **2** insert *ramA* ATCC, **3** *ramA* EA27, **4** *ramR* ATCC, **5** *ramR* EA27 and **6** *ramR* CM64. Lowered expression of *omp36* due to overexpression of *ramA* marked with stars *.

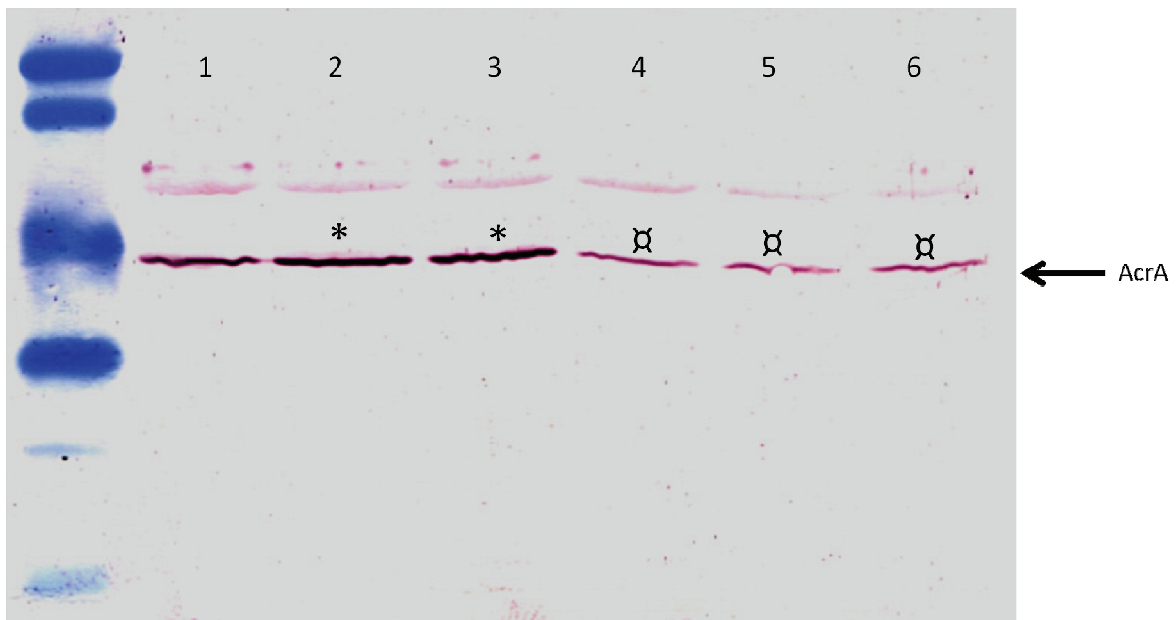

Figure 6a: expression of AcrA in *Enterobacter aerogenes* strain EA289 harboring different plasmids. Lines 1-6: **1** empty plasmid pDriveIV, **2** insert *ramA* ATCC, **3** *ramA* EA27, **4** *ramR* ATCC, **5** *ramR* EA27 and **6** *ramR* CM64. Increased expression of *acrA* due to overexpression of *ramA* marked with stars *, decrease due to *ramR* marked with ¤.

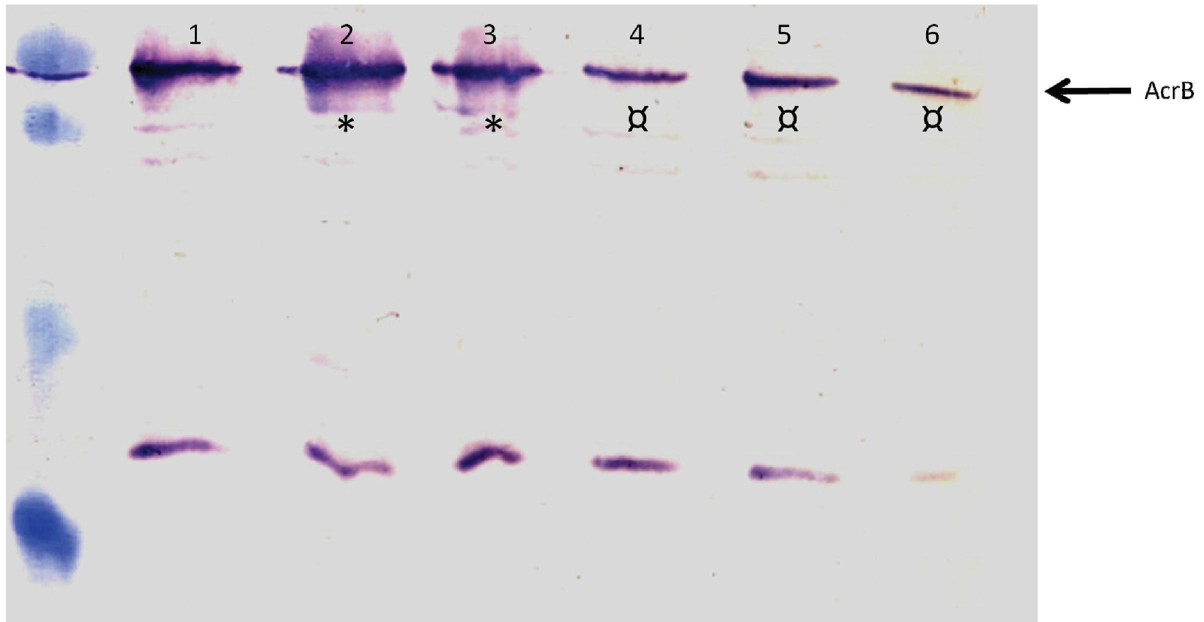

Figure 6b: expression of AcrB in *Enterobacter aerogenes* strain CM64 harboring different plasmids. Lines 1-6: **1** empty plasmid pDriveIV, **2** insert *ramA* ATCC, **3** *ramA* EA27, **4** *ramR* ATCC, **5** *ramR* EA27 and **6** *ramR* CM64. Increased expression of *acrB* due to overexpression of *ramA* marked with stars *, decrease due to *ramR* marked with ¤.

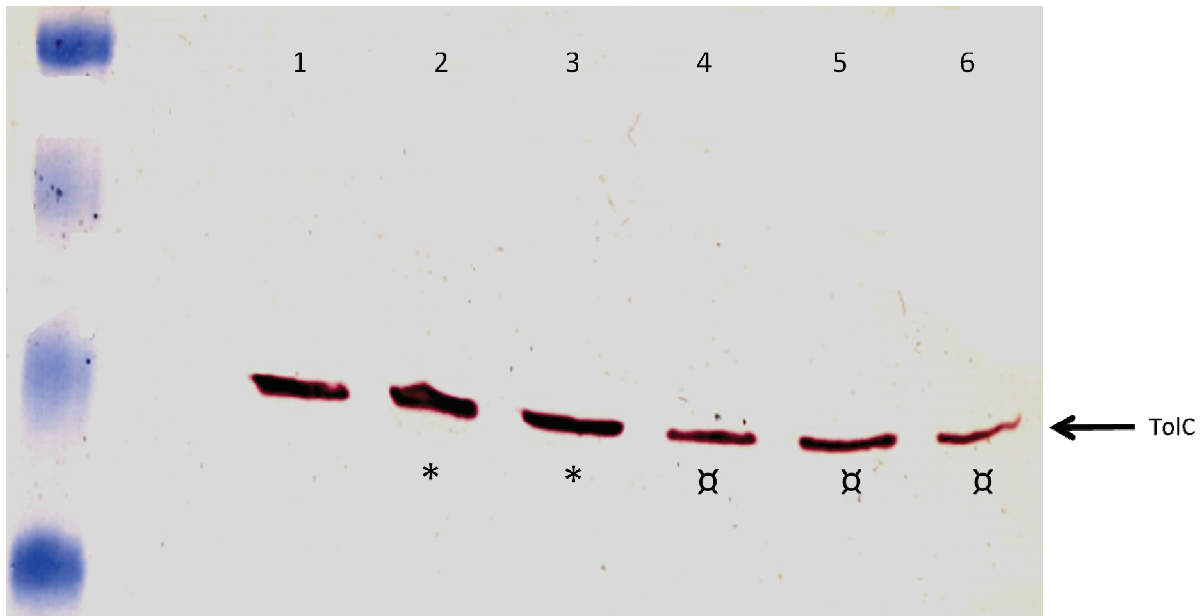

Figure 6c: expression of TolC in *Enterobacter aerogenes* strain IPM240 harboring different plasmids. Lines 1-6: **1** empty plasmid pDriveIV, **2** insert *ramA* ATCC, **3** *ramA* EA27, **4** *ramR* ATCC, **5** *ramR* EA27 and **6** *ramR* CM64. Increased expression of *acrB* due to overexpression of *ramA* marked with stars *, decrease due to *ramR* marked with ¤.

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Strain	MIC (µg/ml)								Porine	Efflux	Reference
	CI	NX	NA	TC	CL	IP	PM	TZ			
Laboratory reference strains:											
ATCC13048	---	0.125	4	2	4	0.25	0.125	0.5	yes	no	
ATCC15038	---	0,125	---	---	2	0.25	0.5	0.5	yes	no	
Laboratory strains 'trained' with antibiotics:											
CM64	---	2	128	16	256	0.25	0.5	1	yes	yes	Ghisalberti et al., 2005
IPM5	---	16	---	8	16	4	1	---	yes	no	Bornet et al., 2003
IPM20	---	16	---	8	32	8	8	---	yes	yes	Bornet et al., 2003
IPM40	---	16	---	8	64	16	16	---	no	yes	Bornet et al., 2003
IPM60	---	16	---	8	64	16	16	---	no	yes	Bornet et al., 2003
IPM70	---	16	---	8	64	16	16	---	no	yes	Bornet et al., 2003
IPM80	---	16	---	16	64	16	16	---	no	yes	Bornet et al., 2003
IPM120	---	16	---	16	64	16	16	---	no	yes	Bornet et al., 2003
IPM160	---	16	---	16	64	32	32	---	no	yes	Bornet et al., 2003
IPM240	---	16	---	16	64	32	32	---	no	yes	Bornet et al., 2003
clinical isolates:											
EA117	32	256	>256	8	512	0.25	64	>512	yes	yes	Gayet et al., 2003
EA119	16	256	>256	8	16	0.125	32	>512	yes	no	Gayet et al., 2003
EA27	32	256	512	16	512	8	64	512	no	yes	Pradel et al. 2002
EA289	32	256	512	8	512	8	64	512	no	yes	Pradel et al., 2002
EA5	---	256	256	8	512	4	64	512	no	yes	Ghisalberti et al., 2005

EA103	---	---	---	---	---	16	16	---	no	no	Charrel et al., 1996
EA111	---	---	---	---	---	8	16	---	no	---	Charrel et al., 1996
EA110	---	---	---	---	---	16	64	---	no	---	Charrel et al., 1996
EA102	---	---	---	---	---	16	32	---	no	---	Charrel et al., 1996
EA3	---	256	---	---	512	4	64	>512	yes	yes	Chevalier et al., 2008
NEA7	---	64	---	---	256	1	4	256	yes	yes	Chevalier et al., 2008
NEA14	---	512	---	---	1024	2	32	>2048	yes	yes	Chevalier et al., 2008
NEA19	---	512	---	---	1024	1	64	>2048	yes	yes	Chevalier et al., 2008
EA54	---	64	>512	2	16	2	64	---	---	yes	Chevalier et al., 2008
EA108	---	64	>512	2	<4	2	1	---	---	no	Chevalier et al., 2008
EA6582	---	256	>512	4	256	4	4	---	---	yes	Chevalier et al., 2008
EA121653	---	256	>512	2	256	1	1	---	---	no	Chevalier et al., 2008
EA1061701	---	256	>512	8	>256	2	1	---	---	yes	Chevalier et al., 2008
EA109688	---	128	>512	2	16	1	4	---	---	yes	Chevalier et al., 2008
EA106206	---	128	>512	4	256	4	64	---	---	yes	Chevalier et al., 2008
EA131102	---	256	>512	8	>256	4	2	---	---	yes	Chevalier et al., 2008
EA131538	---	256	>512	8	>256	>4	32	---	---	yes	Chevalier et al., 2008
EA137464	---	512	>512	8	>256	>4	64	---	---	yes	Chevalier et al., 2008
EA103280	---	<4	16	8	8	4	1	---	---	yes	Chevalier et al., 2008
EA112978	---	256	>512	2	<4	4	4	---	---	no	Chevalier et al., 2008
EA45377	64	---	---	4	256	4	4	---	---	---	Bornet et al., 1999 (unpublished)
GIM63001	64	---	---	8	256	2	4	---	yes	---	Bornet et al., 1999

GIM59705	128	---	---	4	512	1	2	---	no	---	Bornet et al., 1999
GIM59704	128	---	---	4	256	16	32	---	no	---	Bornet et al., 1999
GIM53292	128	---	---	16	512	32	32	---	no	---	Bornet et al., 1999
GIM54584	128	---	---	4	256	4	16	---	yes	---	Bornet et al., 1999
GIM55621	128	---	---	16	512	8	128	---	no	---	Bornet et al., 1999
GIM55625	64	---	---	2	256	1	2	---	yes	---	Bornet et al., 1999
GIM59627	128	---	---	8	512	8	64	---	no	---	Bornet et al., 1999
MOK72691	256	---	---	8	256	16	128	---	no	---	Bornet et al., 1999
MOK73694	256	---	---	4	128	16	32	---	yes	---	Bornet et al., 1999
MOK75586	256	---	---	4	128	16	32	---	no	---	Bornet et al., 1999
MOK76500	256	---	---	4	128	1	2	---	yes	---	Bornet et al., 1999
PAP11668	2	---	---	1	8	1	1	---	yes	---	Bornet et al., 1999
PAP13165	32	---	---	4	256	16	64	---	no	---	Bornet et al., 1999
PAP12698	32	---	---	8	256	2	16	---	yes	---	Bornet et al., 1999
PAP12586	32	---	---	8	256	2	8	---	yes	---	Bornet et al., 1999
PAP12515	64	---	---	16	256	16	64	---	no	---	Bornet et al., 1999
RAB73698	64	---	---	8	256	2	4	---	yes	---	Bornet et al., 1999
RAB73482	64	---	---	4	256	1	2	---	yes	---	Bornet et al., 1999
RAB76089G	64	---	---	8	256	8	128	---	no	---	Bornet et al., 1999
RAB76089P	32	---	---	8	512	8	512	---	yes	---	Bornet et al., 1999

