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Post-transcriptional regulation of porin expression in *Escherichia coli* and its impact on antibiotic resistance

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Résumé

La résistance aux antibiotiques est une menace sérieuse et grandissante pour la santé publique, causant approximativement 700 000 décès annuels. Chez les bactéries à Gram-négatif, l'imperméabilité de la membrane externe et ainsi l'incapacité des antibiotiques à pénétrer l'enveloppe bactérienne pour atteindre leur cible est un facteur majeur contribuant au développement de la résistance. Chez *Escherichia coli*, les porines OmpF et OmpC sont des protéines de la membrane externe qui forment des canaux pour la diffusion de petites molécules hydrophiles tels que les antibiotiques. Les modifications des porines telles que la diminution de leur expression ou des altérations structurales se retrouvent dans de nombreux isolats cliniques résistants, limitent la translocation des antibiotiques, diminuent leur concentration intracellulaire et leur activité. L'expression des porines est soumise à une régulation complexe à plusieurs niveaux. Notamment, la régulation transcriptionnelle de *ompF* et *ompC* est bien connue et fait intervenir le système à deux composants EnvZ-OmpR en réponse à l'osmolarité du milieu. Au niveau post-transcriptionnel, plusieurs études ont également montré le rôle des petits ARN non-codants (sRNAs, *small RNAs*). Parmi ceux-ci, MicF et MicC modulent l'expression respective de OmpF et OmpC. Ils fonctionnent par appariement de bases avec le site de liaison du ribosome du messager cible, bloquant ainsi l'initiation de la traduction. De manière intéressante, les gènes codant ces deux sRNAs sont adjacents à deux gènes codant des porines — *micF-ompC* et *micC-ompN* — suggérant une co-régulation.
Dans ce cadre, et en utilisant *E. coli* comme bactérie modèle, les objectifs de mon travail de thèse étaient : (1) de caractériser la régulation du sRNA MicC et la co-régulation putative de la porine quiescente OmpN; (2) d'examiner l'effet global de MicC sur le transcriptome; (3) d'analyser l'impact de l'expression de MicC sur la sensibilité aux antibiotiques. Dans un premier temps, nous ainsi avons étudié le rôle de plusieurs facteurs environnementaux et des voies de régulation connues pouvant conduire à une augmentation de l'expression de MicC. Pour cela, nous avons mesuré l'activité β-galactosidase d'une fusion transcriptionnelle *micC-lac*Z dans de nombreuses conditions de croissance et dans de nombreux contextes génétiques. Nous avons également optimisé le test du gène rapporteur à un format microplaque afin de cribler plusieurs collections de molécules fournis par la compagnie Biolog. Les résultats obtenus montrent l'induction de MicC en présence d'antibiotiques de la famille des β-lactamines (spécifiquement les carbapénèmes et les céphalosporines) ainsi qu'en déplétant le facteur de transcription sigma spécifique au stress de l'enveloppe, σ^E. Ces mêmes conditions activent aussi l'activité d'une fusion *ompN-lacZ*, indiquant une régulation transcriptionnelle commune de *micC* et *ompN*. De plus, la production de OmpN a été confirmée par une analyse en immunoblot avec des anticorps spécifiques. Ainsi, MicC pourrait agir conjointement avec σ^E pour contrôler l'expression de OmpC et OmpN en

réponse à la présence de β-lactamines, une famille d'antibiotiques qui cible justement la synthèse du peptidoglycane et l'intégrité de l'enveloppe. Etant donnée la conservation de MicC chez les entérobactéries, nous avons effectué une étude par RNASeq pour déterminer l'impact de la surexpression de MicC sur le transcriptome d'*E. coli* et identifié 60 ARNm régulés par MicC en plus de sa cible initiale *ompC*. L'identification des spectres cibles globaux des sRNAs est importante pour comprendre leur importance dans la physiologie bactérienne, ici celui de MicC dans la résistance aux antibiotiques. Les travaux à venir viseront à étudier cet aspect en détail ainsi que le lien putatif entre la résistance aux β-lactamines, la perte d'OmpC et la surexpression de MicC dans des isolats cliniques d'entérobactéries.

Mots clés : Entérobactéries, enveloppes bactériennes, perméabilité membranaire, résistance aux antibiotiques, porines, petits ARNs régulateurs non-codants.

Abstract

Antimicrobial resistance (AMR) is a serious and growing health threat as it has been estimated that 700,000 people die every year from drug resistant bacteria. A major factor contributing to AMR is the inability of antibiotics to penetrate the bacterial OM (OM) to reach their requisite target for being effective. In Gram-negative bacteria, such as *Escherichia coli*, the two classical porins OmpF and OmpC are among the most abundant OM proteins and form water filled channels for the diffusion of small hydrophilic molecules including antibiotics. Porin modifications, in the form of decreased expression or structural modifications are found in several resistant clinical isolates, limit antibiotic uptake and decrease their intracellular concentration and activity. Given the importance of the OM, it is not surprising that the expression of porins is under complex regulation at multiple levels. Regulation of *ompF* and *ompC* at the transcriptional level is well studied, and involves the EnvZ-OmpR two component systems in response to external osmolarity changes. Research has also shown that enterobacteria use small regulatory RNAs (sRNAs) to fine tune porin expression at the post-transcriptional level. Among these, MicF and MicC are the two major sRNAs that modulate the expression of OmpF and OmpC, respectively. They suppress porin expression by base pairing with the ribosome binding site of targeted porin mRNA, thereby blocking translation. Interestingly, these two sRNAs are encoded next to porin gene, i.e. *micF-ompC* and *micC-ompN*, suggesting a dual regulation.

In this work, our goals were: (1) to characterize the regulation of the sRNA MicC and the putative co-regulation of the quiescent porin OmpN in *E. coli*; (2) to examine the global effect of MicC on the *E. coli* transcriptome; (3) to analyze the impact of MicC expression on antibiotic susceptibility. First, we have investigated the factors like external growth conditions and regulatory pathways that lead to increased production of MicC by measuring the β-galactosidase activity of a *micC-lacZ* transcriptional fusion. For this search, we optimized the reporter gene assay into a 96-wells format and screened collections of compounds provided by the Biolog phenotype Microarray™. Our work shows that the expression of *micC* was increased in the presence of β-lactam antibiotics (specifically carbapenems and cephalosporins) and in an *rpoE* depleted strain. Interestingly, the same conditions enhanced the activity of an *ompN–lacZ* fusion, suggesting a dual transcriptional regulation of *micC* and *ompN*. Increased levels of OmpN in the presence of sub-inhibitory concentrations of chemicals could not be confirmed by Western blot analysis, excepting when the sigma factor σ^E was depleted. We suggest that the MicC sRNA acts together with the σ^E envelope stress response pathway to control the OmpC/N levels in response to β-lactam antibiotics. We also performed RNA sequencing to determine the impact of MicC overexpression on *E. coli* transcriptome. This identified 60 mRNA targets negatively regulated by MicC apart from its original

target *ompC*. Identification of the global target spectra of MicC is of importance to understand its importance on the overall bacterial physiology, and more specifically on AMR. Preliminary results showed that *E. coli ΔompF* overexpressing MicC exhibit reduced susceptibility to β-lactams, probably due OmpC shutdown. Future studies will aim to investigate the putative connection between β-lactam resistance, loss of OmpC and overexpression of MicC in clinical isolates of *Enterobacteriaceae*.

Keywords: Enterobacteria, bacterial envelopes, membrane permeability, antibiotic resistance, OM porins, small regulatory RNAs.

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List of abbreviations

1-Ara4N	4-amino-4-deoxy-l-arabinose
ABC	ATP- binding cassette
AMP	Adenosine Monophosphate
ATB	Antibiotic
ATP	Adenosine Triphosphate
BLM	Black Lipid Membranes
CAP	Covalently Attached Protein
CCCP	Cyanide M-Chlorophenyl Hydrazone
cDNA	Complementary DNA
CR	Carbapenem-Resistant
CRE	Carbapenem-Resistant *Enterobacteriaceae*
DHFR	Dihydrofolate Reductase
DHPS	Dihydropteroate Synthase
DNA	Deoxyribonucleic Acid
DUV	Deep Ultraviolet
EDTA	Ethylenediaminetetraacetic Acid
ESBL	Extended Spectrum B-Lactamases
ESR	Envelope Stress Responses
HGT	Horizontal Gene Transfer
H-NS	Histone-like Structuring Nucleoid protein
HTS	High Throughput Screening
IM	Inner Membrane
IMP	Integral Membrane Protein
ISC	Iron-Sulfur Cluster
KCl	Potassium Chloride
LC-MS	Liquid Chromatography–Mass Spectrometry
LP	Lipoprotein
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MATE	Multidrug And Toxin Extrusion
MDR	Multidrug-Resistance
MFS	Major Facilitator Superfamily
MIC	Mininmum Inhibitory Concentration
mRNA	Messenger RNA
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
OM	Outer Membrane

OMP	Outer Membrane Protein
PABA	P-Aminobenzoic Acid
PACE	Proteobacterial Antimicrobial Compound Efflux
PAβN	Phenylalanine-Arginine B-Naphthylamide
PBP	Penicillin-Binding Proteins
PCR	Polymerase Chain Reaction
pETN	Phosphoethanolamine
PMF	Proton Motive Force
PRP	Pentapeptide Repeat Protein
RBS	Ribosome Binding Site
RIP	Regulated Intramembrane Proteolysis
RNA	Ribonucleic Acid
RND	Resistance- Nodulation-Cell Division
ROS	Reactive Oxygen Species
SMR	Small Multidrug Resistance
SPU	Self-Promoted Uptake
TA	Teichoic Acids
TCA	Tricarboxylic Acid
TCS	Two-Component Systems
TDR	Totally Drug-Resistant
TMP	Trimethoprim
tRNA	Transfer RNA
UTR	Untranslated Region
WTA	Wall Teichoic Acids
XDR	Extremely Drug-Resistant

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Chapter 1: Introduction

1. The antibiotic resistance crisis

1.1. Overview

In 1900, infectious diseases were the leading cause of death in the world. The selective action exerted on pathogenic bacteria and fundi by "wonder drugs", i. e. microbial secondary metabolites, ushered in the antibiotic era, which has been of great importance of human beings ever since. Antibiotics are low molecular weight compounds, most of which are produced by microorganisms or derived from natural products, which are active at relatively low concentrations against other microorganism. Beginning with the discovery of penicillin by Alexander Fleming in 1928, antibiotics have revolutionized the field of medicine (1). They have saved millions of lives, alleviated pain and suffering, and have been used prophylactically for the prevention of infectious diseases in human and veterinary medicine. Soon after, in the 1940s, Selman Waksman introduced the first simple and successful discovery platform: soil-derived *Streptomyces* were screened for antimicrobial activity against a susceptible test microorganism by detecting zone of growth inhibition on an overlay plate (2). This systematic screen led to the discovery of streptomycin as the first effective compound against *Mycobacterium tuberculosis* and the first aminoglycoside. This approach was widely adopted by the pharmaceutical industry and produced the major classes of antibiotics over the next 20 years (3). In parallel to their discovery, resistance to antibiotics by the target microorganisms was also emerging, but chemical modifications to existing antibiotics still produced active analogues. An example is the class of fluoroquinolones, which was developed in the 1960s to optimize nalidixic acid and seemed to limit the spread of resistance. Since then, the platform has been abandoned and no class of broad-spectrum compounds has been reported (**Figure 1**). Resistance traits to multiple classes of antibiotics, resulting in strains with multidrug-resistance (MDR) phenotypes, have progressively narrowed the available therapeutic options for some pathogens. Although resistance can be associated with decreased fitness and/or virulence, some MDR strains retain a remarkable ability for infected and spreading in the clinical settings, and can experience a rapid epidemic diffusion (the so-called high-risk MDR clones) (4). With some Gram-negative bacteria, resistance may also involve most or even all the available antimicrobial agents, resulting in extremely drug-resistant (XDR) or totally drug-resistant (TDR) phenotypes, which recreate situations typical of the pre-antibiotic era (5). This so-called "antibiotic resistance crisis" has been compounded by the lagging in antibiotic discovery and development programs in recent

years. This has recently drawn the attention of scientific societies, public health agencies and political bodies (6-9).

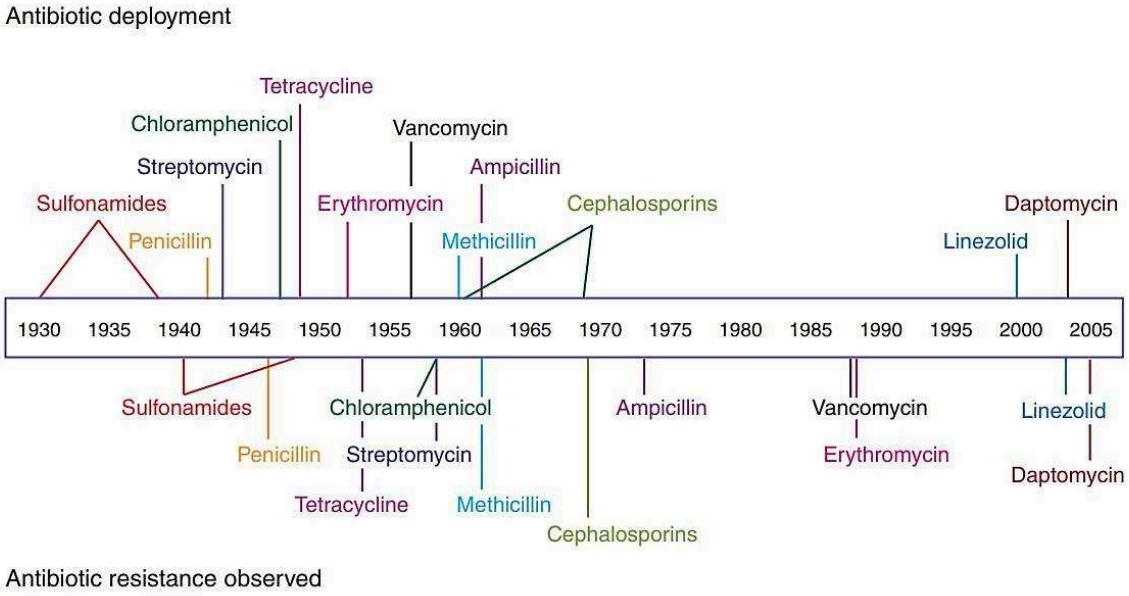

Figure 1: Timeline of antibiotic development and the evolution of antibiotic resistance. The year each antibiotic was deployed is depicted above the timeline, and the year resistance to each antibiotic was observed is depicted below the timeline (figure acquired from Clatworthy *et al.*, 2007).

Infectious diseases are now the second most life threatening cause in the world, number three in developed countries, and four in the United States. Worldwide, 17 million people die each year from bacterial infections. In the United States, each year approximately 2 million people are infected with antibiotic resistant bacteria, of which 23,000 will subsequently die as result of these infections. This global threat challenges decades of progress in medicine, food security and public health. Failure to tackle this problem immediately will have vast implications by 2050 with estimated 10 million lives every year at risk along with a total economic loss of 100 trillion USD (8).

Recent reports using data from hospital-based surveillance studies as well as from the Infectious Diseases Society of America have begun to refer to a group of nosocomial pathogens as "ESKAPE pathogens" (1, 11). ESKAPE is an acronym for the group of bacteria, encompassing both Gram-positive and Gram-negative species, made up of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. These bacteria are common causes of life-threatening nosocomial infections among critically ill and immunocompromised patients and are characterized by drug resistance mechanisms. This situation is especially troubling with respect to the Gram-negative species of the ESKAPE pathogens, such as *P. aeruginosa, A. baumannii* and members of the *Enterobacteriaceae* family. Improved understanding of the factors that render these

pathogens difficult to target is a key step in addressing the rising unmet medical need in this area (13, 14).

1.2. Main classes of antibiotics against Gram-negative bacteria and bacterial adaptation

1.2.1. Introduction to the bacterial cell envelopes

The bacterial cell envelope is a complex multilayered structure that serves to protect these organisms from their unpredictable and often hostile environment. The cell envelopes of most bacteria fall into one of two major groups (**Figure 2**). Gram-negative bacteria are surrounded by a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane (OM) containing lipopolysaccharide (LPS). Gram-positive bacteria lack an OM but are surrounded by layers of peptidoglycan many times thicker as compared to that in the Gram-negatives. Threading through these layers of peptidoglycan are long anionic polymers, called teichoic acids (TA). The composition and organization of the Gram-negative envelope layers will be described in details in section 2. However, one can already note that the presence of the OM is a defining feature of Gram-negative bacteria, which acts as an effective barrier that limits penetration of existing antibiotic compounds and explains the paucity of novel penetrating compounds.

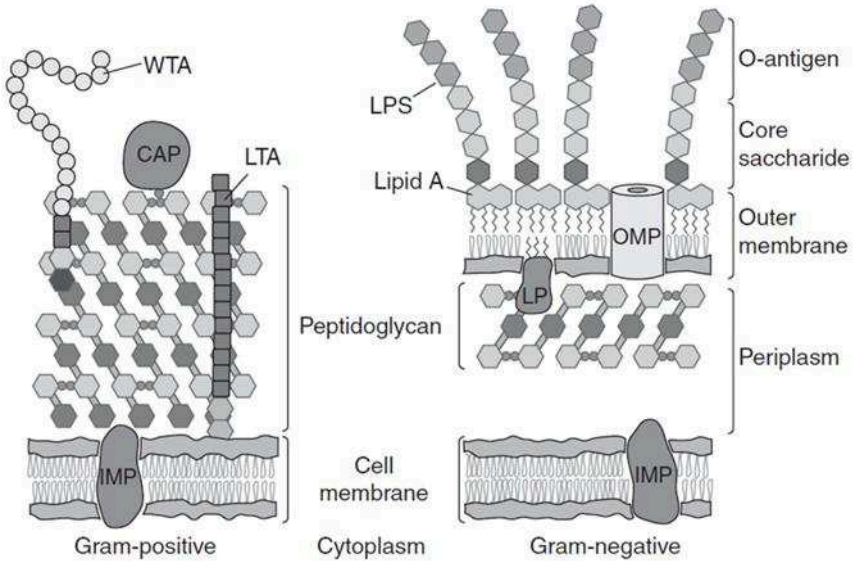

Figure 2: Depiction of the Gram-positive and Gram-negative cell envelopes. The various abbreviated components are CAP, covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, OM protein; WTA, wall teichoic acid (picture acquired from Silhavy *et al.*, 2010).

1.2.2. Antibiotic classes and their targets

As of now, there are four proven targets presently known for the main antibacterial drugs (**Figure 3**):

(1) Protein synthesis
(2) RNA synthesis
(3) DNA replication and repair
(4) Envelope structure and synthesis
(5) Folic acid metabolism

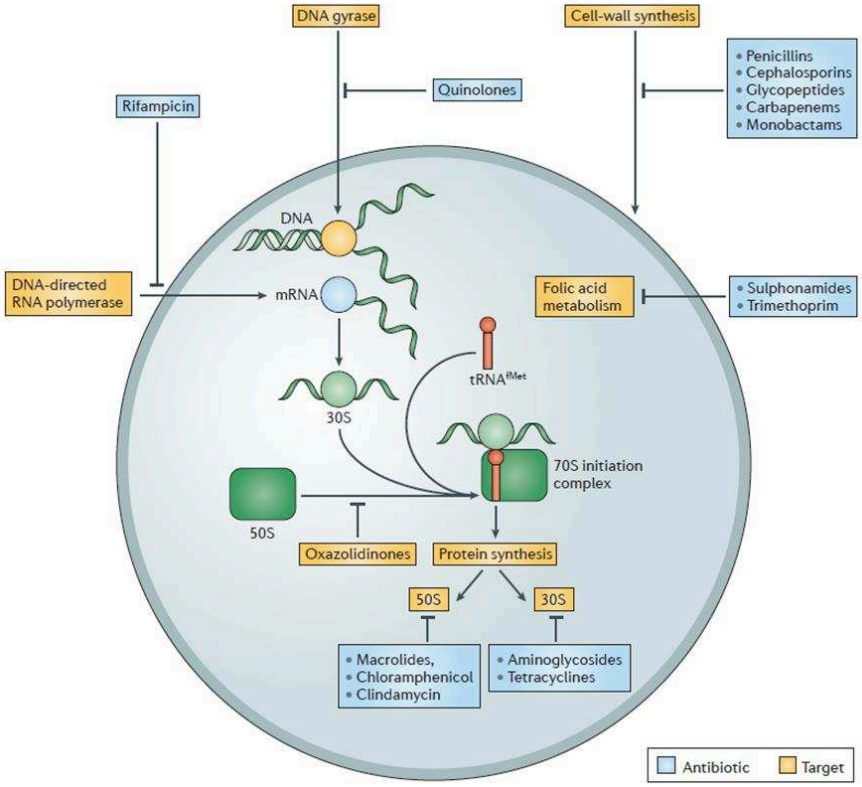

Figure 3: Major antibacterial drug targets. There are three main antibacterial drug targets in bacteria: cell-wall synthesis, DNA replication and repair and protein synthesis. The figure shows the antimicrobial agents that are directed against each of these targets. In the case of protein synthesis, aminoglycosides and tetracyclines inhibit 30S RNA, and macrolides, chloramphenicol and clindamycin inhibit 50S RNA (figure acquired from Lewis, 2013).

1.2.2.1. Protein synthesis

The process of mRNA translation occurs over three sequential steps — initiation, elongation and termination — that involve the ribosome and a range of cytoplasmic accessory factors (16). The ribosome is composed of two ribonucleoprotein subunits;

the 50S and the 30S, which assemble, during the initiation step, following the formation of a complex between the mRNA, the N-formylmethionine-charged aminoacyl tRNA, several initiation factor and a free 30S subunit (17). Drugs that inhibit protein synthesis are among the broadest classes of antibiotics and can be divided in two classes whether they are 50S- or 30S-inhibitors. 50S ribosome inhibitors include macrolides (such as erythromycin), lincosamides (such as clindamycin), streptogramins, phenicols (such as chloramphenicol), and oxazolidinones (such as linezolid) (18, 19). 50S inhibitors function by physically blocking either the initiation of protein translation (as in the case of oxazolidinones) or translocation of peptidyl tRNAs, which serves to inhibit the peptidyltransferase reaction that elongates the nascent peptide chain. The model for this mechanism involves blocking the access of peptidyl tRNAs to the ribosome, subsequent blockage of the peptidyltransferase elongation reaction by steric inhibition and eventually triggering dissociation of the peptidyl tRNA (20, 21). This model also explains the observation that these classes of antibiotics lose their activity when elongation has progressed beyond a crucial length (22). 30S ribosome inhibitor includes tetracyclines and aminocyclitols. Tetracyclines and aminocyclitols targets two different steps of protein synthesis. Tetracyclines work by blocking the access of aminoacyl tRNAs to the ribosome (23). The aminocyclitol class includes spectinomycin and aminoglycosides (such as kanamycin and gentamycin), which binds to the 16S rRNA component of the 30S ribosome subunit and interfere in another step (**Figure 4**). Spectinomycin interferes with the stability of the peptidyl tRNA binding to the ribosome by inhibiting translocation catalyzed by the elongation factor, but does not cause protein mistranslation (24, 25, 26). By contrast, the interaction between aminoglycosides and the 16S rRNA induces an alteration in the conformation of the complex formed by an mRNA codon and its cognate charger peptidyl tRNA at a ribosome. This promotes tRNA mismatching, which often results in protein mistranslation (27, 28, 29). Among ribosome inhibitors, aminoglycosides are the only class that is broadly bactericidal.

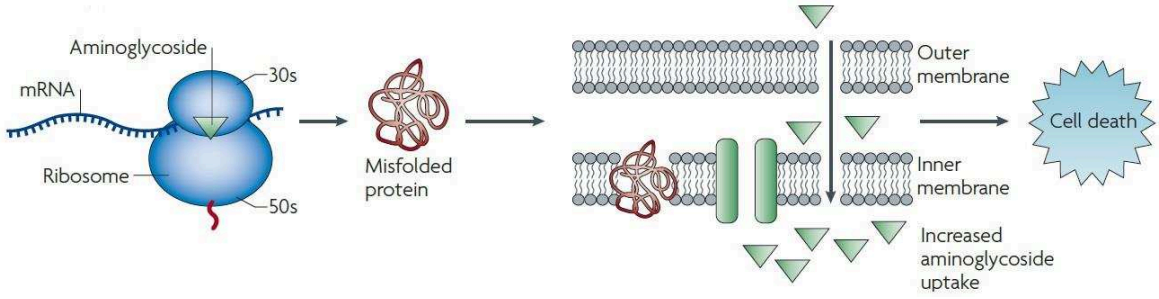

Figure 4: Aminoglycosides bind to the 30S subunit of the ribosome and cause disincorporation of amino acids into elongating peptides. These mistranslated proteins can misfold, and incorporation of misfolded membrane proteins into the cell envelope leads to increased drug uptake. This, together with an increase in ribosome binding, has been associated with cell death (figure acquired from Kohanski *et al.*, 2010).

1.2.2.2. RNA synthesis

Rifamycins are semi-synthetic antibiotics, which were first isolated from the Gram-positive *Streptomyces mediterranei* in the 1950s (31, 32). The rifamycin class of antibiotics inhibits DNA-dependent transcription, by stably binding with high affinity to the β-subunit (encoded by *rpoB*) of a DNA-bound and actively transcribing RNA polymerase (33, 34, 35). The β-subunit is located in the channel that is formed by the RNA polymerase-DNA complex, from which the newly synthesized RNA strand emerges (34). Rifamycins are bactericidal on Gram-positive bacteria but bacteriostatic against Gram-negative bacteria — a difference that has been attributed to drug uptake and not to affinity of the drug with the β-subunit of the RNA polymerase (36). Notably, rifampycins are among the first-line therapies used against mycobacteria, because they efficiently induced cell death (37). However, rapid emergence of rifamycin resistant *rpoB* mutants necessitates its use in combinatorial therapies (38).

1.2.2.3. DNA replication and repair

DNA synthesis, mRNA transcription and cell division require the modulation of chromosomal supercoiling through topoisomerase-catalysed breakage and rejoining reactions (39, 40). These reactions are exploited by the synthetic quinolone class of antibiotics, including the clinically relevant fluoroquinolones, which target DNA-topoisomerase complexes (41, 42, 43). Quinolone interfere with the maintenance of chromosomal topology by targeting topoisomerase II — also known as DNA gyrase — and topoisomerase IV, trapping these enzymes at the DNA cleavage stage and preventing strand re-joining (43, 44, 45). As a result of quinolone–topoisomerase–DNA complex formation, the DNA replication machinery becomes arrested at blocked replication forks, leading to inhibition of DNA synthesis, which immediately leads to bacteriostasis and eventually cell death (43, 46, 47, 48) (**Figure 5**). The introduction of double-stranded DNA breaks following topoisomerase inhibition by quinolones also induces the reaction to DNA stress (SOS response), in which RecA is activated by DNA damage. RecA promotes self-cleavage of the LexA SOS repressor protein, inducing the expression of the SOS response genes such as DNA repair enzymes (49). Consistently, preventing the induction of the SOS response enhances killing by quinolones (50, 30).