Table 1: laboratory strains and clinical isolates with corresponding MICs in µg ml^{-1}; CI Ciprofloxacin, NX Norfloxacin, NA Nalidixic acid, TC Tetracycline, CL Chloramphenicol, IP Imipenem, FX Cefoxitin, XM Cefuroxime, PM Cefepime, TZ Ceftazidime; Porins no/yes indicates if antibody testing showed negative or positive results; Efflux no/yes indicates if active efflux could be proven or not; --- stands for not determined; reference indicates were the strain has been described and characterized earlier

Strain	Sequence	AA N°

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ATCC13048   VARPKSEDKKQALLEAATAAFAQSGIAASTSAIARSAGVAEGTLFRYFATKDELLNELYLAIKMRLVQTMIAGLNPDEKRPKENARNIWNSYIDWGMRNP   100
ATCC15038   ----------------------------------------------------------------------------------------------------   100

IPM         ----------------------------------------------------------------------------------------------------   100
CM64        ----------------------------------------------------------------------------------------------------   100

EA103280    ----------------------------------------------------------------------------------------------------   100
EA112978    -----------------------------------------------------------------------D----------------------------   100

NEA7        -----------------------------------------------------------------------D----------------------------   100
NEA19       -----------------------------------------------------------------------D---------------------------S   100

GIM59704    -----------------------------------------------------------------------D---------------------------S   100
MOK75586    -----------------------------------------------------------------------D---------------------------S   100
PAP12586    -----------------------------------------------------------------------D---------------------------S   100
RAB73482    -----------------------------------------------------------------------D---------------------------S   100

EA27        -----------------------------------------------------------------------D---------------------------S   100
            .......................................................................*...........................*

ATCC13048   MEYRAIRRMALSERITDETRIQVKESFPELNEMCQLSVKAVFLSDAYRAFGDALFLSLAETTIEFASHDPQRAREIIALGFEAMWNALHENES   193
ATCC15038   --------------------S---------------------------------------------------------------------S--   193

IPM         ---------------------------------------------------------------------------------------------   193
CM64        -----------------------------------------------------  --------------------------------------   191

EA103280    ---------------------------------------------------------------------------------------------   193
EA112978    --------------------S---------------------------------------------------------------------S--   193

NEA7        --------------------S---------------------------------------------------------------------S--   193
NEA19       --------------------S----------------------------------------------------------------ECAA   189

GIM59704    --------------------S----------------------------------------------------------------ECAA   189
MOK75586    --------------------S----------------------------------------------------------------ECAA   189
PAP12586    --------------------S----------------------------------------------------------------ECAA   189
RAB73482    --------------------S----------------------------------------------------------------ECAA   189

EA27        -------------------------------------------------------------------------------------ECAA   189
            ....................*................................**..............................********
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Figure 2: Comparison of amino-acid sequence of RamR in clinical isolates and laboratory strains. IPM represents the 9 strains IPM5-IPM240. Strains GIM59704, MOK75586, PAP12586, RAB73482 and EA27 represent 43 of 47 (91.5%) tested clinical isolates

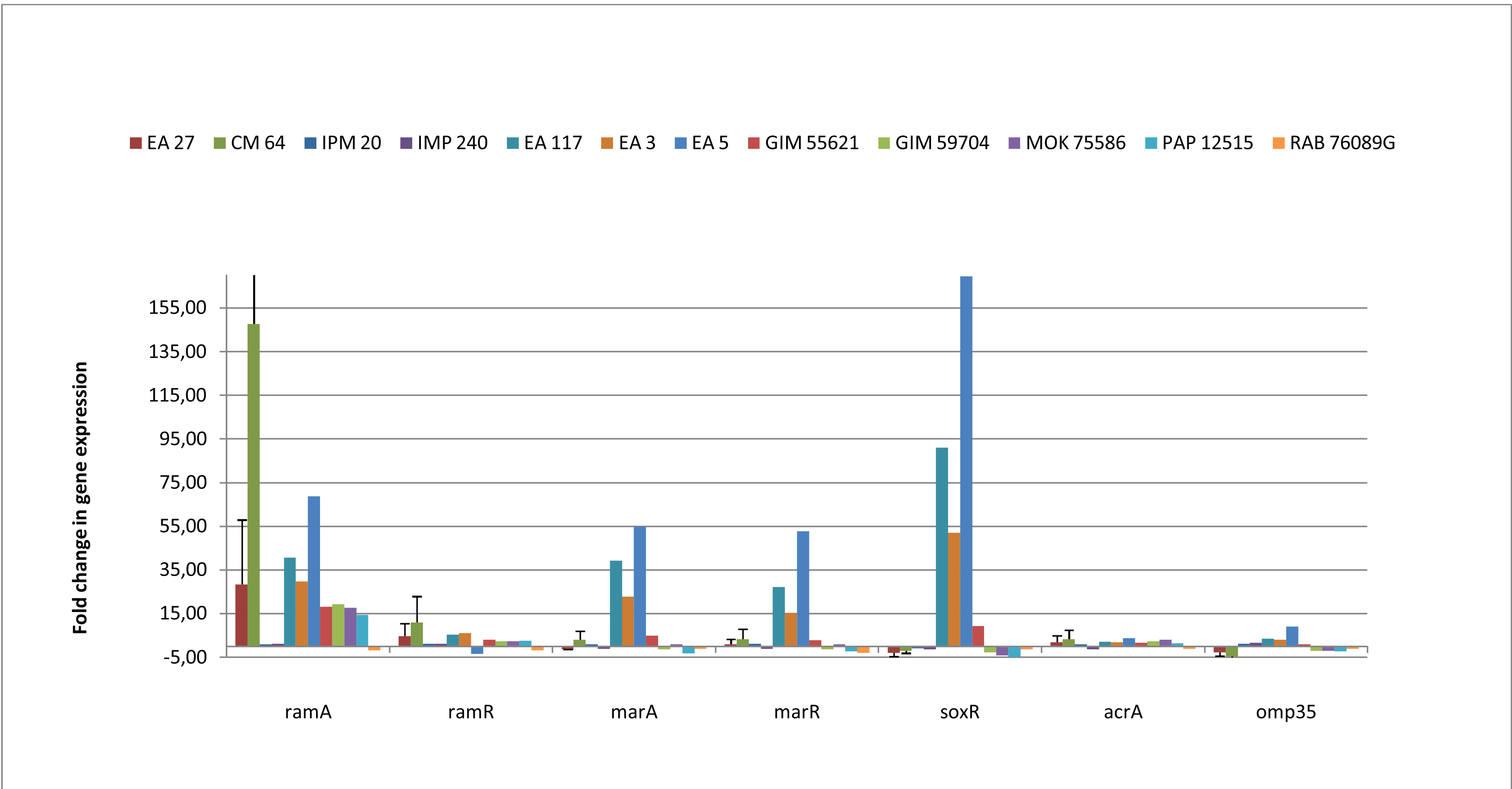

Figure 4: expression of *ramA*, *ramR*, *marA*, *marR*, *soxR*, *acrA* and *omp35* in clinical isolates compared to laboratory strain ATCC13048

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How β-Lactam Antibiotics Enter Bacteria: A Dialogue with the Porins

Chloë E. James[1]‡, Kozhinjampara R. Mahendran[2]‡, Alexander Molitor[1], Jean-Michel Bolla[1], Andrey N. Bessonov[2], Mathias Winterhalter[2], Jean-Marie Pagès[1]*

1 UMR-MD-1, Transporteurs membranaires, Chimiorésistance et Drug Design, Faculté de Médecine, IFR 88, Université de la Méditerranée, Marseille, France, **2** School of Engineering and Science, Jacobs University Bremen, Bremen, Germany

Abstract

Background: Multi-drug resistant (MDR) infections have become a major concern in hospitals worldwide. This study investigates membrane translocation, which is the first step required for drug action on internal bacterial targets. β-lactams, a major antibiotic class, use porins to pass through the outer membrane barrier of Gram-negative bacteria. Clinical reports have linked the MDR phenotype to altered membrane permeability including porin modification and efflux pump expression.

Methodology/Principal Findings: Here influx of β-lactams through the major *Enterobacter aerogenes* porin Omp36 is characterized. Conductance measurements through a single Omp36 trimer reconstituted into a planar lipid bilayer allowed us to count the passage of single β-lactam molecules. Statistical analysis of each transport event yielded the kinetic parameters of antibiotic travel through Omp36 and distinguishable translocation properties of β-lactams were quantified for ertapenem and cefepime. Expression of Omp36 in an otherwise porin-null bacterial strain is shown to confer increases in the killing rate of these antibiotics and in the corresponding bacterial susceptibility.

Conclusions/Significance: We propose the idea of a molecular ''passport'' that allows rapid transport of substrates through porins. Deciphering antibiotic translocation provides new insights for the design of novel drugs that may be highly effective at passing through the porin constriction zone. Such data may hold the key for the next generation of antibiotics capable of rapid intracellular accumulation to circumvent the further development MDR infections.

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* E-mail: Jean-Marie.PAGES@univmed.fr

‡ These authors contributed equally to this work.

Introduction

One major biological challenge is to understand how the cell controls the exchange of solutes with its environment and to decipher the role of membrane transporters in this process [1,2]. This aspect of membrane physiology is a key issue in the field of infectious diseases. Antibiotic molecules used in clinical regimens, must penetrate the outer membrane of Gram negative bacteria to reach their target sites and kill the pathogen [3,4]. Rapid delivery to achieve the required concentrations of antibiotic molecules at their internal targets is now an acute objective due to the threat associated with re-emerging infectious diseases that are resistant to multiple antibiotics. Multi-drug resistant (MDR) bacterial infections have become a worldwide problem, particularly in hospital settings [5–7]. Among the most urgent is the opportunistic pathogen, *Enterobacter aerogenes*, responsible for nosocomial infections able to rapidly develop a MDR phenotype within 5 days of antibiotherapy [8]. In order to unlock new therapeutic options/solutions, it is crucial to understand how and how fast antibiotics interact with bacterial cells and the mechanisms that lead to such high levels of resistance. There are 3 major characteristics that an effective antibiotic must exhibit. 1) Rapid and stable accumulation at the target site; 2) Strong target binding; 3) Stability against enzymatic attack [9].

The first step of antibiotic interaction with Gram-negative bacteria is to cross the outer membrane, which forms a protective barrier against hostile environments [3,4]. The exact mechanism of uptake across this lipid bilayer by hydrophobic compounds is poorly understood. The membrane is punctuated by porins, which are major outer membrane proteins (OMPs) that form water-filled channels allowing diffusion across the membrane. Clinical studies show that the general diffusion porins of many enterobacteriaceal species serve as a major gateway for the passage of β-lactams and fluoroquinolones [3,4]. Furthermore, alteration of outer membrane permeability, including modification of porin expression has emerged as a major MDR mechanism in *E. aerogenes* and other enterobacterial pathogens [8,10–13]. To improve the translocation efficiency of future antibiotics, it is vital to understand the underlying molecular mechanisms of transport.

The crystal structures of several porins have been determined and the conserved internal loop 3 constitutes a crucial part of the porin channel involved in the influx of antibiotics [4,14–17]. Mutations in this region of Omp36 from *E. aerogenes* and OmpF and OmpC from *E. coli* have been shown to confer altered permeability and susceptibility to various antibiotics [4,16,18–22]. Analysis of these loop 3 mutations has indicated that certain substitutions induce drastic changes in channel properties due to the presence of bulky or differentially charged residues [20–22].

Investigation of antibiotic transport through porin channels can be carried out by insertion of purified porins into planar lipid bilayers. Quantification of the molecular dialogue between antibiotic molecules and porin channels can be achieved *via* analysis of ion current noise in the presence of antibiotics [17,23]. Measuring the ion current through purified porins reconstituted into planar lipid bilayers provides information about a number of structural and functional properties such as pore size and selectivity [24]. Moreover, the passage of large molecules through the channel interrupt the ion current causing fluctuation or even transient blockages of conductance [17,23,25]. Therefore, addition of various antibiotics to the system can cause interaction dependent fluctuations in the ion current and report on the electrophysiological parameters of translocation [17,25].

In this study transport properties through a major *E. aerogenes* porin, Omp36 (homologous to *E. coli* OmpC and to *Klebsiella pneumoniae* OmpK36) were investigated. Physiological conditions within the patient body favor the expression of Omp36 belonging to the OmpC-family, over OmpF-type porins [10,16,26,27]. This is therefore the more relevant porin type to consider during antibiotherapy [4,13]. Here the aim was to quantify the influx of representative β-lactams through Omp36. The porin was purified and ion flow through a single trimer reconstituted into a planar lipid bilayer was measured. The presence of antibiotics caused ion current fluctuation in a concentration dependent manner. Analysis of these fluctuations, induced by penetration of the antibiotics into the channel, allowed crucial information to be obtained about the transport mechanism. In addition Omp36 was expressed in the outer membrane of a porin-null *E. coli* mutant (BL21Δ*omp*). Minimum inhibitory concentration assays were used to assess β-lactam susceptibility conferred by Omp36 as the sole porin. Information about the rate of translocation through this porin for delivery to target sites was further provided by measuring the rate of decline of colony forming units following exposure to inhibitory levels of β-lactams.

Results

Evidence and quantification of antibiotic translocation through Omp36

The *omp36* gene was cloned and expressed in the porin-null *E. coli* strain BL21Δ*omp* [28] (see **Text S1**, **Table S1** and **Fig. S1**). Omp36 was purified using ion-exchange chromatography and a single trimeric porin was reconstituted into artificial lipid membranes [17,23]. Application of a transmembrane voltage established an ion current through the channel and, in the absence of antibiotics, no visible current blockage was detected up to a voltage of <150–200 mV **(Fig. 1a)**. Addition of antibiotics to this system caused fluctuations in the ion current reflecting the possible channel-drug interactions. Ertapenem which is a negatively charged carbapenem [9] caused spontaneous blockage of the ionic currents **(Fig. 1b)**. The presence of antibiotic caused rapid blockages of the monomers. These ion current fluctuation increased with increasing concentration (see **Text S1** and **Fig. S2**). Furthermore, analysis at higher time resolution clearly indicated complete monomer channel blockages. On average 0.5 mM ertapenem caused single monomer blockages and at an increased concentration of 15 mM two monomers were blocked. Interactions with cefepime, a zwitterionic cephalosporin [9], were also detected, but the blockage events were shorter and less frequent than those caused by ertapenem **(Fig. 1c)**. In contrast ceftazidime **(Fig. 1d)** and ampicillin **(Fig. 1e)** caused no significant blockage of the ionic current indicating negligible interaction with the channel. Similar characterization of OmpC, for which a high-resolution structure has recently been resolved [15], showed the same pattern of interaction with ertapenem, cefepime, ceftazidime and ampicillin (data not shown).