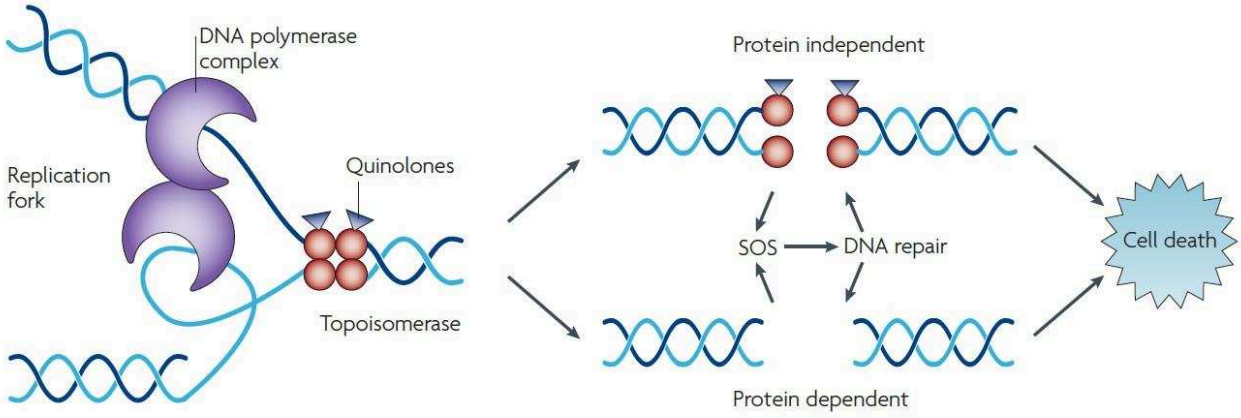

Figure 5: Quinolone antibiotics interfere with changes in DNA supercoiling by binding to topoisomerase II or topoisomerase IV (picture from Kohanski *et al.*, 2010).

1.2.2.4. Envelope structure and synthesis

- **Inhibition of cell wall synthesis**

Peptidoglycan (also known as murein) is a meshwork of peptide and glycan strands that are covalently cross-linked, which is essential for cell viability and provides a protective barrier against environmental stresses (51, 52). Not surprisingly, it represents a major target for approved antibiotics and the development of new antibacterial molecules (53). Layer of peptidoglycan are maintained by the mutual activity of transglycosylases and penicillin-binding proteins (PBPs; also known as transpeptidases), which add disaccharide pentapeptides to extend the glycan strands of existing peptidoglycan molecules and cross-link adjacent peptide strands of immature peptidoglycan units, respectively (54) (**Figure 6**).

β-lactams and glycopeptides are among the classes of antibiotics that interfere with specific steps in the cell wall biosynthesis (55) (**Figure 6**). On one hand, β-lactams, including penicillins, cephalosporins and carbapenems block the crosslinking of the peptidoglycan units by inhibiting the peptide bound formation, which is normally catalyzed by PBPs (56, 57, 51). β-lactams act as pseudo-substrates, analogous to the terminal D-alanyl-D-alanine dipeptide of the peptidoglycan, by acylating the active site of PBPs, thereby disabling their function (58, 59). This results in changes to cell shape and size, induction of stress responses and ultimately cell lysis. By contrast, glycopeptides such as vancomycin inhibit peptidoglycan synthesis by binding to peptidoglycan units (at the terminal D-alanyl-D-alanine dipeptides) and by blocking

transglycosidase and PBP activity (60). As such, glycopeptides generally act as steric inhibitors of peptidoglycan maturation and reduce the mechanical strength of the cell.

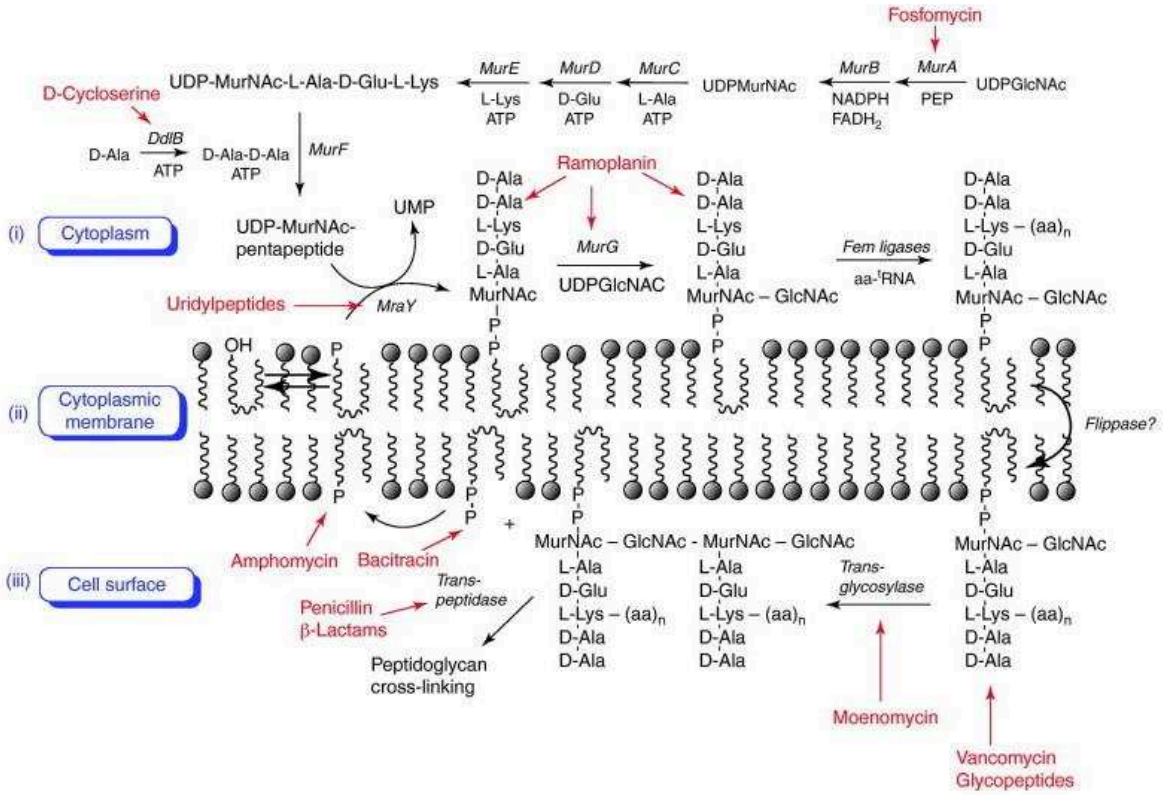

Figure 6: The peptidoglycan biosynthetic pathway showing sites of action of inhibitors. The pathway involves three stages: (i) cytoplasmic steps, leading to the peptidoglycan precursor UDPMurNAc-pentapeptide; (ii) lipid-linked steps, involving lipid carrier undecaprenyl phosphate; and (iii) polymerization and crosslinking of the cell wall on the cell surface. Abbreviations: GlcNAc, N-acetyl-glucosamine; MurNAc, N-acetyl-muramic acid (figure from Bugg *et al.*, 2011).

It is worthwhile to note that glycopeptides are only effective against Gram-positives due to low permeability, whereas β-lactams can be used to treat infections caused by Gram-positive and Gram-negative bacteria. In particular, the standard antibiotic regimen for treating infections caused by *Enterobacteriaceae* was the latest generation cephalosporins — ceftazidime and cefotaxime; and cefepime are well-known examples of third and fourth generations cephalosporins used in the clinics — and carbapenems as important classes of β-lactam antibiotics. Translocation of β-lactams across OM porins and porin associated mechanisms of resistance, which synergizes the activity of periplasmic β-lactamases, towards this specific class of antibiotics will be discussed in detail throughout subsequent sections of this manuscript.

- **Polypeptides antibiotics**

Colistin (also known as polymyxin E) is a polypeptide antibiotic that was originally isolated in 1947 from the soil bacterium *Paenibacillus polymyxa* subsp. *colistinus* (61). Colistin and polymyxin B belong to the class of polymyxins, which is one of the primary classes of antibiotics with activity against most Gram-negative bacteria. The chemical structure of polymyxins is similar to that of cationic antimicrobial peptides (CAMPs) (defensins and gramicidins), which represent the first line of defense against bacterial colonization in eukaryotic cells (62). Polymyxins are cationic polypeptides that consist of a cyclic heptapeptide possessing a tripeptide side chain acylated at the N terminus by a fatty acid tail (63, 64, 65) (**Figure 7**).

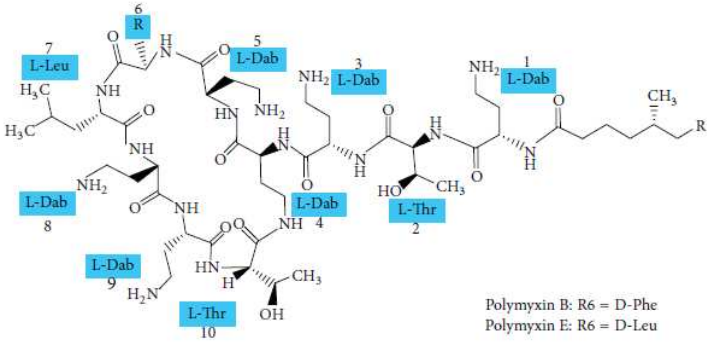

Figure 7: Structures of polymyxins B and E (colistin). Dab, diaminobutyric acid ; Thr, threonine ; Phe, phenylalanine ; L, levogyre ; D, dextrogyre. (figure taken from Yu *et al*, 2015).

The target of polymyxins is the OM of Gram-negative bacteria. The electrostatic interaction that occurs between the α,γ-diaminobutyric acid (Dab) residue of the positively charged polymyxin on one side and the phosphate groups of the negatively charged lipid A membrane on the other side, displaces divalent cations (Ca^{2+} and Mg^{2+}) from the negatively charged phosphate groups of membrane lipids (67). This facilitates the formation of lipopolysaccharide (LPS) destabilized areas through which polymyxin will cross the OM. Polymyxin then destroys the physical integrity of phospholipid bilayer of inner membrane, leading to leakage of the cytoplasmic content and ultimately causing cell death (**Figure 8a**). An alternative mechanism called vesicle-vesicle contact has also been proposed (**Figure 8b**), in which polymyxin can mediate the contacts between periplasmic leaflets of the inner and OMs and promote the exchange of phospholipids between vesicles. The resulting loss of specificity of phospholipid composition potentially causes an osmotic imbalance, leading to cell lysis (68, 69). Finally, another mode of action of polymyxins is the inhibition of essential respiratory enzymes (inhibition of type II NADH-quinone oxidoreductases [NDH-2]) present in the bacterial inner membrane (70).

Polymyxins have a narrow antibacterial spectrum, mainly against common Gram-negative bacteria. They are active against most members of the *Enterobacteriaceae*

family, including *E. coli*, *Enterobacter* spp. and *K. pneumoniae*, and common nonfermentative Gram-negative bacteria such as *A. baumannii* and *P. aeruginosa* (71, 72). Nonetheless, polymyxins have recently regained significant interest as a consequence of the increasing incidence of infections due to MDR Gram-negative bacteria. Of concern, they are being reconsidered as last-resort antibiotics in many areas where MDR is observed in clinical medicine. In parallel, the heavy use of polymyxins in veterinary medicine is currently being reconsidered due to increased reports of polymyxin-resistant bacteria.

Polymyxin resistance in Gram-negative bacteria is primarily due to chemical modifications of the LPS (72). Both intrinsic and transferable mechanisms of polymyxin resistance have been characterized and will be discussed in a following section (1.2.4.3. Permeability barrier).

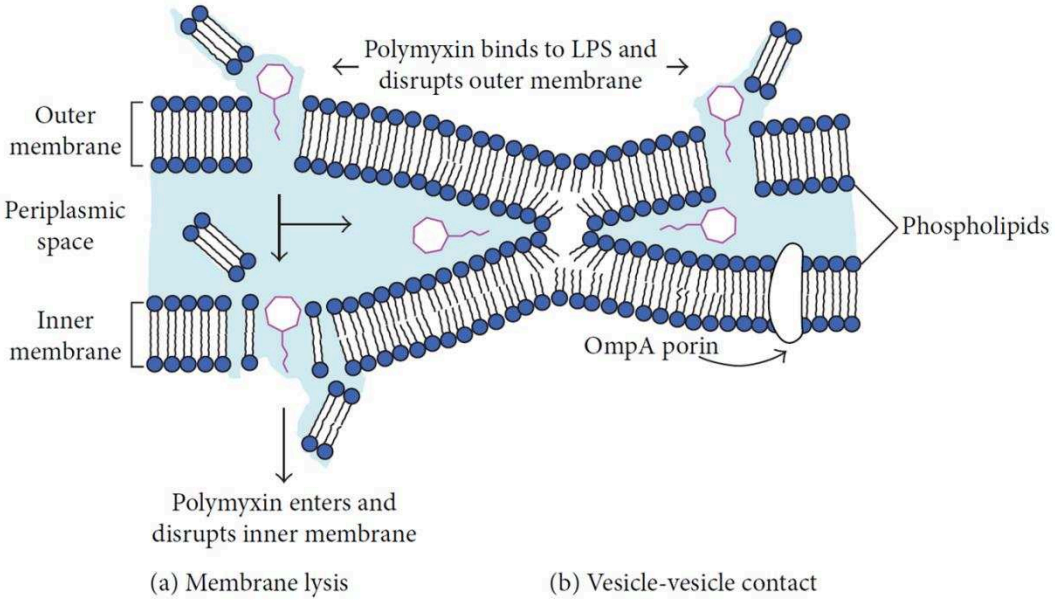

Figure 8: Antibacterial mechanisms of polymyxins: (a) classic mechanism of membrane lysis; (b) alternative mechanism of vesicle-vesicle contact. The polymyxin is colored as magenta. LPS stands for lipopolysaccharide (figure acquired from Yu *et al.*, 2015).

1.2.2.5. Folic acid metabolism

Bacteria cannot utilize pre formed folic acid (also referred as folate or vitamin B9) so they synthesize their own. Folate is an essential cofactor required for many one-carbon transfer reactions and is a critical precursor for the biosynthesis of purines, pyrimidines, and amino acids. Tetrahydrofolate (the activated form of the vitamin) is a precursor for the synthesis of glycine, methionine, thymidine triphosphate, and purines and and the enzyme dihydrofolate reductase (DHFR) maintains its cellular levels. Consequently, the inhibition of DHFR activity depletes the cell of essential metabolites for protein, RNA, and DNA biosynthesis, resulting in bacterial inactivity (73, 74, 75).

Trimethoprim (TMP) is a pyrimidine inhibitor of bacterial DHFR by binding with it in complex with nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) (76).

The first synthesized antimicrobial agent Prontosil targets dihydropteroate synthase (DHPS) of the folic acid pathway (77). DHPS catalyzes the condensation of ρ-aminobenzoic acid (ρABA) with 7, 8-dihydropterin-pyrophosphate to form 7,8-dihydropteroate. Sulfanilmide drugs are structural analogs and competitive inhibitors of ρABA at the DHPS active site. Like TMP, the inhibition of DHPS critically depletes cellular folate levels inducing 'thymineless' death which is induced by starving of thymidine triphosphate (78). Today, DHFR-DHPS inhibitor combinations continue to be used as a first-line therapy in the prophylaxis and treatment of HIV-associated pneumonia infections.

1.2.3. How antibiotics kill bacteria?

Understanding the basic mechanism(s) of bacterial death could provide essential guidance for designing new antibacterial compounds and/or improving the efficacy of existing antibiotics.

As described in the above sections, it has been recognized that the different classes of antibiotics have specific targets and that bacterial death results from target inhibition (i. e. the loss of the cell wall integrity by β-lactams through the inhibition of PBPs or the blocking of DNA replication through the inhibition of topoisomerases by fluoroquinolones). Conversely, renowned results from J. J. Collins and colleagues showed that the mode of action of bactericidal antibiotics rather converges to a deadly spiral by the production of free radicals (30, 79-89). This model has generated enormous interest in the community because of its novelty and plausibility. In addition, it offers the opportunity to consider new antibacterial targets. However, this model is not unanimous. Six years after Collins' first publication, results from three independent groups (Imlay and colleagues, Lewis and colleagues, and Barras and colleagues) provided conflicting experimental evidence. Rather than a sterile dialogue, all these studies still show us the complexity of bacterial physiology. These results are a real goldmine for understanding how "pathogenic" bacteria die (or not) from the action of antibiotics.

To identify contributors to bacterial death resulting from topoisomerase poisoning, reconstitution of stress response networks was first carried out following treatment of *E. coli* with lethal concentrations of norfloxacin, a potent fluoroquinolone (79). This primary work identified an oxidative damage-mediated cell death pathway, which involves generation of reactive oxygen species (ROS) and breakdown in iron regulatory dynamics following norfloxacin-induced DNA damage. Specifically, norfloxacin treatment was found to promote superoxide generation soon after topoisomerase II poisoning and to ultimately result in the generation of highly toxic hydroxyl radicals through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$). Under these conditions, the Fenton reaction was found to fuelled by superoxide destabilization

of Fe-S cluster catalytic sites, repair of these damaged Fe-S clusters, and related changes in iron-related gene expression (79).

Building on this work, it was later shown that all major classes of bactericidal antibiotics (including β-lactams, aminoglycosides and quinolones) promote the generation of lethal hydroxyl radicals formation in both Gram-positive and Gram-negative bacteria, regardless of the differences in their primary drug to target interactions (30). Stress response network analysis methods used in this study suggested that antibiotic-induced hydroxyl radical formation is the end product of a common mechanism wherein alterations in central metabolism related to NADH consumption, which results from an increased tricarboxylic acid cycle and respiratory activity, are crucial to superoxide-mediated Fe-S cluster destabilization and stimulation of the Fenton reaction. These results were validated by additional phenotypic experiments, biochemical assays and gene expression measurements, confirming that lethal levels of bactericidal antibiotics trigger a common oxidative damage and cell death pathway.

Work by Ling *et al.* supported this mechanism in that the overexpression of the alkyl hydroperoxide reductase subunit F (a protein defending bacteria against hydrogen peroxide), but not its inactive mutant, suppressed aggregated protein formation upon streptomycin treatment and increased aminoglycoside resistance (90). As such, cellular defense against hydrogen peroxide lowered the toxicity of mistranslation. Further evidence came from the direct correlation between intracellular hydroxyl radical formation and bacterial persistence as reported by Kim *et al.* (91). Persisters are slowly growing or non-growing phenotypic variants that stochastically emerge in susceptible bacterial populations and that survive in the presence of lethal doses of antibiotics (89, 92). In this study, flow cytometric analysis revealed distinct bacterial populations in terms of intracellular hydroxyl radical levels, morphology and viability. Upon antibiotic treatment, a small sub-population of *E. coli* survivors do not overproduce hydroxyl radicals and maintain normal morphology, whereas most bacterial cells were killed by accumulating hydroxyl radicals and displayed filamentous morphology. These results suggest that, while they probably induce different antibiotic stress responses, bacterial persisters all commonly show lowered hydroxyl radical formation and enhanced tolerance to antibiotics.

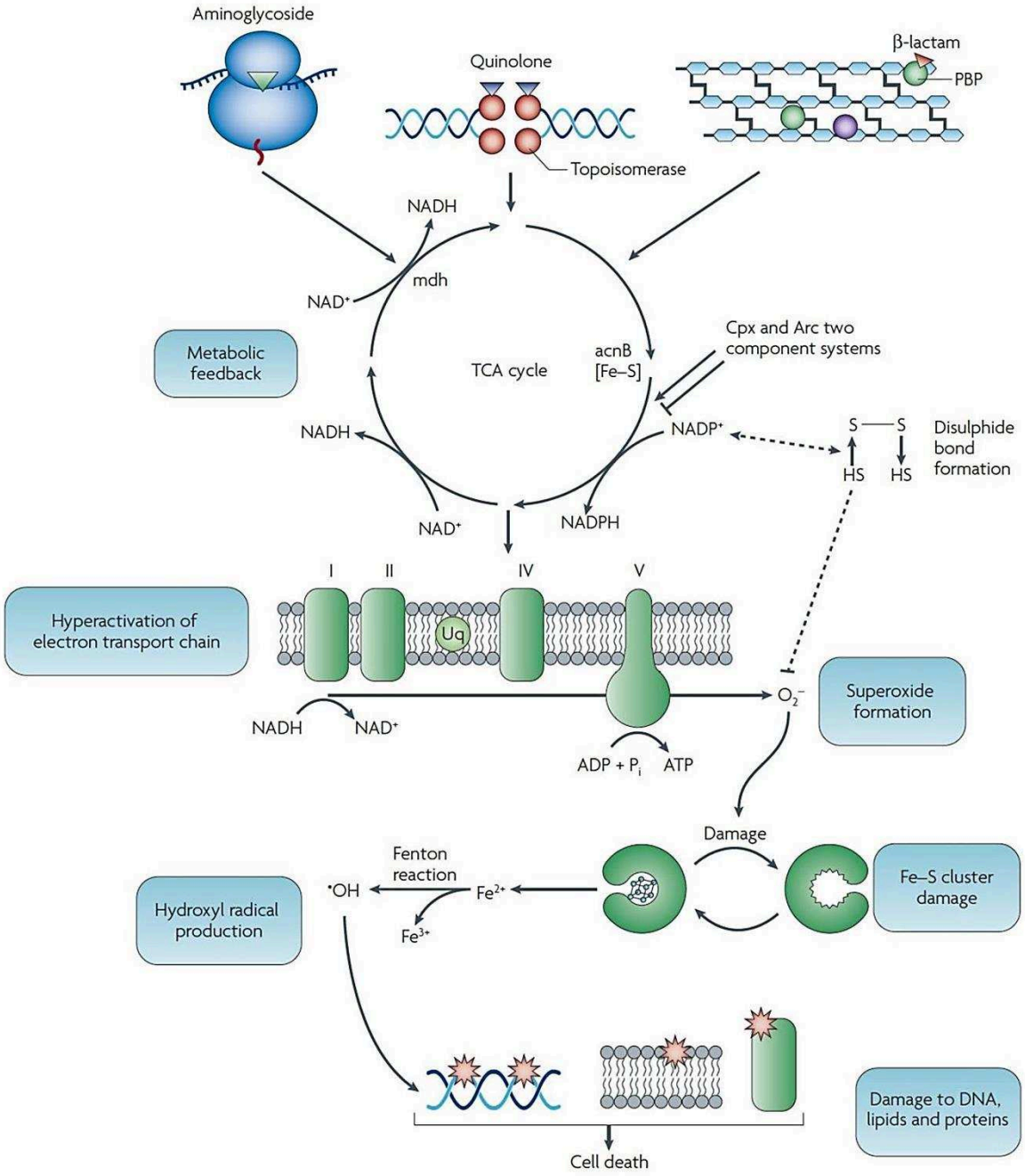

Figure 9: Common mechanism of cell death induced by bactericidal antibiotics. The primary drug–target interactions (aminoglycoside with the ribosome, quinolone with topoisomerase, and β-lactam with penicillin-binding proteins (PBPs)) stimulate the oxidation of NADH through the electron transport chain, which is dependent on the tricarboxylic acid (TCA) cycle. Hyperactivation of the electron transport chain stimulates superoxide (O_2^-) formation. Superoxide damages Fe–S clusters, making ferrous iron available for oxidation by the Fenton reaction. The Fenton reaction leads to the formation of hydroxyl radicals (•OH), which damage DNA, lipids and proteins. This contributes to antibiotic-induced cell death. Quinolones, β-lactams and aminoglycosides

also trigger hydroxyl radical formation and cell death through the envelope (Cpx) and redox-responsive (Arc) two-component systems. It is also possible that redox-sensitive proteins, such as those containing disulphides, contribute in undetermined way to the common mechanism (dashed lines). *acnb*, aconitase b; *mdh*, malate dehydrogenase; *uq*, ubiquinone (figure acquired from Kohanski *et al*., 2010).

However, this unified mechanism of killing was severely challenged by the direct and simple observation that there was no difference in survival of bacteria treated with various antibiotics under aerobic or anaerobic conditions (93, 94). In these two independent studies, the authors show that antibiotic treatment do not accelerate the formation of hydrogen peroxide in *E. coli* and do not elevate the intracellular level of free iron, as an essential substrate for the generation of lethal hydroxyl radicals through the Fenton reaction. Rather, it appears that one should be cautious in interpreting the oxidation of fluorescein-based dyes such as hydroxyphenyl fluorescein used by Collins and coll. as a marker of ROS generation. Finally, work provided by Ezraty *et al.* also show the ROS response is dispensable upon treatment with bactericidal antibiotics (95). Furthermore, results demonstrate that Fe-S clusters are required for killing only by aminoglycosides but not by fluoroquinolones or β-lactams. In contrast to cells using the major Fe-S cluster biosynthesis machinery, ISC, cells using the alternative machinery, SUF, cannot efficiently mature respiratory complexes I and II, resulting in the breakdown of the proton motive force, which is required for bactericidal aminoglycoside uptake. Similarly, during iron limitation, cells become intrinsically resistant to aminoglycosides by switching from ISC to SUF and down-regulating both respiratory complexes. Therefore, it was concluded that Fe-S proteins promote aminoglycoside killing by enabling their uptake. To date, the role of ROS in killing of bacteria by antibiotics is still under debate.

1.2.4. Bacterial adaptation to antibiotics: resistance versus tolerance

The general bacterial strategies for MDR *Enterobacteriaceae* can be categorized into three types: enzymatic barrier, target protection barrier and membrane barrier (96, 97, 98) (**Figure** 10). In clinical settings, one or more of these strategies working at the same instant are responsible for high level of resistance (99). All of these mechanisms basically accomplish the same goal of preventing the antibiotic to bind to its target.

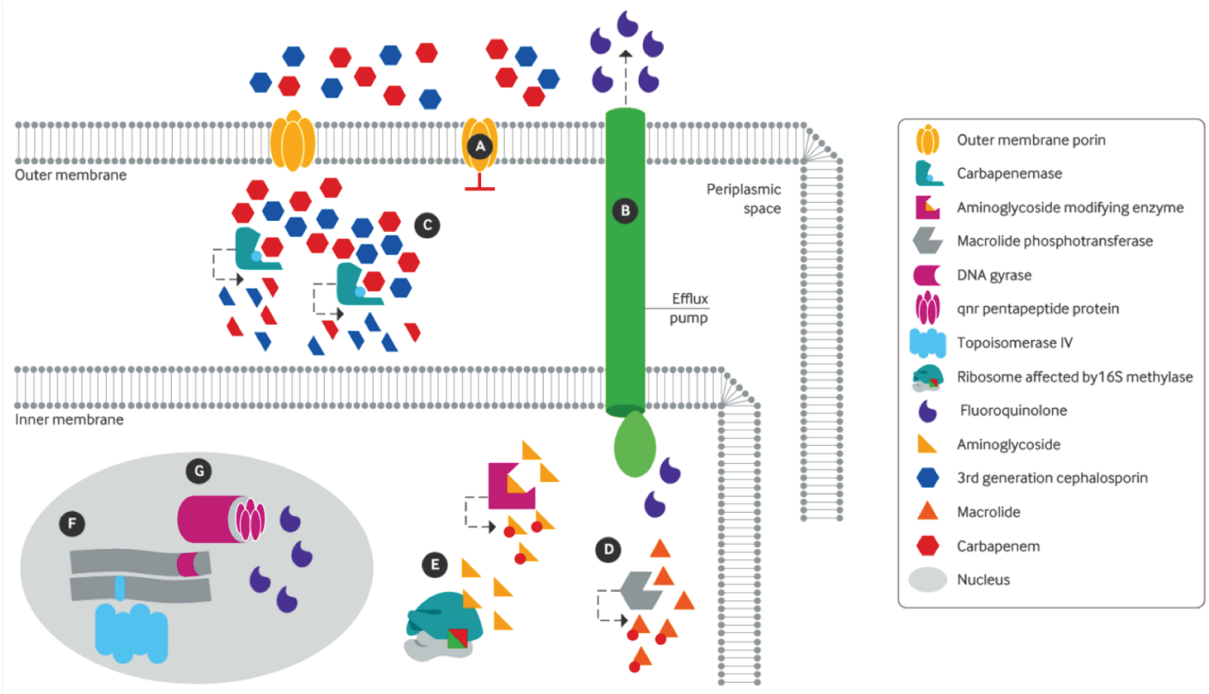

Figure 10: Important mechanisms of antibiotic resistance in *Enterobacteriaceae*. Porin deficiencies or alterations (A) reduce antibiotic access and efflux pumps (B) may actively transport antibiotics out of the cell. β-lactamases (C) acting in the periplasmic space hydrolyze β-lactam antibiotics and thereby prevent disruption of the cell wall. Intracellular (for example, aminoglycoside modifying) enzymes (D) alter antibiotics. 16S rRNA methylases (E) prevent aminoglycoside binding. Mutations in targeted DNA gyrase and topoisomerase IV genes (F) render fluoroquinolones ineffective. Pentapeptide Qnr proteins (G) prevent fluoroquinolones from effectively binding to DNA gyrase through target mimicry (figure acquired from Iredell *et al.*, 2016).