The penetration of antibiotics into the channel can also be measured by analysing the power density spectra of the ion current. In **Fig. 2a**, a typical power density spectra of the ion current fluctuations is shown. The figure shows clearly the effect of different antibiotics: the presence of 10 mM ertapenem increased the ion current noise 15 fold compared to background levels. In contrast a much higher concentration of cefepime (25 mM) caused only a doubling of the noise level. In the case of ampicillin and ceftazidime (see **Text S1** and **Fig. S3**) no excess noise was visible (see Material and Methods for details). As previously shown the excess noise was caused by perturbing the ion current inside the channel in the presence of interacting antibiotics [17,23]. Channel blocking by the antibiotic molecules was also quantified by using a statistical analysis of the channel in its 'un-occupied' and 'occupied' (or blocked) states. The average residence time (τ) of each antibiotic in the Omp36 channel was obtained by single exponential fitting of blockage time histograms with the distribution of dwell time in the blocked state (single channel analysis). The τ was 0.14 ± 0.02 ms for ertapenem and 0.10 ± 0.02 ms for cefepime at 50 mV (**Fig. 2b**) (see Material and Methods for details).

The strength of the ertapenem interactions allowed further quantification of the "molecular dialogue" between this antibiotic and the Omp36 channel. **Fig. 2c** shows the number blocking events, which increase with increasing antibiotic concentration. Following *cis* side addition of ertapenem more intense blocking was observed compared to *trans* side addition. This indicated an asymmetry in accessibility of the binding site and a lower energy barrier from the *cis* side. As ertapenem is negatively charged, *cis* side addition of the antibiotic along with the application of positive voltages should favor translocation through the channel. Clearly, we observed an enhanced rate of translocation when the antibiotic was travelling from the *cis* to the *trans* side. However, blockage events following *trans* side addition of ertapenem were less frequent and voltage independent. This indicated a higher energy barrier on the *trans* side and the electric field did not influence the rate of translocation. In **Fig. 2d**, the average residence time of antibiotic molecules in the channel measured at different voltages is shown. It is important to note that the residence time was independent of the concentration and of which side of the membrane the drug was added. This indicated the presence of a single affinity site in the channel according to the model described by Schwarz *et al.*, 2003 [25].

We simplified the mathematical analysis by assuming the presence of one affinity site within the channel, accessible from both sides of the lipid membrane. The most important step that determines antibiotic translocation is the entrance and exit rates. The kinetic rate for channel entrance and exit allows estimation of the net flux of antibiotics. For example when 1 mM ertapenem was added to the *cis* side of the lipid membrane, the association rate constant (k_{on}) was 9100 ± 1000 $(Ms)^{-1}$ and the binding constant (K) was 1.50 ± 0.05 M^{-1} at an applied voltage of 50 mV.

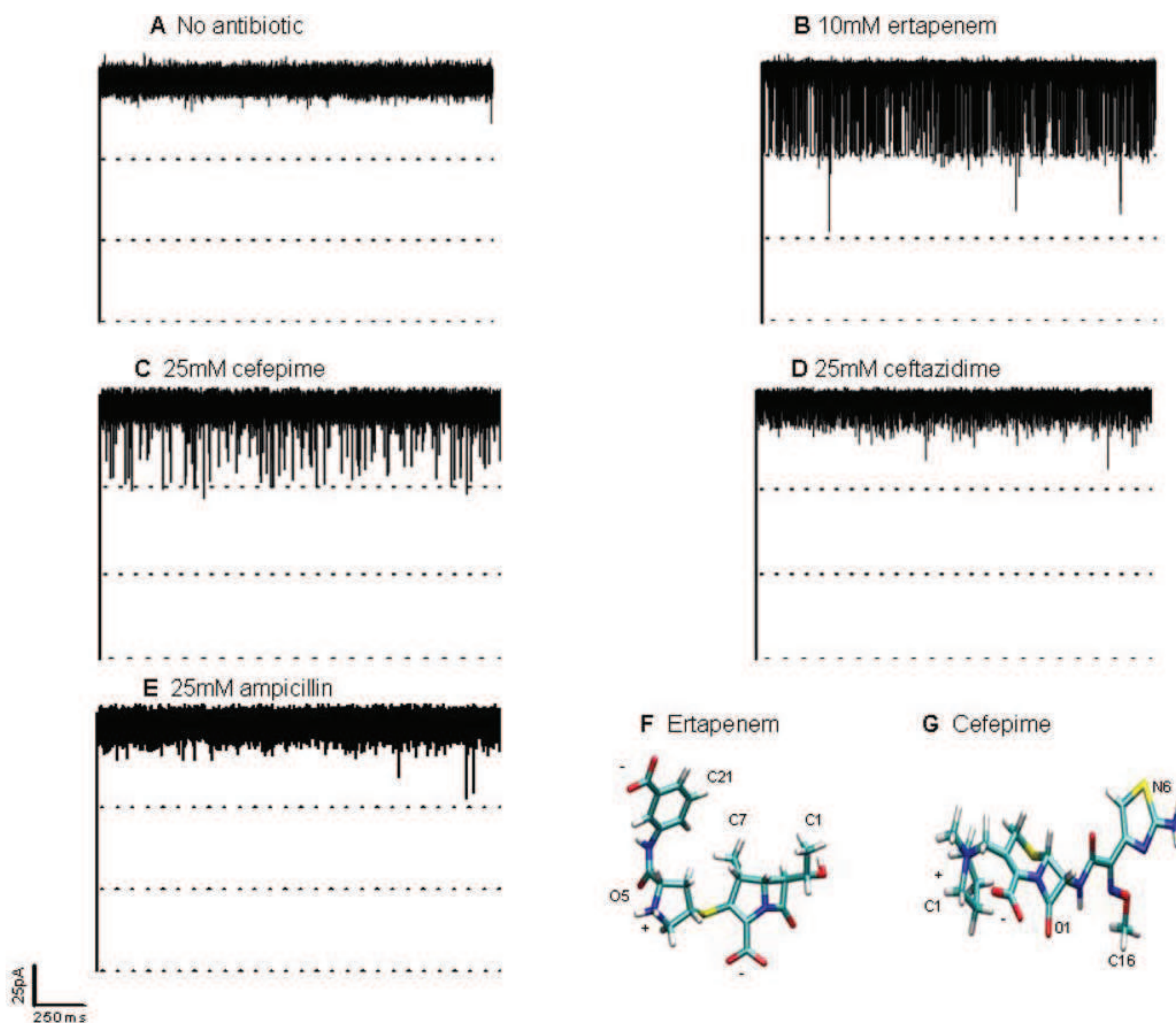

Figure 1. Typical ion current recordings through a single Omp36 trimer reconstituted into planar lipid membranes. a. In the absence of antibiotic, almost no channel closure was visible. **b.** Addition of 10 mM ertapenem on the *cis* side caused rapid closure of one monomer. **c.** Addition of 25 mM cefepime on the *cis* side caused significantly less blocking compared to ertapenem. **d.** Addition of 25 mM ceftazidime on the *cis* side caused no blocking. **e.** Addition of 25 mM ampicillin on the *cis* side caused no blocking. Membrane bathing solution was 1 M KCl (pH 6) and the applied voltage was 50 mV. **Chemical structure of antibiotics**. **f.** Ertapenem **g.** Cefepime. The antibiotics are displayed in ''balls and sticks'' and colored by atom type (oxygens in red, nitrogens in blue, carbons in cyan, sulfur in green, hydrogens in white).
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As previously shown the flux of antibiotic molecules per second is given by [25,29,30] (see Material and Methods for details)

$$J = [k_{on}/2,] \cdot \Delta c$$

This analysis concluded that, using a 1 mM ertapenem concentration gradient across the channel, about 5 molecules were able to translocate each Omp36 monomer per second. Blocking events in the presence of cefepime were weak compared to ertapenem. When 1 mM cefepime was added to the *cis* side of the lipid membrane, the association rate constant (k_{on}) was 1000 ± 100 $(Ms)^{-1}$ and the binding constant (K) was $0.2 \pm 0.02\ M^{-1}$ at an applied voltage of 50 mV. The number of molecules translocated was approximately 0.5 molecules per second per monomer. Inspection of the above equation showed that translocation was proportional to the on-rate [25,29,30]. The strength of an antibiotic interaction with the affinity site of a channel greatly influences the efficacy of its translocation [31,32]. Our data shows that ertapenem interacts more strongly than cefepime with the Omp36 channel (**Fig 1, 2**) and translocates the channel more rapidly.

Rate of β-lactam Action on *E. coli* Expressing Omp36 as the Sole Porin

The ability of β-lactams to traverse the outer membrane *via* Omp36 channels was initially determined here using minimum inhibitory concentration (MIC) assays. Omp36 (or OmpA as a negative control – see **Text S1**) was expressed, on an IPTG inducible plasmid, as the sole porin in an otherwise porin-null *E. coli* strain (BL21Δ*omp*). Expression of Omp36 in the outer

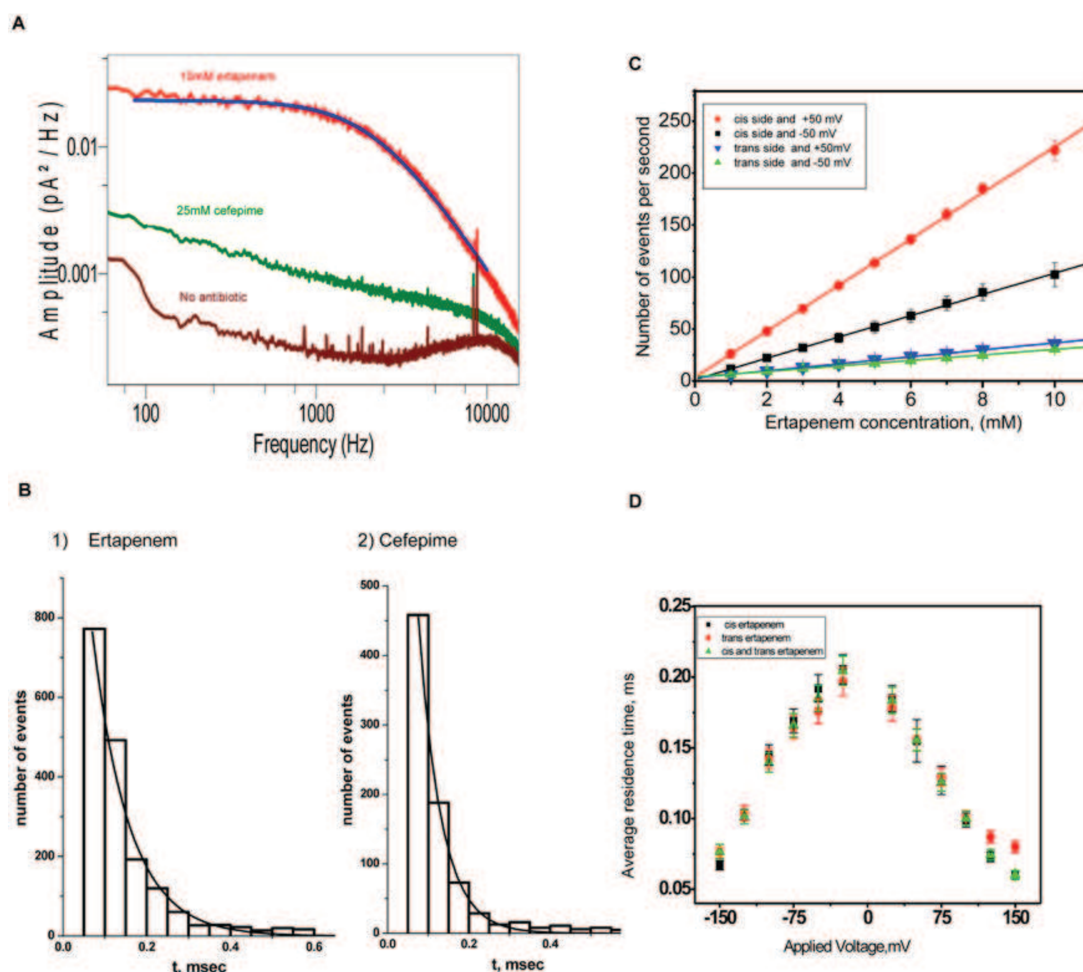

Figure 2. Kinetic analysis of antibiotic transport through Omp36. a. Power spectral densities of the excess noise in the ion current through a single trimeric Omp36 channel in the presence two different antibiotics ertapenem and cefepime added to the *cis* side of the lipid membrane. Smooth solid line through the spectra is Lorentzian fit with $\tau = 120$ µs for ertapenem. **b.** Time histogram of Omp36 channel blockage in presence of 10 mM ertapenem (1) or 25 mM cefepime (2) added to the *cis* side of the lipid membrane. Solid line is the single exponential fit with characteristic time $\tau = 128$ µs(1) and 105 µs(2). **c.** The number of ertapenem blocking events per second was linear to ertapenem concentration and depended on applied voltage and side of antibiotic addition (*cis* or *trans* side). **d.** Ertapenem residence time did not depend on the direction of the drug addition (*cis*, *trans* or both sides) and it depended on the applied voltage. Average residence time decreased with increased applied voltage.
doi:10.1371/journal.pone.0005453.g002

membrane (see **Text S1** and **Fig. S1**) resulted in an 8 fold increase in sensitivity to ertapenem with an MIC of 0.5 µg ml^{-1} in IPTG-induced cultures compared to 4 µg ml^{-1} in non-induced cultures and those harboring vector only (see **Text S1** and **Table S1**). These data confirmed the involvement of Omp36 in β-lactam susceptibility (see **Text S1**). We further compared the efficacy of ertapenem and cefepime action by exposing bacterial cultures to inhibitory concentrations of each β-lactam and observing the percentage decreases in cell number (colony forming units, cfu ml^{-1}) over time (**Fig. 3**). In the presence of either ertapenem or cefepime, BL21Δ*omp* cultures expressing Omp36 as the sole porin were depleted at a dramatically increased rate compared to those expressing OmpA (**Fig. 3**) and, to a lesser extent, vector only (data not shown). The action of ertapenem was observed to be considerably faster than cefepime with a 90% decrease in cfu ml^{-1} of Omp36 expressing cultures within 45 minutes and 90 minutes respectively and a 99% decrease within 60 minutes and 150 minutes. Care must be taken when interpreting this data. The rapid action of ertapenem could be attributed to high target affinity or stability against β-lactamase degradation [33,34] (see Supplementary Data Section). However, with the use of stringent controls imposed here, these results corroborate both MIC and electrophysiological data, suggesting that efficient interactions of ertapenem with an affinity site in the Omp36 channel confer faster influx across the outer membrane *via* this porin, contributing to the faster rate of action.