1.2.4.1. Enzymatic barrier

This strategy of resistance consists in the enzymatic destruction or modification of antibiotic. The classic case is the hydrolytic deactivation of the β-lactam ring by β-lactamases produced in resistant bacteria. The β-lactamase producing bacteria secrete this enzymatic weapon into the periplasm to destroy β-lactam antibiotics before they can reach the PBP targets. It is estimated that a single β-lactamase molecule can hydrolyze 1,000 penicillin molecules per second. Therefore, if 10^5 enzymes are secreted per producing cell, then 100 million molecules of penicillin are destroyed every second, which is clearly an effective strategy.

Hydrolysis of β-lactam antibiotics by β-lactamases is the most common mechanism of resistance for this class of antibacterial agents in clinically important *Enterobacteriaceae*. Because penicillins, cephalosporins, and carbapenems are included in the preferred treatment regimens for many infections, the expression and the

functional features of these enzymes play a critical role in the selection of appropriate therapy. β-lactamase production is most frequently suspected in a Gram-negative bacterial isolate that demonstrates resistance to a β-lactam antibiotic. Due to more sophisticated molecular approaches than were previously available, it has become increasingly easy to obtain nucleotide sequences, with their deduced amino acid sequences, for the genes encoding these enzymes in β-lactam-resistant clinical isolates. Classification of β-lactamases has traditionally been based on either the functional characteristics of the enzymes or their primary structure (100). The simplest classification is by protein sequence, whereby the β-lactamases are classified into four molecular classes, A, B, C, and D, based on conserved and distinguishing amino acid motifs. Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine, whereas class B β-lactamases are metalloenzymes that utilize at least one zinc ion at their active site to facilitate β-lactam hydrolysis. Among typical examples, one can cite:

- Extended spectrum β-lactamases (ESBLs), which mostly belong to class A β-lactamases, are active against most of penicillins and cephalosporins. ESBLs are carried on plasmids and easily transferable in *Enterobacteriaceae*. This characteristic usually explains the rapid spread of epidemic resistant clones. These enzymes can be inhibited by β-lactamase inhibitors such as tazobactam, clavulanic acid and avibactam, which can restore the activity of the antibiotic when used in combination therapy (*e. g.* piperallicin + tazobactam; amoxicillin + clavulanic acid; ceftazidime + avibactam);
- AmpC cephalosporinases, which belong to class C β-lactamase, are present on the chromosome of many *Enterobacteriaceae* and are active against cephalosporins. AmpC is normally expressed at low levels but can be induced upon exposure to certain β-lactams such as imipenem or cefoxitin. AmpC cephalosporinases cannot be inhibited by approved β-lactamase inhibitors.
- Carbapenemases are metalloenzymes, which belong to class D β-lactamase, are active against all clinically-available β-lactams including carbapenems, one of the most potent subclass of β-lactam antibiotics. Over the past 10 years, the emergence of carbapenem-resistant (CR) *Enterobacteriaceae* (CRE) poses a serious threat to public health worldwide (101). In particular, the increasing prevalence of carbapenem-resistant *K. pneumoniae* is a major source of concern (102). *K. pneumoniae* carbapenemases (KPCs) have been reported worldwide (103), including in Europe with high spreading in Italy and Greece (**Figure 11**). Because CRE are usually MDR, high mortality rates have been reported in patients with bloodstream infections caused by CR *K. pneumoniae* (104). Among novel treatment approaches, the use of β-lactamase inhibitors with broad spectrum activity is the most promising such as relebactam and vaborbactam currently in phase 3 clinical developments in combination with imipenem and meropenem, respectively (105).

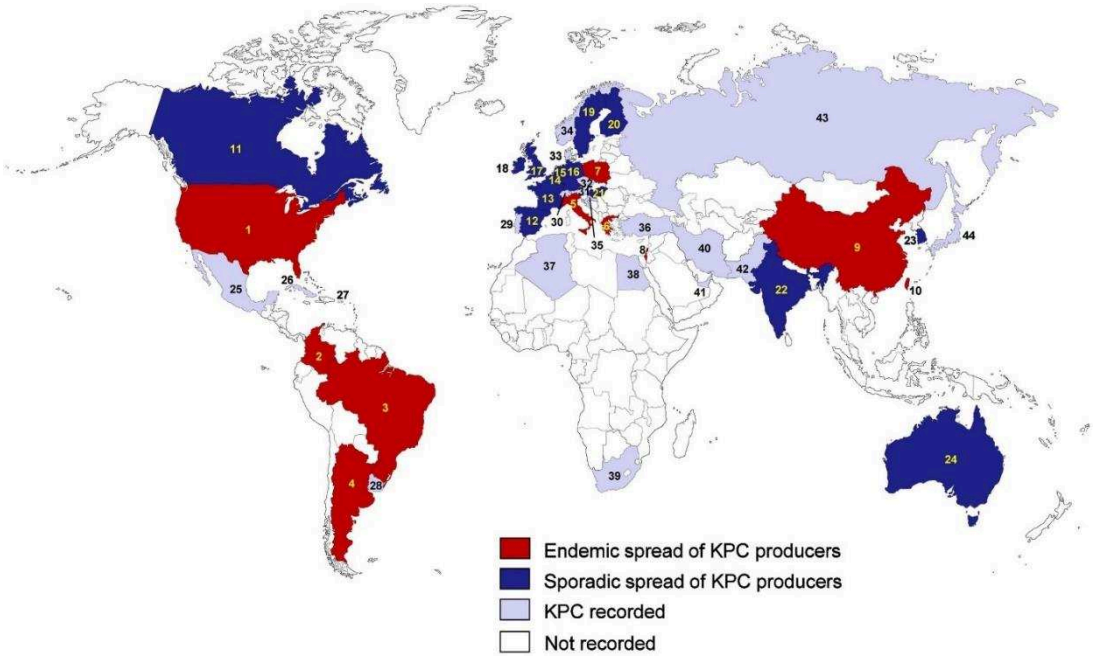

Figure 11: Epidemiological features of KPC-producing *K. pneumoniae*. (1) USA; (2) Colombia; (3) Brazil; (4) Argentina; (5) Italy; (6) Greece; (7) Poland; (8) Israel; (9) China; (10) Taiwan; (11) Canada; (12) Spain; (13) France; (14) Belgium; (15) Netherlands; (16) Germany; (17) UK; (18) Ireland; (19) Sweden; (20) Finland; (21) Hungary; (22) India; (23) SouthKorea; (24) Australia; (25) Mexico; (26) Cuba; (27) PuertoRico; (28) Uruguay; (29) Portugal; (30) Switzerland; (31) Austria; (32) CzechRepublic; (33) Denmark; (34) Norway; (35) Croatia; (36) Turkey; (37) Algeria; (38) Egypt; (39) South Africa; (40) Iran; (41) United Arab Emirates; (42) Pakistan; (43) Russia; (44) Japan (picture has been taken from Lee *et al.*, 2016).

Other antibiotic classes, such as the aminoglycosides, do not contain such hydrolytically labile groups. These protein-synthesis inhibitors are still neutralized by deactivating enzymes that decorate the periphery of the aminoglycosides with three types of chemical substituents that interrupt the binding to the RNA targets in the ribosome. Aminoglycoside resistance enzymes can be adenylyl transferases, which add AMP moieties, phosphoryl transferases, which add -PO_3 groups, or acetyl transferases, which acetylate the amino groups of the antibiotic (107).

1.2.4.2. Drug-target interaction barrier

Evasion of drug target interaction is an effective means of antibiotic resistance (**Figure 12**). This could happen by three means that includes (i) mutations/modifications of target binding site; (ii) protection/masking of target, or (iii) overexpression of the target. For example, amino acid substitutions of the QRDR in DNA gyrase and topoisomerase IV are often found in clinical strains and confer fluoroquinolone resistance. Modification of the target can occur by addition of a

chemical group that prevents antibiotic binding without altering the primary protein sequence. An interesting example of target protection by masking is provided by the *qnr* genes that confer quinolone resistance (108). The *qnr* genes encode pentapeptide repeat proteins (PRPs), which bind to and protect DNA gyrase and topoisomerase IV from the lethal actions of quinolones.The Qnr families of *qnr* resistance genes have been found on plasmid in various pathogens.

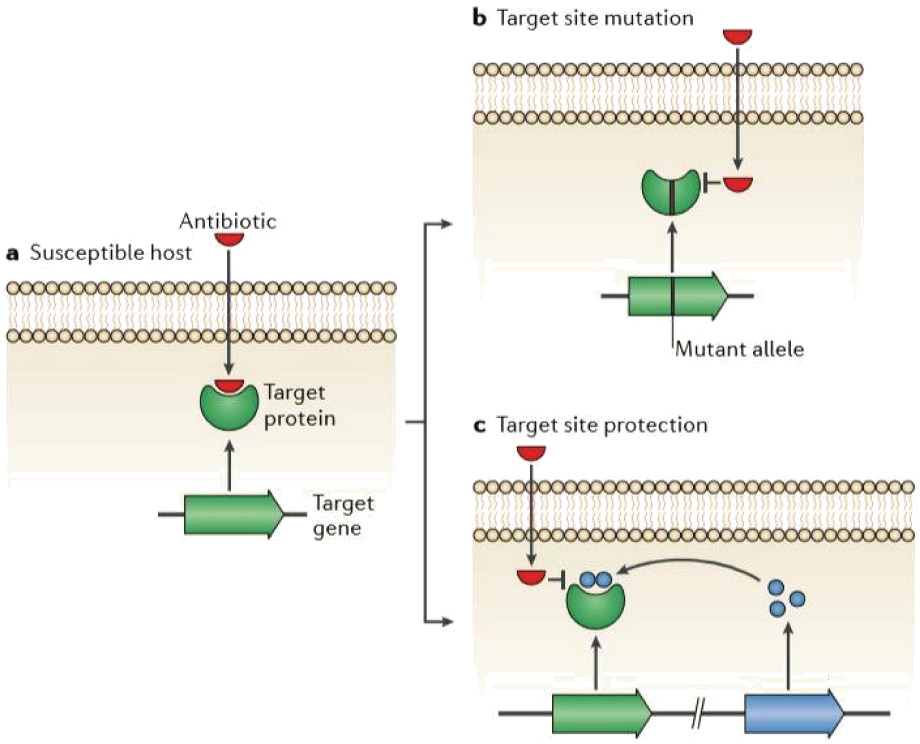

Figure 12: Target protection barrier. a) A susceptible host in which an antibiotic is able to bind tightly to its specific target and exert an inhibitory effect. b) Mutation of the target site (for example, as found in mutations in topoisomerase genes in many species that confer fluoroquinolone resistance) or recombination to provide a mosaic allele (as found in the mosaic penicillin-binding proteins in pneumococci and gonococci that confer β-lactam resistance) results in a functional target with reduced affinity for the antibiotic, which does not bind efficiently and therefore has a reduced or negligible effect. c) Modification of the target by addition of a chemical group can also prevent antibiotic binding without altering the primary protein sequence of the target, which retains its activity. (figure acquired from Blair *et al.,* 2015).

1.2.4.3. Permeability barrier

To be effective, most antibiotics must reach their specific intracellular targets and accumulate at concentrations that can act in some reasonable time frame. In Gram-negative *Enterobacteria*, this implies translocation across the outer and inner membranes (**Figure 13**). Here, changes in the overall ability of drugs to pass through

this diderm envelope due to loss of porins and/or drug removal via upregulation of efflux pumps can lead to MDR (109-112). In addition, this permeability barrier reduces the discovery of new antibiotics effective against Gram-negative bacteria. However, one could expect that the basic principles established by extensive studies of *E. coli* would allow permeation rules, in analogy with Lipinski's rules, and if such existed and were applied to structure–activity relationships or to filtering compound libraries, would yield compounds that permeate some of the Gram-negative barriers. These aspects will be developed in the following sections. Here, I will focus on the exception of polymyxins, which target the LPS of the OM but are also submitted to resistance mechanisms involving modifications of the envelope composition.

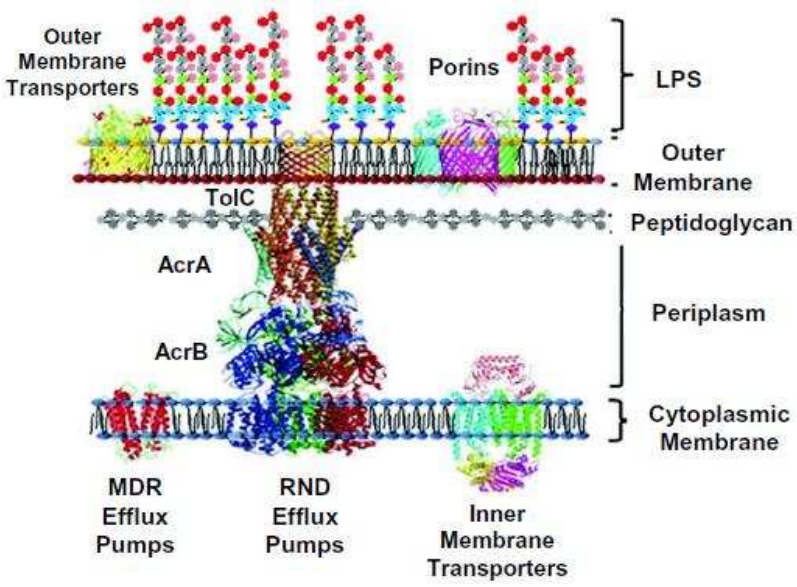

Figure 13: Schematic representation of the cell envelope of Gram-negative bacteria (**Figure** acquired from Manchester *et al.*, 2012; Silver, 2016).