Discussion

This study deciphers a role for the enterobacterial porin, Omp36 in antibiotic transport. Recent clinical studies of *K. pneumoniae* infection observed that exposure to ertapenem promoted drug resistance *via* the loss of OmpK36 [36–38]. Furthermore, many recently evolved metallo-carbapenemases participating in the enzymatic barrier require decreased porin expression to effectively confer high-level carbapenem resistance [33]. Increasing clinical studies report the down-regulation of porin expression, or a shift favoring the expression of smaller or more restrictive

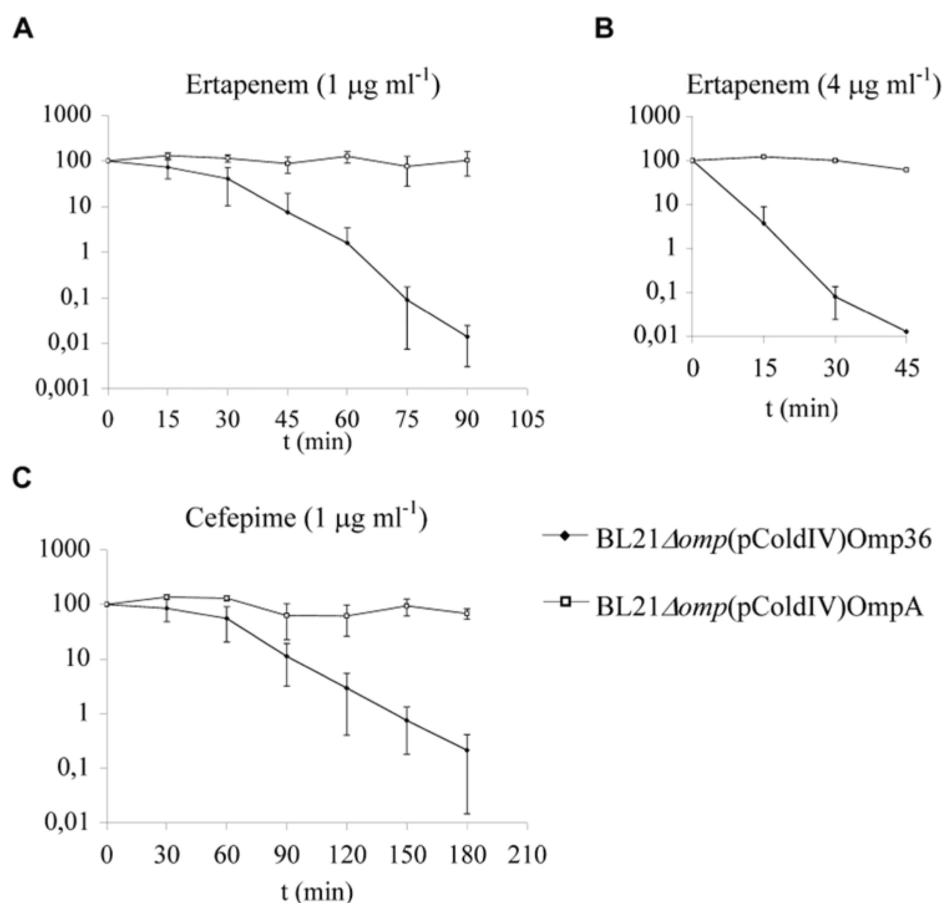

Figure 3. Influence of Omp36 Expression on the Rate of β-lactam Antibiotic Activity. Percentage decrease in cfu ml^{-1} of BL21*Δomp* cultures expressing either Omp36 or OmpA following exposure to inhibitory concentrations of: **a** ertapenem (1 μg ml^{-1}), **b** ertapenem (4 μg ml^{-1}), **c** cefepime (1 μg ml^{-1}). Experiments were repeated three times and error bars were indicated.
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channels, as a response to antibiotherapy [4,13]. This results in reduced membrane permeability that severely limits intracellular drug accumulation, allowing the evolution and/or the acquisition of other resistance mechanisms including target mutations, enzymatic production, etc [13]. Such reports highlight the importance of: 1) efficient influx through porins for β-lactams to reach their target sites, and 2) a detailed understanding of this dynamic and interactive process.

The pathway of the antibiotic molecule through the channel is of crucial importance for the intracellular accumulation of antibacterial drugs. It has become clear that the transport of β-lactams or fluoroquinolones through OmpF-type porins is not by passive diffusion through an inert tube, but involves specific interactions with porin channels [17,19,23]. Due to the detailed knowledge of its crystal structure most studies of antibiotic-porin interactions so far have focused on OmpF from *E. coli* [14,39], which is a major porin type expressed *in vitro* along with homologs Omp35 and OmpK35 in *Enterobacter* and *Klebsiella* spp. However, *in vivo* temperature and salt concentrations, favor the expression of OmpC-type porins including *Klebsiella pneumoniae* OmpK36 and *E. aerogenes* Omp36 [26,27] investigated here. Consequently, these are the dominant porins in the patient body [3,4,13] and represent the key strategic pathways for β-lactams and fluoroquinolones to penetrate the bacterial cell during patient therapy. Our study combines high resolution ion conductance measurements with biological susceptibility assays to explore β-lactam translocation properties through Omp36, a major porin of the MDR pathogen, *E. aerogenes*. Using two representative β-lactam molecules, we demonstrate that interaction with the channel correlates with facilitated translocation through the porin and thus enhances the transport efficiency. We hypothesize that there is a strong interaction, involving hydrophobic and hydrogen bonds, between ertapenem and specific aminoacid residues which constitute the affinity site within Omp36. Ertapenem has a net negative charge and two carboxylic groups are able to form hydrogen bonds with the basic residues of the channel. In the case of cefepime (a zwitterionic compound) we measured a lower channel affinity. This is in agreement with previous molecular modeling of cefepime in the constriction zone of OmpF [19] which is the Omp35 homologue in *E. coli* [4]. For optimal permeation, a balance is required between affinity and repulsion interactions at key sites in the constriction zone. Our MIC data agree with the electrophysiological results, showing stronger activity of ertapenem than cefepime in bacterial cells expressing Omp36 as the sole functional porin. In addition we have demonstrated the rate of ertapenem antibiotic action on these cells to be strongly faster than that of cefepime and that this is partly due to more rapid transport through the porin.

A number of chemical and physical properties of antibiotic molecules, such as size, hydrophobicity, stoichiometry and charge,

have been shown to influence their rate of permeation through porin channels. For example, zwitterionic compounds have been shown to penetrate proteoliposomes very rapidly [40] and have induced increased ion flux perturbations through OmpF in lipid bilayer models compared to other charged compounds. In addition, large molecules, with bulky side-chains, such as azlocillin and piperacillin have shown low permeation rates [17].

Efficient translocation through porins requires favorable channel properties in addition to a streamlined antibiotic molecule. As β-lactam molecules are similar in size to the channel diameter, their passage is not a simple diffusion but rather a gliding process along the pore wall. Within the constriction zone of porin channels, strategically located residues create a strong electrostatic field [15,17,36]. Key exposed residues particularly in the internal loop 3 have been identified that transiently interact with translocating molecules to strongly influence the rate of permeation [17] and the antibiotic efficacy [19]. Site-directed mutagenesis at such sites in *E. coli* OmpF and OmpC has been shown to alter susceptibility to certain antibacterial molecules [21,22,41,42]. OmpC and Omp36 porins harboring loop 3 mutations have been detected in a small number of resistant clinical isolates of *E. coli* and *E. aerogenes* and may represent an emerging bacterial drug resistance strategy in order to restrict antibiotic influx [4,13]. Several biophysical investigations report the interaction between ampicillin and OmpF during drug diffusion in agreement with microbiological evidence [17,19,24]. In contrast, we have shown that ampicillin interaction with Omp36 and OmpC is negligible. Nikaido and Rosenberg [43] showed much restricted penetration of antibiotic molecules with bulky side-chains and negative charges through OmpC than through the wider OmpF channel. The recently resolved OmpC crystal structure suggests that electrostatic pore potential and specific atomic details inside the channel are the key parameters distinguishing OmpC and OmpF rather than size [15]. This reduced permeability through OmpC-type porins could explain the shift from OmpF-type to OmpC-type expression observed in clinical isolates during antibiotherapy as a strategy to limit antibiotic influx [4,13]. Ertapenem and cefepime both possess some of the star qualities required for rapid translocation. They are small and compact, and interact with the channel significantly. The recent description of the OmpC 3D structure [15], presents the opportunity to decipher some of the detailed molecular criteria involved in antibiotic diffusion through this porin group. Future experiments should explore mutagenesis of key sites within the Omp36 L3 loop to decipher exactly which residues are interacting with each drug, and therefore, which aspects of the antibiotic molecular structure drives rapid transport.

Our data suggest that for optimal permeation, a balance is required between affinity and repulsion interactions at key sites in the constriction zone. Consequently, the strength of interaction has a major influence on rates of antibiotic penetration, *ie* intracellular accumulation, and thus antibiotic efficiency [13].

A combination of efficient intracellular accumulation, stability against β-lactamases and target affinity is exhibited by ertapenem for effective antibiotic activity in bacteria. Crossing the outer membrane is the first step in the β-lactam journey to its periplasmic target site ensuring sufficient intracellular concentrations for bacteriocidal activity. We report here that certain molecular characteristics such as compact structure and a particular pattern of ionic charges yet to be deciphered may constitute a 'passport' for rapid travel through the porin demonstrating that drug passive diffusion is in fact an interactive process. Our approach may contribute to the rational design of new antibiotic candidates against MDR pathogens and serve to optimize influx by screening translocation rates of new compounds, to determine whether they hold a valid passport for the most efficient delivery to target sites.

Materials and Methods

Bacterial Strains and Culture Media

Cloning was performed using *E. coli* JM109. Protein expression for purification and MIC experiments was performed in porin-null *E. coli* BL21(DE3)omp8 (Δ*lamB, ompF::Tn5,* Δ*ompA,* Δ*ompC*) referred to in the text as BL21Δ*omp* [28]. Bacteria were grown in Luria bertani (LB) broth (Difco) except during MIC experiments, in which Muller Hinton (MH) broth (Difco) was used. Transformants were selected on Luria Bertani agar (Difco) containing relevant antibiotics (kanamycin (50 μg ml^{-1}) and or ampicillin (100 μg ml^{-1}) (Sigma)).

Cloning and Outer Membrane Expression of *omp36* and *ompA*

The *omp36* (1137 bp) and *ompA* (1085 bp) genes were amplified, including their signal peptide sequences, from *E. aerogenes* ATCC strain 13048 using PCR, and restriction sites were added (underlined in the primer sequence) to each end using primers *5′omp36Bam*HI (5′-GTTAGCGGATCCATGAAAGTTAAAG-TACTGTCCCTC 3′) and *3′omp36Hind*III (5′-GCGTTAG-CAAGCTTCAGCGTGCTTAGAACTGGTA-3′) and *5′ompA-Bam*HI (5′-GTTAGCGGATCCATGAAAAAGACAGCTATC-GC-3′) and *3′ompAHind*III (5′-GCGTTAGCAAGCTTG-GAAACTTAAGCCTGCG-3′) respectively. PrimeSTAR™HS DNA polymerase (Takara) was used to amplify products by PCR according to the manufacturers instructions (cycling conditions; melting at 98°C, 10 s; annealing at 58°C, 10 s, extension at 72°C, 60 s). Purified PCR products were digested using *Bam*HI and *Hind*III (New England Biolabs) and cloned into the expression vector pColdIV (4359 bp) (Takara), using T4 Ligase (NEB) to create pColdIV*omp36* and pColdIV*ompA*. Plasmid constructs were confirmed by sequencing (GenomeExpress), using the primer pair pColdF (5′-ACGCCATATCGCCGAAAGG-3′) and pColdR (5′-GGCAGGGATCTTAGATTCTG-3′) [44] then transformed into BL21Δ*omp*. Transformants were grown to early-exponential phase (OD_{600} 0.4) in LB at 37°C before chilling to 15°C and adding 1 mM IPTG (Eurogentec) for 18 hours. Expression was confirmed by SDS PAGE and immunodetection.

Minimum Inhibitory Concentration Assays

BL21Δ*omp* cultures harboring pColdIV, pColdIV*omp36* or pColdIV*ompA*, were grown to OD_{600} 0.4 in LB containing appropriate antibiotics. Cultures were split into 2 flasks, 1 was induced with IPTG (1 mM) for 1 h and the other was not. Bacteria were then subcultured into MH broth with or without IPTG (0.5 mM) and β-lactamase quenchers tazobactam, clavulanic acid and cloxacillin (4 μg ml^{-1} each) at OD_{600} 0.001 containing no antibiotics. 2-fold dilution series of each antibiotic studied were prepared and added to 1 ml aliquots of bacterial suspensions in MH. Assays were incubated for 18–24 h, 37°C. Each assay was repeated independently 3 times and results were classified according to the Antibiogram Committee of the French Society for Microbiology (http://www.sfm.asso.fr).