In most polymyxin-resistant strains, substituents such as 4-amino-4-deoxy-l-arabinose (l-Ara4N), phosphoethanolamine (pEtN) or galactosamine are enzymatically added to the lipid A or the LPS core; alternatively, the LPS part of the OM may be completely lost in some other isolates (113, 114). By decreasing the net negative charge of phosphate residues, these LPS alterations tend to prevent the binding of polymyxin molecules to the bacterial surface and their further penetration into the cell interior where they are supposed to exert their bactericidal activity. Expression of most of the genes of the LPS modification pathway is under the control of a variety of two-component systems (TCSs) such as PhoP–PhoQ (PhoPQ) and PmrA–PmrB (PmrAB). Each of these phosphorelays is composed of a transmembrane sensor histidine kinase (*e.g.* PhoQ, PmrB), which is subject to autophosphorylation under specific stress conditions, and a cognate cytoplasmic response regulator (*e.g.* PhoP, PmrA), which

modulates the expression of target genes when phosphorylated by the kinase. Some mutations in the genes encoding these TCSs result in constitutive upregulation of the LPS modification pathway and thus polymyxin resistance because of membrane impermeability (**Figure 14**). Polymyxin resistance rates are still low in many countries but are increasing steadily in some others such as Greece and Italy (8). The recent identification of a plasmidborne colistin resistance gene (*mcr-1*) in human, animal and environmental strains of *Enterobacteriaceae* may potentially worsen this situation at the global scale (115). Indeed, reports from all continents multiply on the isolation of *mcr-1*-positive strains (115).

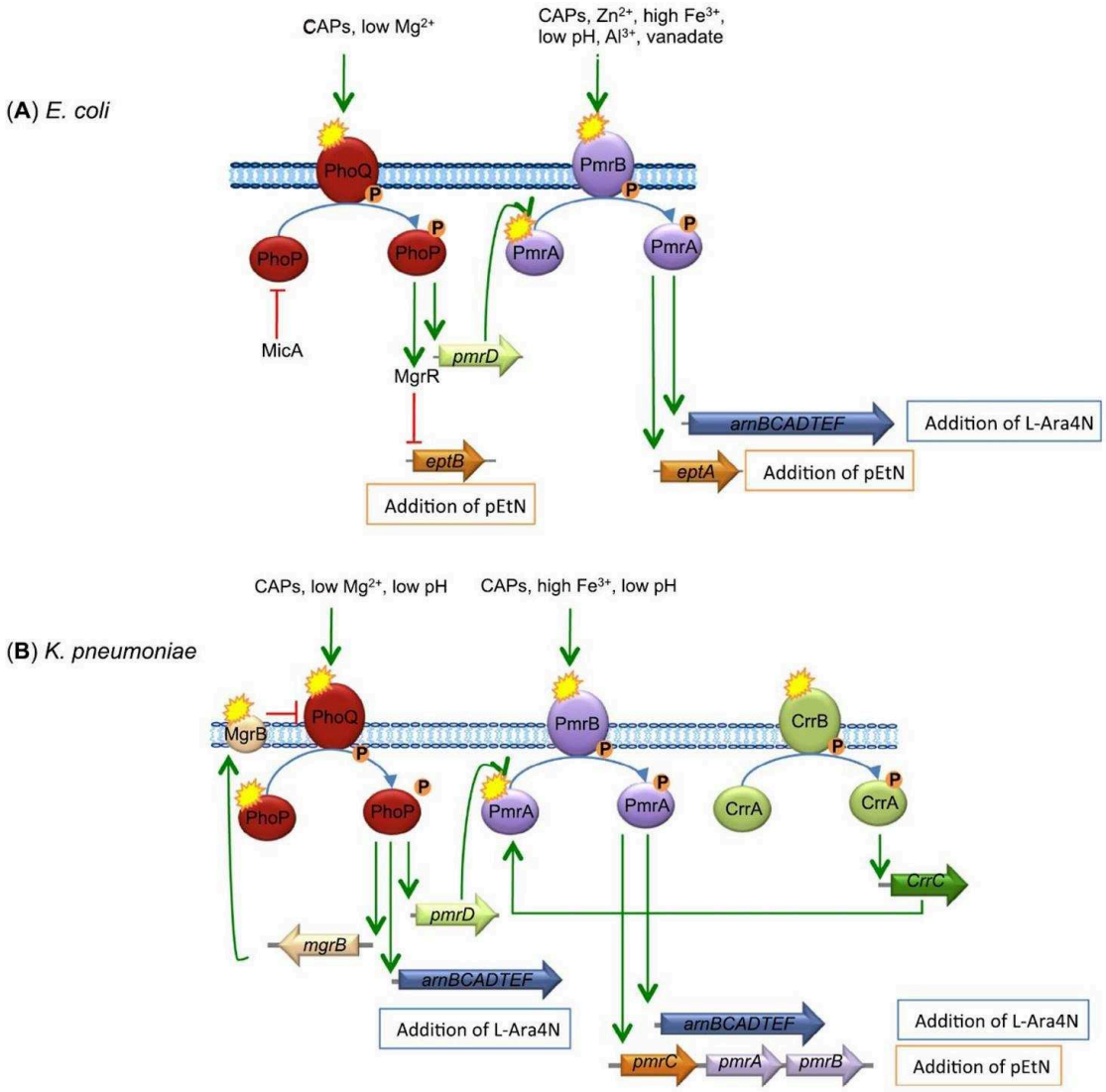

Figure 14: Schematic representation of regulation of genes involved in polymyxin resistance in clinical isolates of (A) *E. coli* and (B) *K. pneumoniae*. In both species, resistance to polymyxins is induced by cationic compounds such as colistin, low Mg^{2+} concentrations, acidic pH and high Fe^{3+} concentrations, which activate the two-component systems (TCSs) PhoPQ and/or PmrAB. Subsequent activation of operon *arnBCADTEF* (also called *pmrHFIJKLM*), the *eptA* gene or the *pmrC* gene triggers the

synthesis and addition of 4-amino-4-deoxy-l-arabinose (l-Ara4N) and phosphoethanolamine (pEtN) to lipid A, respectively. PmrAB is also activated by PhoPQ via the product of the *pmrD* gene. In *K. pneumoniae* (B), the *arnBCADTEF* operon can be directly activated by PhoP. In *E. coli* only (A), a first small RNA, MgrR, directly represses the expression of *eptB*, a gene required for addition of pEtN to the lipopolysaccharide (LPS) core, whilst a second small RNA, MicA, represses the phoP gene. In both *E. coli* and *K. pneumoniae* (A and B) clinical isolates, alterations (represented by yellow asterisks) in histidine kinases PhoQ and PmrB or in the response regulator PmrA lead to constitutive activation of the TCSs PmrAB or PhoPQ. Furthermore, in *K. pneumoniae* (B), inactivation of *mgrB* results in colistin resistance through activation of PhoPQ, whilst mutations in histidine kinase CrrB activate PmrAB through CrrC. CAPs, cationic antimicrobial peptides (including polymyxins) (Figure acquired from Jeannot *et al*., 2017).

1.2.4.4. Drug tolerance

Antibiotics shut down or subvert essential cellular functions, and resistance mechanisms appear to exploit every possible strategy of preventing a drug from hitting its target. The major clinically relevant resistance mechanisms described in the above sections have been studied for a long time and are generally well understood. Again, these include destruction of the antibiotic (for example, by β-lactamases, conferring resistance to β-lactams), target modification (for example, mutation of the 30S ribosomal subunit RpsL conferring resistance to streptomycin), as well as restricted penetration and efflux (for example, multiple drug efflux by the AcrAB-TolC pump).
The same cannot be said about tolerance. Bacterial tolerance to antibiotics is caused by specialized bacterial survivors called persisters (92, 117). Persisters are not mutants, but phenotypic variants of actively dividing cells produced stochastically in a clonal population as a result of fluctuations in gene expression (117-119) and their relative abundance (around 1 %) rises at the late-exponential phase of growth and in biofilms, where cells incur various forms of stress (120, 121) (**Figure 18**). Persisters are non-growing dormant cells. This explains their tolerance to bactericidal antibiotics that depend on the presence of active targets for killing the cells. All of the bacterial pathogens examined so far are able to form persisters, but the mechanism(s) underlying the formation of persisters is still largely unknown. Previous studies have shown that, in the model organism *E. coli*, toxin-antitoxin (TA) modules are a major mechanism of persister formation (118, 120, 122, 123). But recent work by Goormaghtigh *et al*. demonstrated that the deletion of 10 TA systems in *E. coli* does not affect persistence to ofloxacin or ampicillin (124).

Furthermore, in the presence of ciprofloxacin — a potent fluoroquinolone that halts DNA replication — the SOS response induces persister formation through

formation through activating TisB overexpression (122), a member of the toxin family. Similar mechanism could explain the dramatic increase of persisters upon pre-treatment of the cells with rifampin and tetracycline, which halt transcription and translation, respectively (125). Screening a complete bacterial knockout library also identified a number of global regulators involved in persister formation, suggesting that pathways of persister formation are highly redundant (126). For example, overexpression of *ygfA* downregulated overall transcription, and overexpression of *relE* led to a decreased protein synthesis rate (127), both of which assist bacterial drug tolerance through inducing a dormant state of the cell. Owing this, a realistic target for drug discovery has yet to be identified.

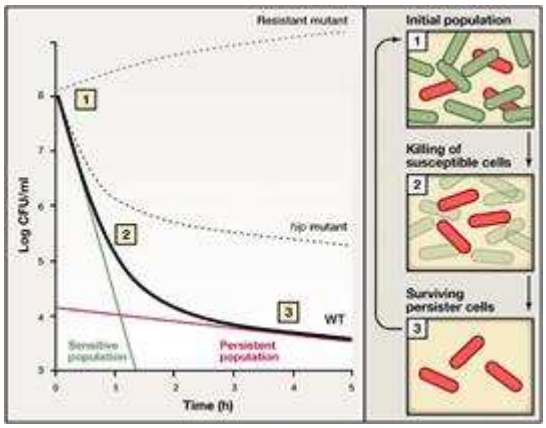

Figure 15: Killing kinetics during treatment with a bactericidal antibiotic. (1) Lethal dose of a bactericidal antibiotic is added at time zero to a growing population of sensitive, genetically identical bacteria. The experiment reveals a characteristic biphasic killing curve. (2) The slope of the initial phase reveals the susceptibility of the bulk of the population. The initial log-linear relationship reveals an exponential killing kinetics (green line). (3) The slope of the second inactivation phase (red line) reveals the existence of a persister subpopulation that is killed with a much slower kinetics. Killing kinetics for a high persister mutant (*hip*) strain producing a highly elevated number of persisters is also shown (dark dashed line). After removal of the antibiotic (pointed by the arrow flanking the right panels), persister cells resume growth and give rise to progeny cells that are genetically identical to the cells of the original population and, therefore, as drug-sensitive as the original cells. The gray dashed line indicates how a drug-resistant mutant strain would support growth under these conditions (figure acquired from Maisonneuve *et al.*, 2014).

Recent reports have also found that indole signaling contributes to persister formation, possibly via upregulation of the efflux pumps through activation of the two-component systems BaeSR or CpxAR and the PspAB (phage shock response) and OxyR (oxidative stress response) pathways as a result of stress response (129, 130). At a

population level, indole molecules produced by highly resistant cells have been shown to provide protection to other more susceptible cells by activating their drug efflux pumps and oxidative-stress protective mechanisms (131). This facilitates the survival of the whole population. Since indole increases antibiotic resistance by enhancing antibiotic efflux (130, 132), a likely mechanism for the rise in persistence upon the reduction in indole concentrations (133, 134) is that the cells become less resistant (due to reduced antibiotic efflux) and thereby less fit to withstand antibiotic stress. Additionally, it has been demonstrated that bacterial persisters, obtained under treatment with β-lactams, show less cytoplasmic drug accumulation as a result of enhanced efflux activity (135). This confirms positive correlation between drug efflux and bacterial persistence.

The significance of persisters and drug tolerance is such that they have been observed in clinical cases and may play a role in the recurrence of chronic infections. Their existence is believed to prolong and exacerbate the treatment of diseases, such as tuberculosis and cystic fibrosis associated lung infections (136-138). Therefore, the importance of persisters in the recalcitrance of infectious diseases raises the bar for drug discovery as there is an urgent need to develop therapies that effectively kill both actively dividing and dormant pathogens.

2. The cell envelope of Gram-negative bacteria

The bacterial cell envelope contains the membrane(s) and other structures that surround and protect the cytoplasm. Unlike cells of higher organisms, bacteria are faced with an unpredictable, dilute and often hostile environment. To survive, bacteria have evolved sophisticated and complex cell envelopes that protect them, but allow selective passage of nutrients from the outside and waste products from the inside. The following discussion concerns the organization, composition, and the functions of the various layers and compartments that make up this remarkable cellular structure. It is easily appreciated that a living organism cannot do what it does without the ability to establish separate compartments in which components are segregated. Specialized functions occur within different compartments because the types of molecules within the compartment can be restricted. However, membranes do not simply serve to segregate different types of molecules. They also function as surfaces on which reactions can occur. Recent advances in microscopy, which are discussed in other articles on this subject, have revealed strikingly nonrandom localization of envelope components. Here, we will highlight recent advances in our understanding of how these extracellular organelles are assembled.