Rate of Antibiotic Action Assays

BL21Δ*omp E. coli* cultures harboring either pColdIV*omp36* or pColdIV*ompA*, were prepared as for MIC assays. In trials performed using the MIC for cultures producing OmpA (4 μg ml^{-1}), Omp36 expressing cultures were depleted to un-

detectable levels within 20 min (see **Text S1** and **Fig. S1**). In order to accurately quantify the rate of action over a number of time points, all induced and diluted cell suspensions (OD_{600} 0.01) were exposed to 2× the MIC for cultures producing Omp36 (1 μg ml^{-1}). At 15–30 min time intervals, 10-fold dilution series of exposed cultures were prepared with LB and spread onto LB agar containing appropriate antibiotics. Plates were incubated overnight at 37°C for 18 h and colonies were counted. Colony forming units (cfu/ml) were calculated for each time point and plotted as the percentage decrease in cfu/ml compared to t = 0. All experiments were repeated independently at least 4 times.

Outer Membrane Extraction

The method for extracting outer membranes (OM) was modified from Bolla [45]. Briefly, induced cultures (1 L) were harvested by centrifugation (10,000×g, 20 min, 4°C). Bacterial cells were disrupted in 50 mM sodium phosphate buffer, (NaPi) pH 7.4 by sonication using the Branson Sonifer 450 (7×2 min, output level 5) on ice and total membranes collected by ultracentrifugation (100,000×g, 1 h, 4°C). Inner membrane proteins were solubilized by agitation with sodium lauryl sarcosinate, 0.15% w/v (sigma) in NaPi (50 mM, pH 7.4, room temperature, 30 min). OM proteins were harvested by ultracentrifugation (100,000×g, 1 h, 4°C). OM expression of Omp36 was assessed using SDS PAGE and immunodetection.

SDS PAGE and Western Blotting

Bacterial protein extracts were analyzed on SDS-PAGE gels containing 10% acrylamide. Samples were denatured in Laemmli loading dye containing 2% SDS and heated 3× to 95°C [11]. Protein size was estimated by comparison with pre-stained low-range molecular weight marker (BioRad). Proteins were stained using Coomassie Brilliant Blue R-250.

For immunodetection, proteins were electrotransfered onto nitrocellulose membranes (Schleicher & Schlull, Keene, NH, USA) in transfer buffer (20 mM Tris, 150 mM glycine, 20% isopropanol, 0.05% SDS). Membranes were blocked using 4% milk in Tris-buffered sodium (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH 8). Polyclonal antibodies were used for detection, anti-F4 antibody directed against a small peptide of the conserved internal loop 3 for porin and anti-OmpA antibody directed against OmpA of *E. coli* for OmpA [11,18]. Detection of antigen-antibody complexes was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove PA, USA) using BCIP and NBT (Sigma) according to the manufacturers instructions.

Purification of Omp36

Purification methods were developed from Bolla [45] and Garavito and Rosenbusch [46]. OM extracts were washed with 0.5% octyl-POE (Bachem AG, Bubendorf, Switzerland) in NaPi (50 mM, pH 7.4). Selective extraction of Omp36 was performed by solubilization from OM preparations using 1% octyl-POE+–NaCl (1 M) at 37°C, 1 h with shaking. Unsolubilized proteins were removed by ultracentrifugation (100,000×g, 1 h at 4°C). Extraction from the pellet was repeated twice using the same conditions. Supernatants were pooled and concentrated using YM-30 centricon filters and NaCl was removed using Hi-Trap desalting columns (GE Healthcare). Omp36 was purified from solubilized protein extracts using a Resource Q ion exchange column (Amersham Biosciences). The column was equilibrated with NaPi, pH 7.4 containing 1.2% POE and 10 mM NaCl. Extracts were loaded at a flow rate of 2 ml min^{-1}, monitoring conductivity and OD at 280 nm at all times using Akta Explorer 10 apparatus. Omp36 was eluted from the column using a linear gradient (12 CV) from 10 mM to 1 M NaCl. Fractions containing Omp36 were verified by SDS-PAGE and immonoblotting.

Single channel measurements and antibiotic interaction

Virtually solvent-free lipid bilayer membranes were formed as previously described by Montal and Mueller [47]. To form planar lipid bilayers with the monolayer opposition technique, we used 1,2-Diphytanoyl-sn-Glycero-3-Phosphatidylcholine (Avantipolar lipids). Two symmetrical compartments of a Teflon chamber each with a solution volume of 0.25 ml of KCl (1 M, pH 6) were separated by a 25 μm thick Teflon film (Goodfellow, Cambridge,UK) containing a round aperture of 60–80 μm diameter. The aperture was pretreated with 1% hexadecane in pentane. Ag/AgCl electrodes were used to detect ion currents (World Precision Instruments, Sarasota FL, USA). The *cis* electrode was grounded while the *trans* electrode was connected to the head stage of an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The applied membrane voltage refers to the difference between the *cis* and *trans* side potentials. The membrane capacitance was 50–100 picofarads. Single channel insertion was achieved by adding 1–2 μl of Omp36 extract (18 ng ml^{-1}) containing 0.6% Octyl POE to the chamber. Single channel insertion was facilitated by applying a membrane voltage of 200 mV and mixing the contents of the chamber. Measurements were performed with an Axopatch 200B amplifier in the voltage clamp mode. Under the applied voltage, protein insertion was easily detected by current increase. The porin was always added to the *cis*-side of the chamber. It is interesting to note that single porin insertion was always asymmetric in contrast to multi-channel recording leading to a more equally distributed orientation. Channel conductance is slightly higher at positive voltage compared to negative voltage in all experiments, which can be used as the test for the direction of channel insertion. Signal was filtered using a low-pass Bessel filter at 10 kHz and recorded to PC at 50 kHz sampling frequency. Data analysis was performed using Clampfit software (Axon Instruments, Inc.). All experiments were carried out at room temperature.

Ion current fluctuations in the presence of various antibiotics were measured at an applied transmembrane voltage. Concentrated aliquots of antibiotic solutions were added to the lipid chamber, mixed very well, and incubated for 10 minutes for complete diffusion in the chamber prior to recording. Antibiotic stock solutions were prepared in 1 M KCl buffered by MES. The pH of the solution was measured and adjusted after the preparation of the stock solution and continuously measured at different concentrations in the course of the experiment and after completing the experiment. Blockage events occurred following addition of antibiotics ertapenem and cefepime to either the *cis* or *trans* side of the artificial membranes. These blockages reveal the current state of the "binding" site and allow analysis of the occupation on a single molecular level. The first step is to analyse the statistic of the time histogram. If the interaction of the antibiotic with the channel can be described by a simple two-state Markovian (no hysteresis) a single exponential decay is observed. The average residence time of antibiotic was calculated using single exponential fitting of blockage time histograms (**Fig. 2b**). At low concentration, $[c] \ll k_{off}/k_{on}$, the characteristic time was close to the average residence time of the drug (τ) thus allowing us to use the following equations: $\tau \approx k_{off}^{-1}$, and $k_{on} = v/(3[c])$ where v is the number of binding events and [c] was the antibiotic concentration. A similar approach was employed for the estimation of ampicillin and moxifloxacin translocation rates through the *E. coli* OmpF channel [24,31].

In the case where single blockage events are less pronounced, the power density spectra is more suited to analyse interactions [29]. Electronically it is rather favorable to average over typical occurring frequencies and the above exponential decay will lead to a Lorentzian in the power density spectra. The experimental parameter is the corner frequency at which a Lorentzian decayed to half of its original value $\omega_c = k_{on}\ [c] + k_{off}$.

The spectrum of ion current fluctuations was fitted to the Lorentzian model: $S(f) = S(0)/(1+(f/f_c)^2)$, where $S(0)$ was the low-frequency spectral density and f_c was the corner frequency, giving the relaxation time constant defined as $\tau = 1/2\pi f_c$. It is interesting to note that the concentration dependent corner frequency obtained from a Lorentzian fit of the power spectrum yielded the same results (data not shown). The corner frequency increased in a concentration dependent manner allowing determination of the on and off rates of ertapenem into the affinity site of the Omp36 channel. In contrast only little increase was visible for cefepime and none for ampicilin and ceftazidime.

Supporting Information

Text S1 Supplementary text S1
Found at: doi:10.1371/journal.pone.0005453.s001 (0.04 MB DOC)

Table S1
Found at: doi:10.1371/journal.pone.0005453.s002 (0.02 MB PDF)

Figure S1
Found at: doi:10.1371/journal.pone.0005453.s003 (0.45 MB EPS)

Figure S2
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Figure S3
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Author Contributions

Conceived and designed the experiments: CEJ KRM MW JMP. Performed the experiments: CEJ KRM AM JMB ANB. Analyzed the data: CEJ KRM MW JMP. Wrote the paper: CEJ KRM MW JMP.

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Towards screening for antibiotics with enhanced permeation properties through bacterial porins: proof of concept.

***Eric Hajjar*[a], *Andrey Bessonov*[c], *Alexander Molitor*[b], *Amit Kumar*[a], *Mahendran K.R.*[c] *Mathias Winterhalter*[c], *Jean-Marie Pagès*[b], *Paolo Ruggerone*[a], *Matteo Ceccarelli*[a,*]**

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[a] Dr Eric Hajjar, Amit Kumar, Prof. Paolo Ruggerone, Dr Matteo Ceccarelli*
Department of Physics, University of Cagliari and SLACS-INFM-CNR, I-09042 Monserrato (CA), Italy.
*Correspondence matteo.ceccarelli@dsf.unica.it

[b] Alexander Molitor, Prof. Jean-Marie Pagès
UMR-MD1, Faculté de Médecine, Université de la Méditerranée, IFR88, Marseille, France.

[c] Dr Andrey Bessonov, Mahendran K.R., Prof. Mathias Winterhalter[c] School of Engineering and Science, Jacobs University, Campus Ring 1, 28759, Bremen, Germany.

Abstract

Gram-negative bacteria are protected by an outer membrane barrier and to reach their periplasmic target, penicillins have to diffuse [1]through outer membrane porins, such as OmpF. Here we propose a structure-dynamics based strategy for improving such antibiotics uptake. Using accelerated molecular simulations we decipher the subtle balance of interactions governing ampicillin diffusion through the porin OmpF. Following an in-depth analysis we draw the complete inventory of the rate-limiting interactions and map them on both the porin and antibiotic structure. This suggests mutagenesis of a hot spot residue of OmpF for which additional simulations lead to drastic changes in the molecular and energetic pathway of ampicillin's diffusion. Inverting the problem, we predict and describe how benzylpenicillin diffuse with a lower effective energy barrier by interacting differently with OmpF. The theoretical predictions have been tested with a number of experiments that were setup to measure the net flux, kinetics of transport and biological activity and altogether allows to validate our theoretical modeling.

Introduction.

The increasing bacterial resistance impairs the function of currently available antibiotics. Besides, we are facing a situation where there are no truly novel active antibacterial compounds in clinical trials[2]. The reasons that have led to this alarming situation are multiple, from the abusive usage of antibiotics, to the shortsighted behavior of pharmaceutical companies that did not diversify antibacterial research, or even abandoned this field for more rentable ones[3]. Today, there is an emergency call for a new way to develop potent antibacterial agents while bringing them to the market faster and at reduced costs. An emerging strategy is to pursue a rational and microscopically founded drug design that starts from the molecular knowledge of resistant mechanisms. In such bottom-up approach, structure-based and computer-assisted drug-design, are expected to play a central role[4]. However, to date, most "virtual screening" strategy has traditionally focused on optimizing the association and efficiency of antibiotics for their target, overlooking all other resistance mechanisms[4]. For example, the permeability to antibiotics – or uptake - is the very first line of defense of Gram-negative bacteria, that are protected by an outer-membrane[5]. Antibiotics have to diffuse passively through outer membrane porins and the

under-expression or mutations of these porins is a well known mechanism of bacterial resistance[5, 6]. In the case of *E.coli*, the uptake of several classes of ß-lactam antibiotics, a prominent group in our current antibacterial arsenal, is largely controlled by the Outer Membrane Protein F (OmpF) porin. A key feature in the structure of porins, as seen from the X-ray structure of OmpF[7], is the presence of the loop L3 that folds back into the channel to form a gate, also called constriction region (see Fig.1). In addition to such spatial constriction, the zone is also characterized by a strong transversal electric field, generated by negatively charged residues D113, E117 (L3 side) that faces a cluster of positively charged residues R42, R82, and R132 (anti-L3 side) (see Fig.1).

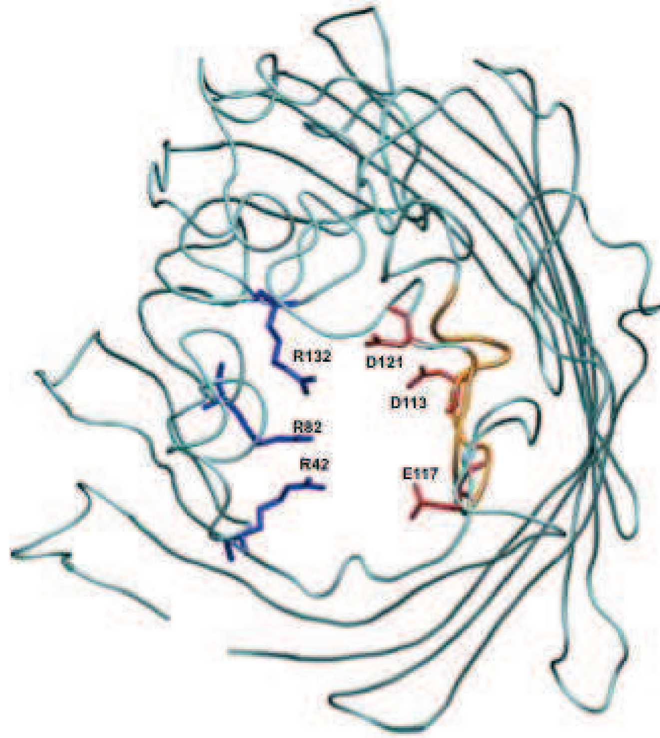


Fig.1: The backbone of OmpF is displayed in cyan cartoons except the loop L3 that is colored in orange. The charged residues at the constriction region are colored by residue type (positively charged in blue, negatively charged in red).

Several studies investigated the role of mutations of residues located at the constriction region of OmpF[8, 9]. Interestingly, the single R132A mutation was found to dramatically increase the uptake of cefepime, a fourth generation cephalosporin β-lactam antibiotic[9]. In a more recent study, Vidal et al. used molecular modeling to illustrate their findings that bacteria

expressing the OmpF mutant D113A were more susceptible to β-lactam antibiotics[10]. Indeed Molecular Modeling is a method of choice to investigate structural effect of mutations, however the simplistic modeling protocol followed by those authors, did not allow them to capture the structural and dynamical consequences of the mutation. An accurate molecular explanation appears necessary before any rational conclusion can be taken from mutagenesis experiments. In principle, standard MD simulations would have the required microscopic accuracy to link the structure and dynamics (of the drug and porin) to the rate of permeation.

However, standard simulations are limited to hundred of nanoseconds at most and they do not allow the study of the reactive pathway that antibiotics follow during passive diffusion, which is on the order of hundreds of microseconds.[1]

To overcome this timescale problem, we propose to use accelerated MD simulation algorithms schemes while keeping an "all atom" description of the systems[11]. In a previous study, we used the metadynamics algorithm to study the diffusion beta-lactam antibiotics through the OmpF channel[12] and this suggested the important role of specific orientation and interactions of the antibiotic for the translocation process. However, due to shortcoming

and limitation in our previous simulation methodology and analysis we were not able to explicitly account for these factors. In the present study we improved the methodology of simulations: by placing the antibiotic well above the constriction region and using a new reaction coordinate to explicitly account for the antibiotic orientation during the simulations.

Taking advantage of the present improvements, the high resolution of the method, in both time and space will allow identifying the structural/dynamical properties (molecular mechanism) of the antibiotic uptake while having access to the effect, at an atomic level, of focused mutagenesis studies.

Very recently we have shown that kinetics properties of the ampicillin diffusion can be obtained either by accelerated molecular simulations or by single molecule experiments, and that results from both methods agreed with spectroscopy[13] and flux measurements[14].

Supported by such agreement we now propose to use for the first time, an adequate in-silico strategy and molecular-based approach, as schematized in the Fig.2, to decipher the antibiotic-porin rate-limiting interactions with the goal of optimizing antibiotics uptake. We first used ampicillin (Amp) as starting model (Fig.2, step1), for which we find the aspartic acid residue

D113 to be a hot spot and tested this hypothesis by performing the simulation of ampicillin diffusion through the mutant OmpF-D113A (Fig.2, step2). In the OmpF D113A porin, Amp diffuses with a lower effective energy barrier and in depth analysis reveal how the mutation affects drastically the physico-chemical, structural and interaction properties between drug and channel. We then invert the problem (Fig.2, step3) and predict and describe how Benzylpenicillin (Bpen), by interacting differently with the residue D113, will diffuse with a lower energy barrier through OmpF, corresponding to a higher flux. Finally, to guide further studies that would aim at tuning antibiotics interactions to improve their flux we draw, for the first time, the complete inventory of the rate-limiting interactions and map such hot-spots on the structure of both porin and antibiotics

To validate our computational, molecular based, approach we present data from three independent experimental assays that were established to measure biological activity, net flux and rates of antibiotics permeation.

Taken together, our molecular based multi-disciplinary approach could be used as a tool to rationalize and accelerate the process of antibacterial discovery and circumvent bacterial resistance.

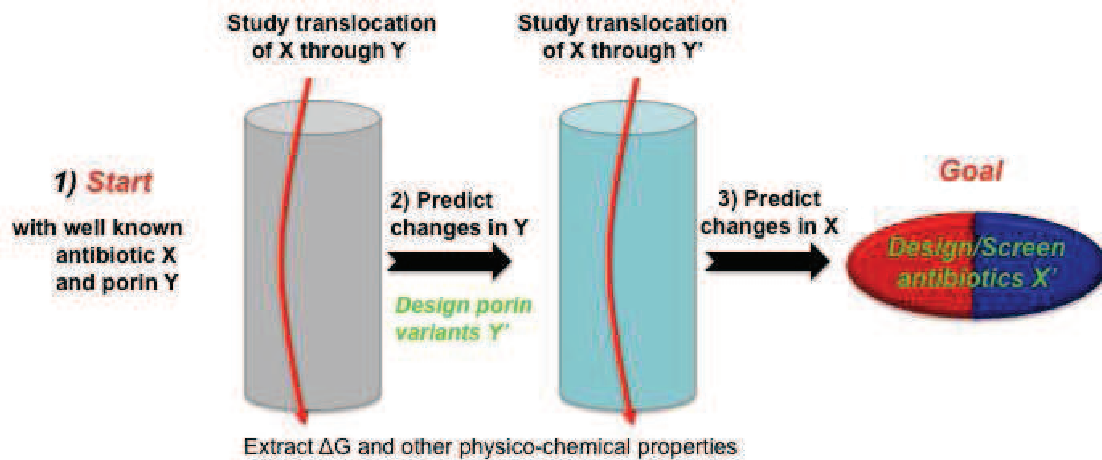


Figure 2: Scheme of the proposed strategy for in silico selection/design of antibiotics (X) with improved permeation properties through porins (Y).
The goal is to minimize the main effective barrier (ΔG) and we are looking for cases where $\Delta G(X,Y) > \Delta G(X,Y') \approx \Delta G(X',Y)$

Results

Following the first step of our methodology (Fig.2) we find that as Amp effectively translocates through the OmpF wild-type (WT) channel, it visits deep energy minima in three distinguishable regions: above, at and below the constriction region. To quantify accurately the diffusion process, additional metadynamics simulations were started from each identified energy minimum (see Materials and Methods) and the calculation of free energy barriers connecting each of them allowed reconstructing the 1D free energy profile corresponding to the antibiotic diffusion (Fig.3A). The profile reveal that Amp has to overcome two large free energy barriers to translocate and that the main effective barrier for this process (ΔG) is of 8 kcal/mol. This effective barrier compares very well with the measured energy barrier recently reported from experiments[15].

We then performed in-depth qualitative analysis along the equilibrium MD simulations started from each minimum. First, we calculated the averaged atomic fluctuations (rmsf) of Amp and found it to be high (although lower than the rmsf value of Amp in a water box) in all minima except in Mini-II located at the constriction zone (Table 1). This difference suggests the importance of entropy-enthalpy compensations to stabilize the affinity site

(Mini-II) at the constriction region and facilitate translocation, as highlighted in a previous study in the context of enzyme-ligand docking[16]. Indeed, such compensation is confirmed by the network of specific interactions that arise between ampicillin and OmpF in Mini-II. As highlighted in the snapshot of Fig.3B, ampicillin makes numerous hydrogen bonds: (i) between its N-terminal positive group and D113 on the L3 side; and (ii) between its C-terminal negative group and R42-R82-R132 on the anti-L3 side. Furthermore, we quantified (see Materials and Method) the presence of "bound water molecules" based on the lifetime of their interaction with Amp (Table 1). Interestingly, water molecules in fast exchange with Amp are found all along the channel, as in the simulations of Amp in bulk water, unlike at the constriction region (in Mini-II) (Table 1). There instead, water molecules are found to exchange slowly while mediating the interactions between Amp and residues at the pore walls.

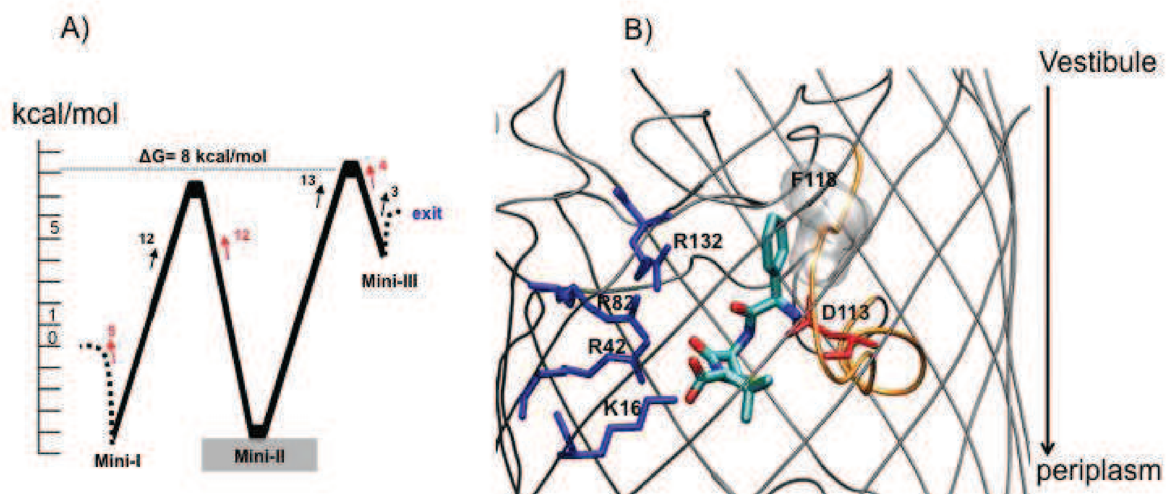


Figure 3: Free energy profile (A) and molecular details (B) for the diffusion of ampicilin through OmpF-wt.
(A) The minimum corresponding to the affinity site at the constriction region is highlighted in grey the energy barriers are reported in kcal/mol.
(B) Snapshot at the affinity site in the constriction region (Mini-II) with the antibiotic in "sticks", colored by "atom type" (blue for nitrogen, red for oxygen, cyan for carbon) and OmpF in grey cartoons (L3 in orange). The residues making significant hydrogen bonds with the antibiotic are colored by residue type (blue for positive, red for negative, green for polar) and those making hydrophobic contacts are displayed by grey molecular surface.

Interestingly, we find the hydrogen bond between the positively charged N-terminal group of Amp and the residue D113 to be maintained as Amp crosses the constriction region. In fact, it is only when this specific interaction is disrupted that the antibiotic can cross the highest energy barrier (Fig.3A) and further diffuses down. We thus hypothesize that this

hydrogen bond with D113 creates a rate-limiting interaction that slows down the ampicillin translocation process. To confirm our hypothesis, we substituted the D113 amino acid by an alanine and repeated the simulation of Amp diffusion (Fig.2, step2). As a result we find a lower effective energy barrier (Fig.4A) for the translocation of Amp through OmpF-D113A (5 vs 8 kcal/mol in the case of the wt).

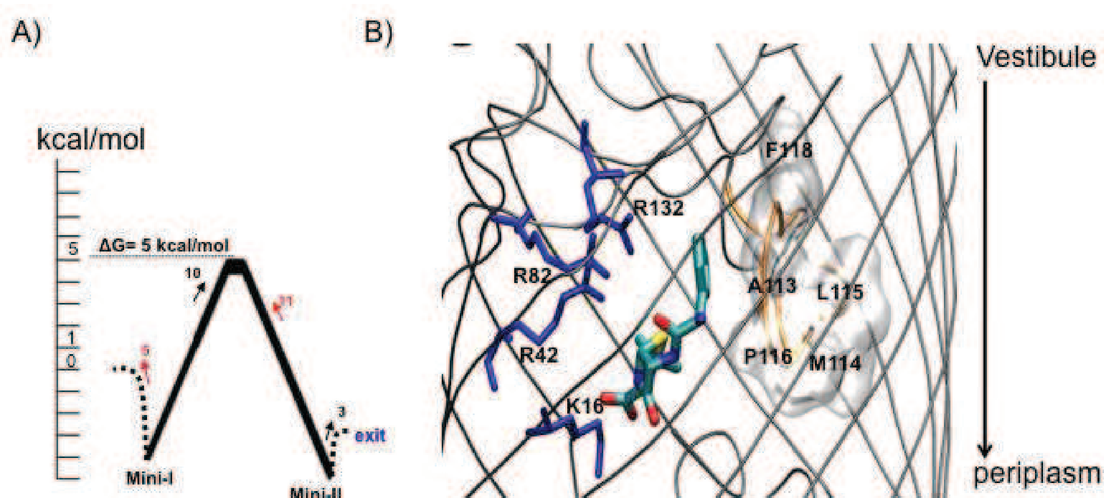


Figure 4: Free energy profile (A) and molecular details (B) for the diffusion of ampicilin through OmpF-D113A (see Figure 2 for legends).

The absence of a deep affinity site at the constriction region suggests that the D113A substitution affects drastically the diffusion of Amp. In this case, the mechanism by which Amp translocates is by crossing only one energy

barrier (10 kcal/mol), to enter in the constriction region (Fig.4A). When it has reached this region of the OmpF channel, the diffusion of Amp further down is then facilitated and the barrier to exit of the channel is rather small (it is of 3 instead of 10 kcal/mol for the wt). The process is thus different from the one described in terms of of “two barriers one binding site”[17], that was observed for Amp through OmpF-wt (Fig.3A). In fact the mutation D113A leads to important structural changes such as the enlargment of the pore (increase of 18 % compared to the OmpF-wt, data not shown) and the opening of a large hydrophobic pocket at the constriction region (Fig.4B). As seen in the Fig.4B, as Amp reaches the constriction region (in Mini-II) the antibiotic positions more towards the L3 loop side where its phenyl group is able to engulf in the hydrophobic pocket newly made available. Such numerous hydrophobic contacts (Fig.4B) on the N-terminal side of the antibiotic are combined to the hydrogen bonds on its C-terminal, as seen in the case of OmpF-wt. This facilitated diffusion of Amp beyond Mini-II constitutes a major difference with the case of OmpF-wt where instead, the minimum at the constriction region was well defined and more stable and the barrier to exit from it and further translocate was very large. The transient state of Mini-II in the case of OmpF-D113A is also validated by

the calculated flexibility of Amp that is twice higher than for the wt and the absence of bound water molecules interacting with Amp in Mini-II (Table 1).

In order to test the theoretical predictions we used high-resolution ion conductance measurements to experimentally access the kinetic rates of ampicillin translocation. The porins were reconstituted into the planar lipid bilayer and the ion current fluctuations through single trimeric OmpF-wt or D113A were studied in the presence of ampicillin. As seen from Fig. 5 in case of D113A mutant, the number of ampicillin binding events is larger compared to WT (the number of events, calculated as explained in the Materials and Method section is 120±15 events/s for OmpF-D113A and 54±6 events/s for OmpF-wt). The second interesting feature is the reduced residence time of ampicillin, which is 170±16 μs in the case of the OmpF-wt and halved for the D113A channel (90±8 μs). Both are concentration independent, which is expected when working at concentration far from saturation.