More than 100 years ago, Christian Gram (1884) developed a staining procedure that classifies nearly all bacteria into two large groups, and this eponymous stain is still in widespread use. One group of bacteria retain the Gram's stain, Gram-positive, and the other do not, Gram-negative. The basis for the Gram's stain lies in fundamental

structural differences in the cell envelope of these two groups of bacteria. For our discussion of the Gram-negative bacterial cell envelope we will refer to the model bacterium *E. coli* and closely related Enterobacteria. Care should be taken in generalizing from examples drawn from particular microorganisms. For example, *E. coli* inhabits the mammalian gut. Accordingly, *E. coli* and other enteric bacteria must have a cell envelope that is particularly effective at excluding detergents such as bile salts. This need not be a pressing issue for other Gram-negative bacteria, and their envelopes may differ in species- and environmentally specific ways. Nonetheless, the ability to use the Gram stain to categorize bacteria suggests that the basic organizational principles we present are conserved. In addition, many bacteria express an outermost coat, the S-layer, which is composed of a single protein that totally encases the organism. S-layers and capsules, which are coats composed of polysaccharides, are beyond the scope of this manuscript.

After more than a decade of controversy, techniques of electron microscopy were improved to the point in which they finally revealed a clearly layered structure of the Gram-negative cell envelope (**Figure 2**) (139). There are three principal layers in the envelope; the OM, the peptidoglycan cell wall, and the cytoplasmic or inner membrane. The two concentric membrane layers delimit an aqueous compartment first termed the periplasm (140). At the same time, biochemical methods were developed to isolate and characterize the distinct set of proteins found in the periplasm (141), and to characterize the composition of both the inner and outer membranes (142, 143). Studies since then have only reinforced their basic conclusions.

2.1. The outer membrane

In this section, I will give an overview of the main constituents of the OM, namely the LPS and outer membrane proteins (OMPs) including OM lipoproteins and integral β-barrel OMPs. I will also describe their biogenesis, how they are synthetized, transported and assembled at the OM. Porins, a major class of integral β-barrel OMPs, as well as porin biogenesis will be detailed in dedicated subsequent sections.

The OM is a distinguishing feature of Gram-negative bacteria. It is an asymmetric lipid bilayer, where phospholipids are present on the inner leaflet, while the outer leaflet is composed of LPS (**Figure 2**) (144). The lipid A is a glucosamine-based phospholipid, and is one of the three parts of an LPS molecule (**Figure 16**) (145). The other two are a relatively short core oligosaccharide (Kdo) and a distal polysaccharide (O-antigen). Lipid A differs from a typical phospholipid by having six saturated fatty-acyl chains rather than two saturated or unsaturated chains. Lipid A domain of LPS is also responsible for endotoxic shock associated with the septicemia caused by Gram-negative bacteria (146). The Kdo2-lipid A biosynthetic pathway may be viewed as having a conserved and a variable component. The conserved (constitutive) enzymes (**Figure 16**) are

intracellular, present in virtually all Gram-negative bacteria, and not generally subject to regulation (145). In contrast, the lipid A modification enzymes, are mostly extracytoplasmic, not conserved among microorganisms, and induced or repressed by external conditions, such as changes in pH, divalent cation concentrations, or the presence of CAMPs (113, 147, 1, 149, 150). Most modification enzymes reside either on the periplasmic surface of the inner membrane or in the OM (151-157). The systematic elucidation of the constitutive pathway for Kdo2-lipid A biosynthesis (**Figure 16**) was enabled by the discovery of 2, 3-diacylglucosamine 1-phosphate (lipid X) (158, 159). Although present at very low concentrations in *E. coli* (160, 161), it accumulates as much as 500-fold, or to about 5% to 10% of the total cell lipid content, in some phosphatidylglycerol-deficient mutants (158, 159). The discovery of lipid X agreed with the correct structure determination (162, 163) and chemical synthesis of lipid A (164). As shown in **Figure 16**, LpxA, -C, and -D are soluble proteins (165, 166, 167), whereas LpxB and LpxH are peripheral membrane proteins (168-170). LpxK, KdtA, LpxL, and LpxM are integral inner membrane proteins (171-175). Their active sites are presumed to face the cytoplasmic surface of the inner membrane, given that their water-soluble co-substrates are cytoplasmic molecules.

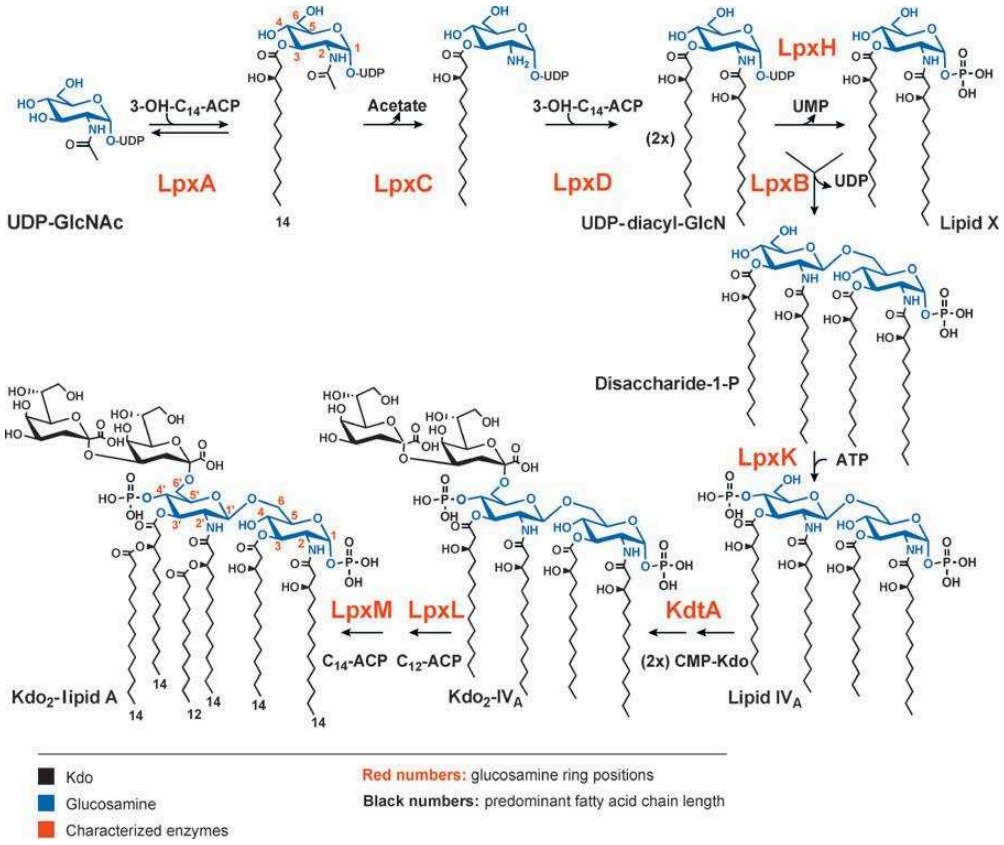

Figure 16: Constitutive pathway for biosynthesis of the Kdo2-lipid A portion of LPS in *E. coli* K-12. Each enzyme of the constitutive Kdo2-lipid A pathway is encoded by a single structural gene (2, 69). The glucosamine disaccharide backbone of lipid A and the Kdo disaccharide are shown. LpxA, -C, and -D are soluble cytoplasmic proteins, whereas LpxH and -B are peripheral membrane proteins. The distal enzymes of the pathway, starting with LpxK, are integral inner membrane proteins, the active sites of which face the cytoplasm. The red numbers specify the glucosamine ring positions of lipid A and its precursors. The black numbers indicate the predominant fatty acid chain lengths found in *E. coli* lipid A. The single molecular species shown at the bottom left represents about 90% of the total lipid A in *E. coli*, with most of the rest bearing a C12 secondary acyl chain at position 3'. The O-antigen polysaccharide chain of variable length is the most distal portion of the molecule and is not produced in *E. coli* K-12 (figure acquired from Raetz *et al.*, 2007).

How *E. coli* lipids cross the inner membrane and are transported to the OM is not fully understood (176, 177). A clue to bacterial lipid transport emerged from studies of *lpxL* mutants and their suppression by multiple copies of *msbA* (178, 179, 180). *lpxL* mutants accumulate tetraacylated lipid A accumulates in inner membranes and show growth inhibition at 42°C (180). LpxL is a lauryl transferase of lipid A and MsbA is an essential ABC transporter closely related to eukaryotic Mdr proteins (178). MsbA overexpression restores the growth of *lpxL* mutants at 42°C without restoring laurate addition, resulting in export of LPS with tetraacylated lipid A to the OM. Several structures of the homodimeric MsbA protein determined by X-ray crystallography and cryo-electron-microscopy support the proposed flippase function of MsbA. These studies revealed that LPS binds deeply inside MsbA at the height of the periplasmic leaflet, establishing extensive hydrophilic and hydrophobic interactions with MsbA and suggest the existence of multiple conformational states (181, 182, 183, 184, 185, 186). When reconstituted in proteoliposomes, MsbA is able to bind nucleotides and various putative substrate, behaves as an ATPase and a lipid flippase (187, 188).

In *E. coli* the Lpt (lipopolysaccharide transport) complex is composed of seven essential proteins (LptABCDEFG) (189-191) that are located in every cellular compartments: cytoplasm, inner membrane, periplasm, and OM (**Figure 17**). The Lpt complex provides energy for LPS extraction from the inner membrane and mediates transport across the periplasm, its insertion and assembly at the OM (15, 192, 193). The Lpt machinery may be divided in three sub-complexes: LptBFGC, LptA, and LptDE, which are located at the inner membrane, in the periplasm, and at the OM, respectively. LptBFG (194-196) constitute an inner membrane ABC transporter that is associated to an atypical subunit, the bitopic inner membrane protein LptC (197), whose function in the ABC transporter is still unclear (196) LptB is the ATP binding domain of this transporter (198) and LptF and LptG represent the transmembrane subunits. At the OM, the LptDE translocon composed by the β-barrel protein LptD and the lipoprotein LptE is responsible of the final stage of LPS assembly at the cell surface (199). The periplasmic

protein LptA connects the two sub-complexes, somehow coordinating their functions (195, 200). The Lpt machinery appears to work as a single device as depletion of any components leads to similar phenotypes, i.e. the blockade of LPS transport and its accumulation to the periplasmic side of the inner membrane where it is decorated with colanic acid by the WaaL ligase (194, 197). Overall, the seven Lpt proteins physically interact and form a trans-envelope complex that spans both inner and outer membranes (199).

Because LPS is an amphipathic molecule, its transport across the periplasm requires at least two energy inputs: one for its extraction from the lipid environment of the inner membrane, and one to facilitate the transit of the hydrophobic lipid A portion through the aqueous environment of the periplasm. Two working models have been considered: the chaperone-mediated transit across the periplasm and the transport through a trans-envelope proteinaceous bridge between the inner and the OM. According to the first model, LptA is the soluble carrier that accepts LPS from LptBFGC, forms a soluble complex shielding its hydrophobic moiety during the diffusion across the periplasm and, ultimately, delivers it to the OM complex LptDE. In fact, LptA binds LPS *in vitro* and *in vivo* (201, 202) and can displace it from LptC, consistently with protein subcellular locations and the unidirectionality of the transport (201, 202, 203). However, physiologically expressed LptA is not found as a soluble periplasmic protein but fractionates with both inner and outer membranes after sucrose density gradient centrifugation (199), suggesting that the protein does not function as a soluble carrier but form oligomeric structures spanning the width of the periplasm. This agrees with previous evidence that LPS transport occurs in spheroplast, where the periplasmic soluble content has been drained, and that soluble LPS-protein complexes have never been isolated from periplasm (204). The second model for LPS transport suggests the existence of a molecular machine made up of individual protein components located in each cellular compartment thus connecting the inner and OM. These molecular machines are not unusual in Gram-negative bacteria, exemplified by the multidrug efflux pumps and secretion systems. The most important evidence supporting this model is that depletion of any Lpt factor leads to accumulation of *de novo* synthesized LPS at the periplasmic side of the inner membrane (197). This suggests that the Lpt proteins constitute a molecular machine that operates as a single device downstream of MsbA-mediated LPS flipping across the inner membrane (see above). This is also in line with the previous observation that Lpt proteins co-fractionate in sucrose density gradient centrifugation in a lighter OM fraction containing inner and OM components and that these proteins all physically interact to form a trans-envelope bridge (199). Interestingly, the trans-envelope model of transport is reminiscent of a model postulated more than 40 years ago by Manfred E. Bayer (205, 1). To date, the two models are still under debate.

LPS plays a critical role in the barrier function of the OM. It is a glucosamine disaccharide with six or seven acyl chains, a polysaccharide core, and an extended

polysaccharide chain that is called the O-antigen (207). The nonfluid continuum formed by the LPS molecules is a very effective barrier for hydrophobic molecules. This coupled with the fact that the porins limit diffusion of hydrophilic molecules larger than 600 Daltons, make the OM a very effective yet, selective permeability barrier (208 and see 3. Porin channels).