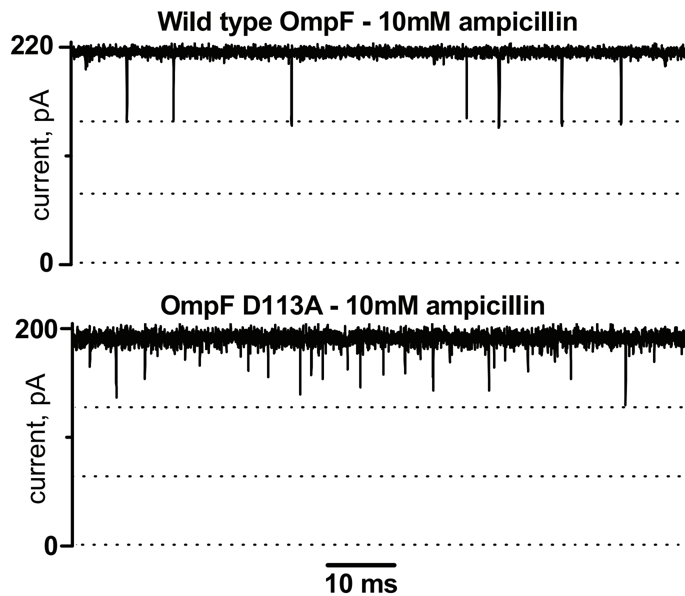
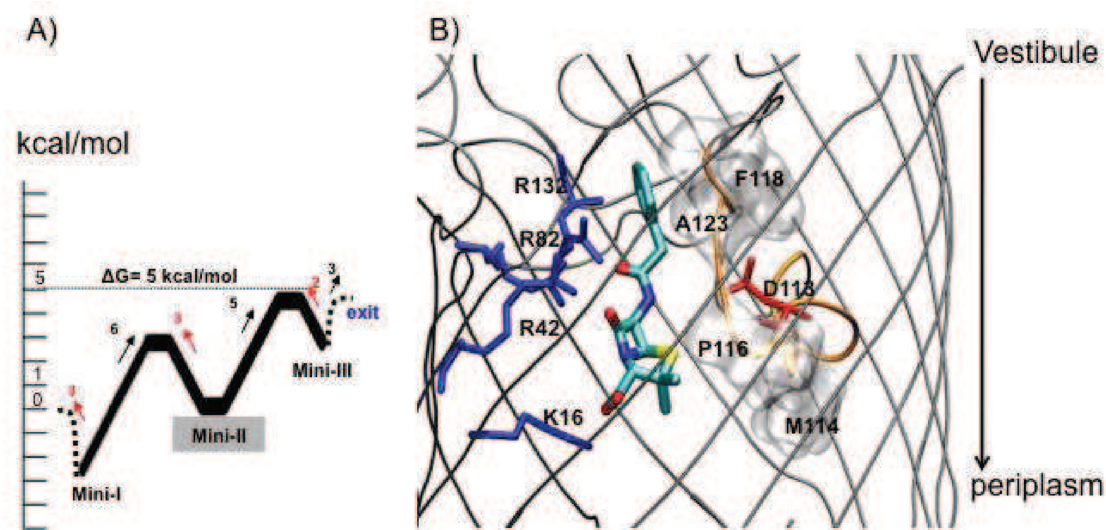


Figure 5. Ion current tracks through single trimeric OmpF-wt or D113A mutant reconstituted into planar lipid membranes in the presence of ampicillin and in 1 M KCl at 25°C. Most of the time the trimeric channel is fully open. Addition of ampicillin causes temporal blocking of about one third. The dotted horizontal lines correspond to the conductance level of one or two fully open channels.

The results from electrophysiology, supporting our computational models, report a clear increase in the kinetics of ampicillin translocation and a lower residence time for the OmpF mutant D113A compared to the WT.

Altogether, our data confirm that properly optimized interactions of ampicillin with the channel in the constriction zone can modulate its translocation. Guided by these findings, and in particular by the determinant

role of D113, we then predict that Benzylpenicillin (Bpen), whose chemical structure only differs from that of Amp by the absence of the N-terminal $NH3^+$ group, would translocate with a lower energy barrier through OmpF



(Fig.2, step3).

Figure 6: Free energy profile (A) and molecular details (B) for the diffusion of Bpen through OmpF-wt (see Figure 3 for legends).

The simulations predict that the diffusion of Bpen through OmpF-wt requires a lower effective barrier of 5 kcal/mol (Fig.6A). In fact, we find the effective barrier of Bpen in OmpF-wt ($\Delta G_{(Bpen,\ OmpF\ wt)}$) to be similar to that of Amp in OmpF-D113A (Fig.4A) and lower to that of Amp in OmpF-wt (Fig.3A).

In other words, our computational analysis yield:

$\Delta G_{(Bpen,\ OmpF\ wt)} \approx \Delta G_{(Amp,\ OmpF\ D113A)} < \Delta G_{(Amp,\ OmpF\ wt)}$.

Furthermore, the 1D energy profile of the translocation of Bpen shows that we recover here the “two barriers one binding site” scheme (Fig.6A), though with energy barriers significantly lower than for the Amp diffusion through OmpF-wt. Such lower barriers can also be related to the different solvation pattern as we note an absence of bound water molecules in the minima at the constriction region (Table 1).

Table 1: Structural details of the antibiotic obtained from the equilibrium MD simulations.
“Bound waters” were defined as the interacting molecules whose residence time is greater than 300ps (see Materials and Methods).

	Averaged Atomic Fluctuations (rmsf in Å)			**Interaction with “bound waters” (% of the total number of interacting water molecules is shown)**		
Region of Analysis	**AMP-WT**	**AMP-D113A**	**BPEN-WT**	**AMP-WT**	**AMP-D113A**	**BPEN-WT**
Water Box	0.99	0.99	1.15	0	0	0
Mini I (above constriction region)	0.74	0.86	0.83	0	0	0
Mini II (at constriction region)	0.29	0.42	0.48	10	6	0
Mini III (below constriction region)	0.40	-	0.72	4	-	0

Furthermore, we find the interaction network of Bpen at the constriction region (Mini-II) to be optimal for a faster translocation. As seen in Fig.6B, Bpen interacts with both walls of OmpF: via Hbonds with its carboxylic group and polar oxygens and via Hcontacts with its phenyl and dimethyl group. As expected, the hydrogen bond between D113 and the polar nitrogen of Bpen is present but it is not durable, the lifetime of this interaction is of 4% of the simulation time compared to a lifetime of 48% in the case of Amp in OmpF-wt. Thus, this specific interaction does not persist as the antibiotic crosses the constriction region, unlike in the case of Amp.

We then used ion conductance measurements to assess the interaction of Bpen with OmpF reconstituted in the lipid bilayer measured at different temperatures. As seen in Fig.7A, at room temperature the presence of 10mM of Bpen caused no significant blockage of ionic current through a single trimeric OmpF channel revealing negligible interaction with the channel.

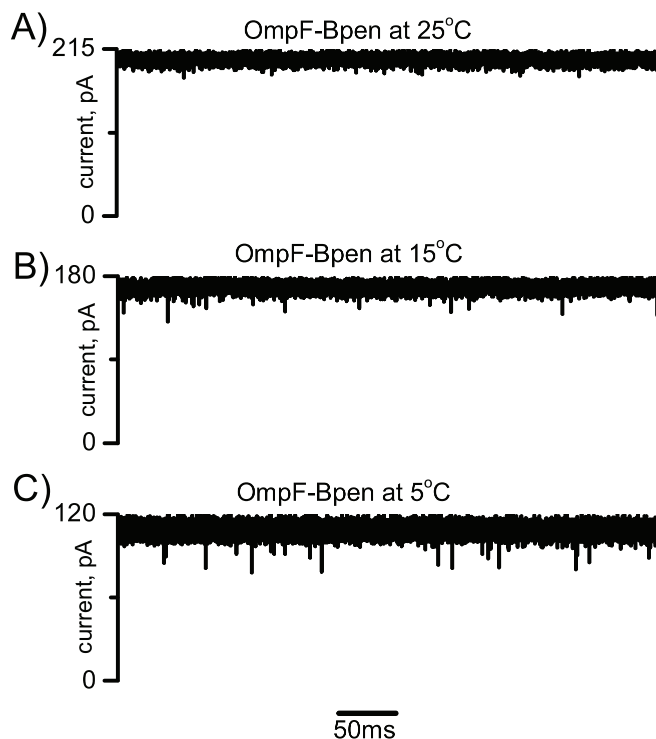


Figure 7: Typical ion current tracks through OmpF-wt in the presence of 10 mM Bpen measured at 25°C (A), 15°C (B) and 5°C (C). 1 M KCl , pH 6 and applied voltage 50mV

We then lowered the temperature to slow down the kinetics, which may increase the resolution at which ion-current blockages by antibiotics can be seen. Interestingly, at low temperature (5^0C), Bpen showed very short and partial ion current blockages **(**Fig.7C**)**. Unlike ampicillin, which blocks complete monomer of a single OmpF trimer, in the case of Bpen, only partial monomer closures are observed. We hypothesize that Bpen translocates through OmpF very fast and that we are not able with this

technique to resolve binding events so that quantitative analysis is not possible in this case.

In order to probe for the net flux of antibiotics through OmpF channels we apply a second method, called liposome swelling assay[18]. Wild type OmpF was reconstituted into the liposomes and permeability of ampicillin and Bpen in a 4-6 pH range was studied (Fig. 8). The obtained results show that permeation of ampicillin and Bpen occurs through the OmpF in pH dependent manner with an increase of the rate towards the isolelectric points of the antibiotics (Fig. 8) [19, 20]. These swelling results are in good agreement with our hypothesis of a faster translocation of Bpen compared to Amp through OmpF-wt.

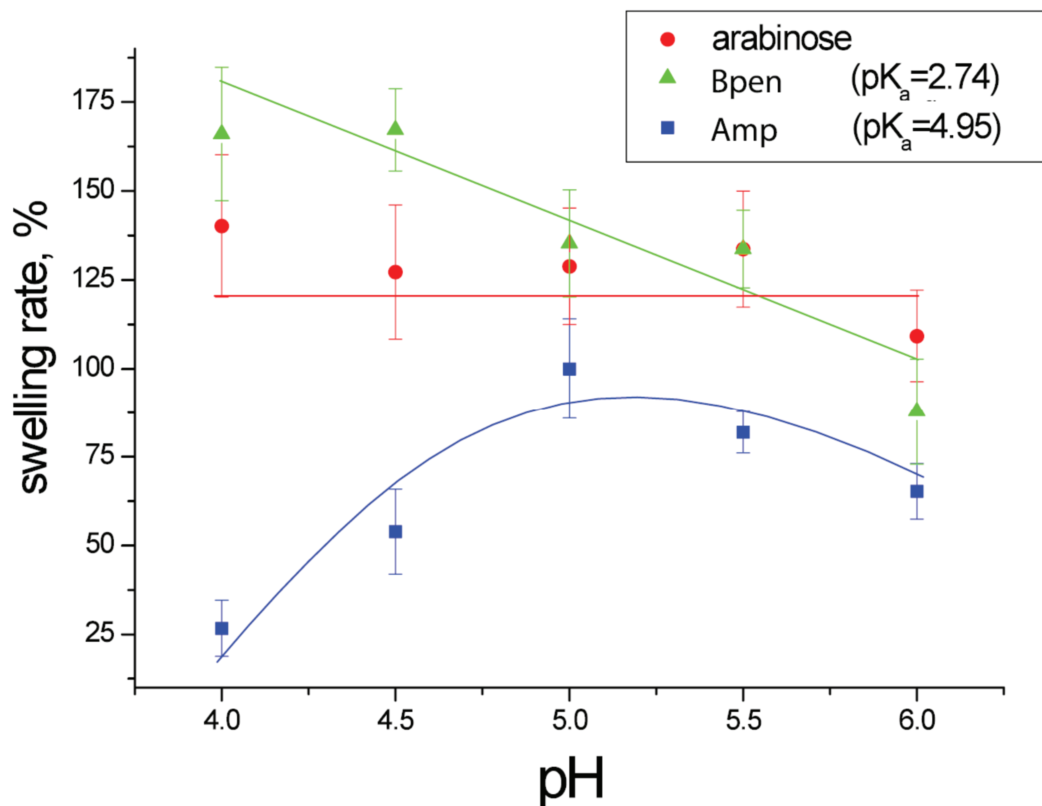


Figure 8: Swelling rates for penicillin G and ampicillin are pH dependent while for arabinose (used as a control) this is not the case.

Taking advantage of the microscopic details that the molecular simulations provide we conclude by drawing in Fig.9 the complete inventory of the identified interactions, mapped on both antibiotics and porin structures.

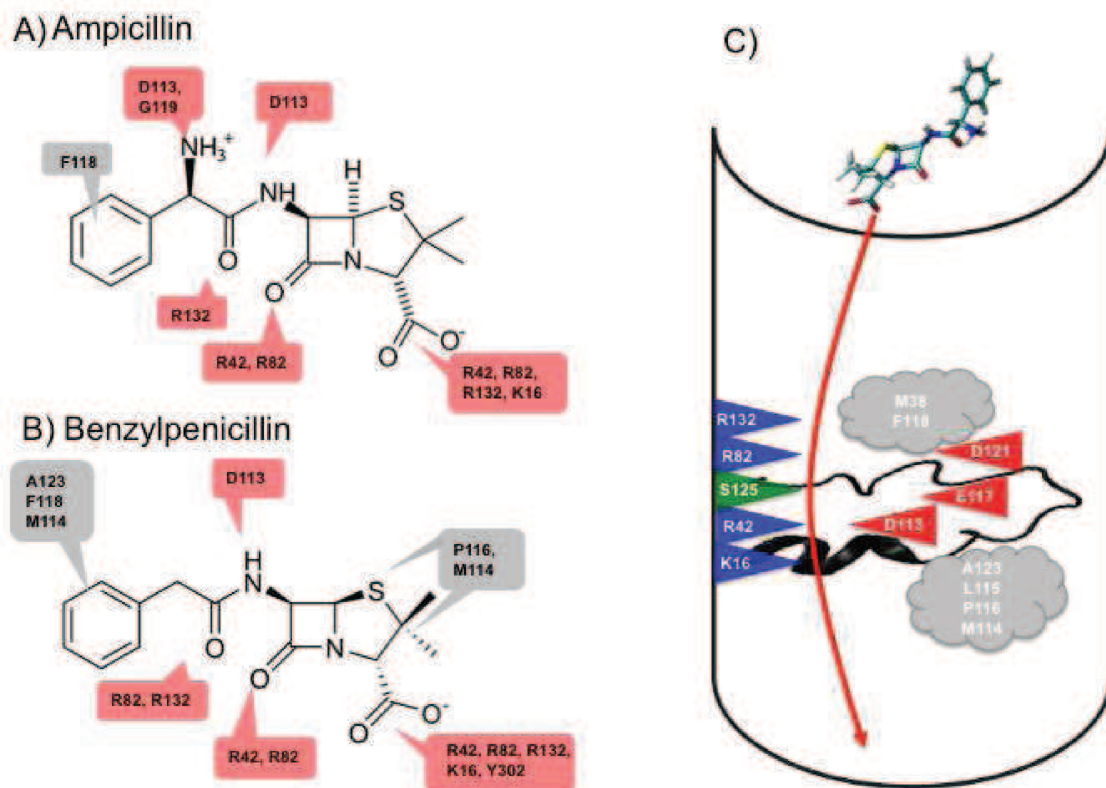


Figure 9: Key interactions mapped on the functional groups of (A) Amp, (B) Bpen and (C) on a scheme representing OmpF (loop L3 defining the constriction region is colored in black). We considered significant interactions from the equilibrium MD simulations at the affinity site of the constriction region. The hydrogen bonds are highlighted in red (A-B) or by a rectangle colored by residue type (C) and hydrophobic contacts are in grey shadding (A-B-C).

Figures 9 A-B highlight the most conserved interactions for each antibiotic functional group. For example, the strongly conserved salt bridge between the antibiotics carboxylic group and either of R132, R82, R42, K16 is expected to be a key determinant as it properly orients the antibiotic with its C-terminal pointing down. We find Bpen to make more hydrophobic

contacts than Amp, in particular its dimethyl and phenyl group match well with the two hydrophobic pocket located at the L3 side of the constriction region. Finally, the Fig.9C sums up the diverse key residues that the antibiotic have to deal with as it crosses the constriction region, highlighting the inner complexity of the channel and revealing the localization of well-defined basic/acidic clusters and hydrophobic pockets.

Biological activity

To assess the rate of β-lactam action on *E. coli,* we expressed OmpF channels as the sole porin in the outer membrane of a porin-null *E. coli* strain and the ability of both ampicillin and Bpen to traverse the outer membrane via OmpF channels were determined using minimum inhibitory concentration (MIC) assays. Further to approach the role of D113 in the biological activity of penicillin class, we studied the susceptibility of the same porin deleted strain that produce either normal wild type OmpF (D113) or mutated OmpF D113A[9]. From our results (reported in Table 2) it is quite clear that the substitution D113A increase the susceptibility for the two antibiotics, ampicillin and Benzylpenicillin. Due to the expression of ß-lactamase in the strain it is not possible to quantify the absolute impact of

the mutation. However, we can conclude on the relative effect of the mutation for each antibiotic. As seen in Table 2, the effect of the mutation on Benzylpenicillin influx, where the MIC value is reduced by a factor 4 as it goes from 256 mg/L for WT to 64 mg/L for D113A, is less marked compared to the corresponding one on ampicillin influx, for which the MIC value is reduced by a factor of 16 as it goes from 16 mg/L for WT to 1 mg/L for D113A. This suggests that the Benzylpenicillin is less dependent to an interaction with D113 during its translocation through OmpF, compared to ampicillin. Such finding as well as the drastic reduction of the MIC value in the case of ampicillin is in good agreement with the computational results

Table 2. Penicillin susceptibility for OmpF wt and D113A mutant

Inhibitors comprise tazobactam and clavulanic acid.

Penicillin	MIC (mg/L)	
	OmpF WT (113D)	Mutant (113A)
Penicillin A group		
Ampicillin	>512	>512
Ampicillin + inhibitors	16	1
Penicillin G group		
Benzylpenicillin	>512	>512
Benzylpenicillin + inhibitors	256	64

………………………………………………………………………

Discussion and Conclusions

Here we used all-atom molecular simulations to follow the paradigm for selecting antibiotics with better permeation properties. This is only made possible recently due to development of accurate accelerated algorithms[11] and computer power. The high resolution in time and space that metadynamics simulations provide was used to characterize the molecular basis of translocation of antibiotics through OmpF-wt and mutants. However, to treat patients, it is not possible to modify the porins of bacteria and in this study, we propose a methodology to invert the problem (Fig.2). The goal is to help predict modifications of a given antibiotic (X') so that it penetrates faster through porins of interest (Y).

We further made a point to support this proof of concept using computer simulations by setting up three independent experimental setups. First, electrophysiology measurements report a clear increase in the kinetics of ampicillin translocation and a lower residence time when changing from OmpF-wt to the mutant D113A. On the other hand, in the case of Bpen, only partial monomer closures were observed and the binding kinetics of translocation could not be calculated. The higher translocation rate of Bpen compared to Amp was then validated using liposome swelling that allows

measuring net fluxes. Finally, biological assays measuring antibiotic susceptibilities showed a much more marked dependence of the residue D113 for ampicillin compared to Bpen. Keeping in mind that the design of potent antibiotics is a very difficult problem we propose that our approach could be efficiently used in conjunction with strategies tackling other effects/mechanism to better circumvent bacterial resistance[4].

Altogether, our study support the idea that the diffusion of the antibiotic through OmpF is governed by a subtle balance of interactions, we then identified the interaction with the residue D113 as rate-limiting, which is in agreement with the study of Vidal *et al.*[10] who found it to be the "most likely interacting residue". In fact, this residue sits in the middle of a potential hydrophobic cluster and only Bpen but not Amp is able to exploit these hydrophobic partners. In the case of Bpen, hydrophobic interactions play an important contribution as a driving force for faster translocation and such possible contribution from hydrophobicity was already raised in the pioneer work of Nikaido[18]. Interestingly, when D113 is mutated to an alanine it opens a large hydrophobic pocket and there is a disruption of the affinity site for Amp at the constriction region. This drastic structural effect induced by the mutation suggests using caution when extrapolating the

effects of experimental mutations, as it has been done often in the past, without support from techniques giving microscopic resolution and providing the molecular mechanisms. Molecular simulations can, in contrast, give an accurate description of the changes induced by mutations on the transport properties, and such mechanistic information should be taken into account to rationalize experimental findings [8-10].

It is of interest to mention that the D113 residue participates actively to the screening of certain charged molecules translocating through OmpF. Such key activity contributes to the efficiency of the permeability barrier as first defense line against toxic compounds [21]. Consequently, another outcome of our study is that the identified "hots spots", both on the antibiotics (Fig.9A-B) and on the porin (Fig.9C) side, could be integrated in a pharmacophore model and used for in silico screening. The strategy of "using old drugs for new uses", by integrating the requirements for optimal translocation with an efficient screening of existing drugs, might lead to the discovery of new potent drugs at a reduced cost [4, 22]. Our approach also opens the way to rational chemical modifications of antibiotics with the goal of improving uptake through bacterial porins and combat bacterial resistance. Finally, our methodology can be conveniently employed to study porin-antibiotic

interactions in other enterobacterial pathogens of interest [5, 6].

Experimental section / Materials and Methods

Molecular Simulations

The starting structure for the simulations of OmpF (crystal structure of pdb code: '2OMF') and antibiotics were prepared as described earlier[12].

The process of antibiotics translocation occurs on a timescale of about 100ms, which cannot be reached by standard MD simulations with an all-atom representation. In this study, the antibiotic translocation could be simulated thanks to the incorporation of the metadynamics simulation algorithm, which is based on an history dependent biasing potential that discourage the system to re-visit the configurations already sampled[11].

Based on previous findings [10, 12, 23], we have chosen the following collective variables for simulating antibiotic translocation using metadynamics[11]: (i) the distance Z: defined as the difference between the coordinates of the centers of mass of the antibiotic and of the porin along the z-axis; (ii) the angle θ: defined as the orientation of the long axis of the molecule with respect to the z-axis.

■■

Due to the complexity of the process studied, we calculated the free energy after obtaining the first and only translocation path. This first crossing is considered to be the most probable path because it is passing through the lowest saddle point. We used the reconstructed free energy in the subspace of the metadynamics collective variables [11] to select the regions of energy minima. Additional metadynamics simulations were launched starting from each minimum and this enabled reconstructing the 1-dimensional free energy profile for the translocation of ampicillin through OmpF-wt and the two mutants. The error bars associated with the energy barrier calculations were assessed as done previously[12] and are of 2kT at most. Furthermore, equilibrium MD simulations were started from each minima to analyze the (i) atomic fluctuations, (ii) hydrogen bonds and hydrophobic interactions of ampicillin using VMD [24], (iii) residence time of water molecules interacting with Amp [25] and (iv) cross sectional solvent accessible surface (SAS) area calculations as done previously[13].

Electrophysiology experiments

Reconstitution experiments in planar lipid bilayer and noise analysis of the ion current have been performed as described in detail previously (12-14).

Lipid monolayer opposition technique was employed to form planar lipid bilayers (Montal and Mueller, 1972). DPhPC was used for membrane formation. A Teflon cell with an approximately 50-μm diameter aperture in the 25-μm-thick Teflon partition and silver-silver chloride electrodes was used. Small amounts of porin (OmpF, or OmpF mutant) from a diluted stock solution of 1 mg/ml containing 1% (v/v) of Octyl-POE, was added to the cis-side of the chamber. Spontaneous channel insertion was usually obtained while stirring under applied voltage. Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in the voltage clamp mode. Signals were analysed Clampfit (Axon Instruments, Foster City, CA) software.

Liposome swelling assay

Wild-type OmpF (1 mg/ml) in 1% Octyl-POE were reconstituted into liposomes as described in[26]. *E. coli* total lipid extract (Avanti Polar lipids, Alabaster, AL) was used for liposome formation and 17% Dextran (MW 40000, Fluka) was used for the liposome filling. After incubation multilamellar liposomes were formed by sonication in water bath sonicator. Size of formed liposomes was checked using Nano-ZS ZEN3600 zetasizer

(Malvern Instruments, UK). Control liposomes were prepared in a same manner without porin addition. The isotonic concentration was determined by diluting the proteoliposomes into different concentrations of raffinose (Sigma) with Osmomat 30 osmolarimeter (Gonotec). Each batch was separated in smaller aliquots assuming a homogeneous distribution. One aliquot of each batch was tested for arabinose, a smaller molecule for which we expect maximum permeation and this swelling rate was set as 100% for the respective batch. Normalizing each batch separately allows reducing the effects of variable reconstitution efficiencies of different preparation. Changes in the optical density was monitored at 400 nm, using Cary 100 Scan spectrophotometer (Varian). The swelling rates, which were means from at least three different sets of experiments, were calculated as described by Nikaido and Rosenberg.

Bacterial Strain, Culture Medium, Minimun Inhibitory Concentration Assays

Bacteria, *E. coli* BL21 (DE3)Δ*omp*[27] harboring either pColdIVompFWT or pColdIVompF113A were grown in Muller Hinton (MH) broth (Difco) containing relevant antibiotic (kanamycin, 50mg/L) (Sigma). At OD_{600} 0.4,

cultures were induced with IPTG (1 mM) for 1 H at 37°C. Bacteria were then subcultured into MH broth including IPTG and, without and with ß-lactamase inhibitors (tazobactam, clavulanic acid, 4 mg/L respectively) to quench the activity of ß-lactamase expressed by *amp* gene present on plasmid[27]. 2-fold dilution series of each antibiotic studied were prepared and added to 1 ml aliquot of bacterial suspension in MH. Assays were incubated for 18H at 37°C. Each assay was repeated independently four times. In order to check the correct expression of wild type and mutated porins, SDS-PAGE and immunoblotting analyses were performed using appropriate antibodies[27].

■■

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Keywords: (OmpF porin, noise analysis, antibiotics, rate-limiting interactions, molecular dynamics)

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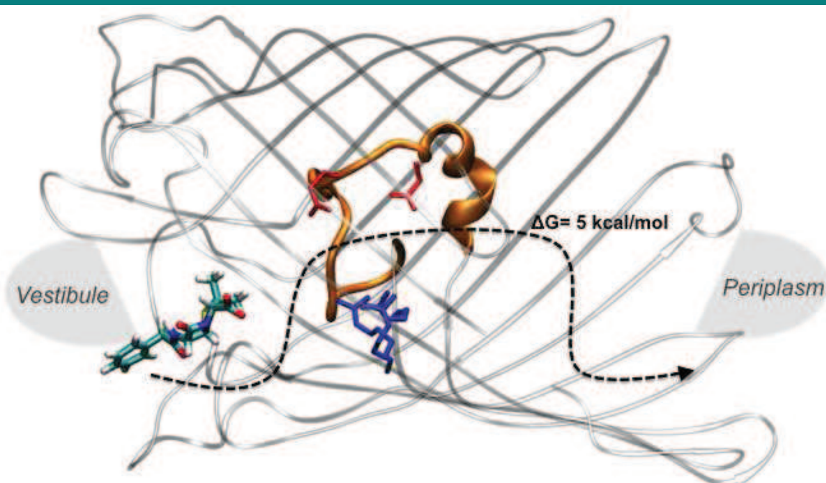

Authors:

Eric Hajjar, Andrey Bessonov, Alexander Molitor, Amit Kumar, Mahendran K.R. , Mathias Winterhalter, Jean-Marie Pagès, Paolo Ruggerone, Matteo Ceccarelli*

Page No. – Page No.

Title:

"Towards screening for antibiotics with enhanced permeation properties: proof of concept using molecular simulations"

Legend: 3D structure of the Benzylpenicillin antibiotic (displayed in "sticks" and colored by atom names) at the mouth (vestibule) of the OmpF homotrimer channel