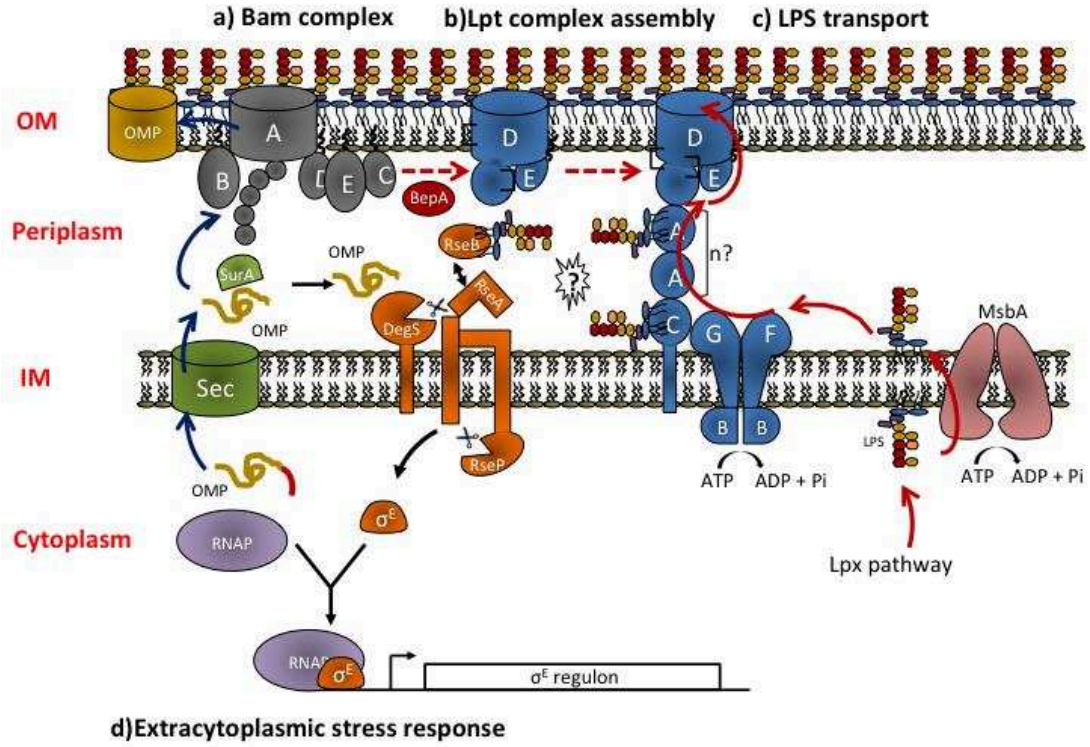

Figure 17: Overview of OMPs and LPS biogenesis pathways and extracytoplasmic stress response. (a) OMPs are synthesized in the cytoplasm and translocated across the inner membrane by the Sec translocon. After translocation, the signal sequence (indicated in red) is cleaved. In the periplasm, chaperone proteins such as SurA assist OMPs folding and deliver them to the BamABCDE complex for assembly at the OM. Blue arrows show the sequence of events occurring during OMPs biogenesis. (b) LptD is an OMP and follows the chaperone/Bam folding pathway. Correct LptD folding requires its association with the lipoprotein LptE and interdomain disulfide bridges isomerization. LptD-LptE interaction at the Bam complex is favored by the chaperone/protease BepA. Red dashed arrows show the sequence of events occurring during LptDE complex assembly. (c) LptDE complex is the LPS OM translocon. LPS is synthesized in the cytoplasm by the Lpx pathway, flipped to the periplasmic face of the IM by MsbA and transported through the periplasm to the outer leaflet of the OM by the Lpt machinery.

Continuous red arrows show the sequence of events occurring during LPS biogenesis. (d) Mislocalized or misfolded OMPs and defects in the LPS export pathway trigger the σ^E envelope stress response (black arrows). Misassembled porins bind to DegS, cleaving RseA and initiating the σ^E stress response. The current model suggests that defects in Lpt assembly/function result in RseB binding to LPS possibly released by the Lpt machinery. RseB bound to LPS frees RseA that can then be cleaved by OMP-activated DegS and by RseP thus activating the σE stress response (Figure acquired from Alessi & Sperandeo, 2017).

With few exceptions, the proteins of the OM can be divided into two classes, lipoproteins and β-barrel proteins. Lipoproteins contain lipid moieties that are attached to an N-terminal cysteine residue (209). It is generally thought that these lipid moieties embed lipoproteins in the inner leaflet of the OM. There are about 100 OM lipoproteins in *E. coli*, and the functions of most of these are not known (210). As it is the case for all extracytoplasmic proteins, lipoproteins are made in the cytoplasm with an amino-terminal signal sequence, and are translocated across the inner membrane by the Sec machinery (see 2.3 The inner membrane). However, the signal sequence is removed by a different signal peptidase, i.e. signal peptidase II also called LspA (211). Signal sequence processing of lipoproteins requires the formation of a thioether diglyceride at a cysteine residue, which will become the N-terminus of the mature lipoprotein. This step occurs at the periplasmic side of the inner membrane and is catalyzed by the diacylglyceryl transferase called Lgt (212). Once the signal sequence is removed, an additional fatty acyl chain is added to the cysteine amino group by the action of the apolipoprotein N-acyl transferase called Lnt (213, 214). These lipid moieties tether the newly formed lipoprotein to the outer leaflet of the inner membrane. Some lipoproteins remain in the inner membrane, and their biogenesis is complete after signal sequence processing and lipid addition. However, most of the lipoproteins *in E. coli* are destined for the OM. Inner membrane lipoproteins have a "Lol avoidance" signal, which is the presence of an aspartate residue at position two of the mature lipoprotein (215). The Lol system, comprising five proteins, catalyzes the localization of lipoproteins to the OM of *E. coli* (216). Briefly, an ATP-binding cassette (ABC) transporter, LolCDE, releases OM-specific lipoproteins from the inner membrane, causing the formation of a complex between the released lipoproteins and the periplasmic molecular chaperone LolA. When this complex interacts with OM receptor LolB, the lipoproteins are transferred from LolA to LolB and then localized to the OM (**Figure 18**).

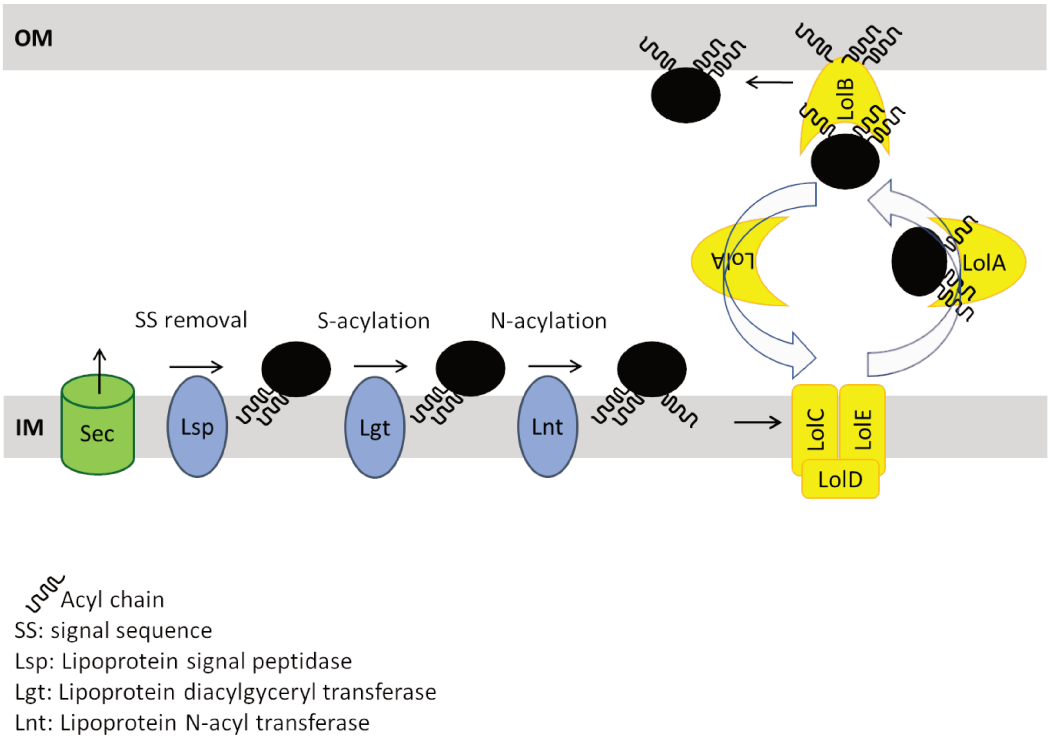

Figure 18: Lipoprotein maturation and export pathway. Lipoprotein (black) is synthesized in the cytoplasm with the N-terminal SS which targets it for translocation across the inner membrane by the Sec translocon. The lipoprotein remains anchored in the inner membrane by its SS and Lgt adds a diacylglyceryl moiety to the Cys residue. LspA cleaves the SS and Lnt adds another acyl chain to the newly formed N-terminus. The lipoprotein is then recognized by inner membrane LolCDE complex which powers extraction of the lipoprotein from the inner membrane using the energy of ATP. The lipoprotein is released to the periplasm in a complex with the chaperone LolA. LolA delivers lipoprotein to the OM acceptor lipoprotein Lol, which inserts it in the inner leaflet of the OM. Empty LolA returns to LolCDE and is recycled.

Nearly all of the integral, transmembrane proteins of the OM assume a β-barrel conformation. These proteins are β-sheets that are wrapped into cylinders, and I will refer to these OM proteins as OMPs (217-219). Not surprisingly, some of these OMPs, such as the classical porins OmpF, and OmpC, function to allow the passive diffusion of small molecules such as mono- and disaccharides and amino acids across the OM. These porins have 16 transmembrane β-strands. They exist as trimers (220, 221) and are abundant, representing approximately 250,000 copies/cell. Other trimeric OMPs such as LamB (18 transmembrane β-strands) and PhoE (16 transmembrane β-strands) function in the diffusion of specific small molecules such as maltose, maltodextrins and phosphate anions respectively (222, 223) and are induced under maltose or phosphate starvation, respectively. OmpA is another abundant OMP. It is monomeric, and it is unusual in that it can exist in two different conformations (222). OmpA has only eight transmembrane β-strands, but also contains a large periplasmic domain that interacts

with the peptidoglycan, thereby performing a structural role. TonB-dependent transporters (TBDTs) are bacterial OM proteins that bind and transport ferric chelates called siderophores (*e.g. E. coli* FepA, FecA, FhuA and CirA transport enterobactin, citrate, ferrichrome and catecholates, respectively) as well as vitamin B12 (*e.g. E. coli* BtuB), hemophores (*e.g. Serracia marcescens* HasR), nickel complexes, and carbohydrates. TBDTs share a common architecture with a large β-barrel made of 22 β-strands that spans the OM, large extracellular loops that function in ligand binding. In standard growth conditions the N-terminal plug domain that gates the central pore, while in the presence of iron deficiency, substrate binding induce large conformational changes that eventually lead to the periplasmic release of the TonB-box in the periplasm, which necessary for energy transduction in the form of protonmotive force from the IM complex ExbB-ExbD-TonB (225-227). TBDTs are present at a copy number of 200–400 molecules/cell but subjected multiple levels of regulation (227). Certain enzymes are also present in the OM. Notably, phospholipase (PldA), protease (OmpT) and LPS modifying enzyme (PagP) (228-230). The active site of most of these enzymes is located in the outer leaflet, or it faces the exterior of the cell (OmpT). TolC is a minor but functionally important OMP in *E. coli*. It is a multifunctional protein that is involved in secretion of toxins and efflux of a wide range of xenobiotics including antibiotics, biocides, bile salts and organic solvents (231). Mutants lacking any of these enzymes exhibit no striking phenotypes. The only known function of the OM is to serve as a protective barrier, and it is not immediately obvious why this organelle is essential. But what a barrier it is! *Salmonella*, another enteric bacterium, can live at the site of bile salt production in the gall bladder (232), and it is generally true that Gram-negative bacteria are more resistant to antibiotics than are their Gram-positive cousins. Indeed, some Gram-negative bacteria, such as *Pseudomonas*, are notorious in this regard. OMP biogenesis will be developed in subsequent sections as it occurs in different steps including the translocation across the inner membrane *via* the Sec or Tat machineries for (see 2.3 The inner membrane); and a ride from periplasmic chaperones to membrane insertion with the β-barrel protein assembly machinery (BAM) (see 3.1.3 Porin biogenesis).

2.2. The periplasmic space

The outer and inner membranes delimit an aqueous cellular compartment called the periplasm. The periplasm is densely packed with proteins and it is more viscous than the cytoplasm (233). Proteins that inhabit this compartment include the periplasmic binding proteins, which function in sugar and amino acid transport and chemotaxis, as well as chaperone-like molecules that perform quality control and shuttle functions for the Lpt (234), the Bam (235, 236, 237, 237) and the Lol (216) machineries, dedicated to LPS, OMPs and lipoproteins biogenesis. It also contains the peptidoglycan, peptidoglycan-synthases and hydrolases, which are tightly coordinated with elements of the bacterial cytoskeleton including the elongasome and the divisome (238).

Noteworthy, the peptidoglycan interacts with other structural proteins such as Lpp, called murein lipoprotein or Braun's lipoprotein (239, 240) and OmpA as well as proteins of the Tol-Pal system (241) for bridging the peptidoglycan to the outer and inner membranes, respectively. Noteworthy, Lpp is the most abundant protein in *E. coli*, with more than 500,000 molecules/cell. The ε-amino group of the carboxyterminal lysine residue of one third of these molecules is covalently attached to the diaminopimelate residue in the peptide crossbridge. Finally, the periplasm contains part of trans-envelope machines such as drug efflux and protein secretion systems, which transport substrates without periplasmic intermediates (178), and some surface appendages, such as flagella, which are required for bacterial mobility (178).

2.3. The inner membrane

The inner membrane separates the periplasm from the cytosol. Also called the plasma or cytoplasmic membrane, the inner membrane is composed of a symmetrical phospholipid bilayer. In *E. coli*, it consists of 70–80% phosphatidylethanolamine, 15–20% phosphatidylglycerol and a small fraction of cardiolipin (244, 245). Inner membrane proteins mostly function in energy production, lipid biosynthesis, molecular transport, including small molecule import and extrusion, protein secretion, extracytoplasmic protein and LPS biogenesis (246).

In this section, I will develop the inner membrane steps of extracytoplasmic protein biogenesis that involve either the Sec or the Tat machinery. All proteins are synthesized in the cytoplasm, but proteins destined for the periplasm or the OM is made initially in a precursor form with a signal sequence at the amino terminus. The signal sequence targets them for translocation from the cytoplasm (242). This translocation reaction is catalyzed by an essential, heterotrimeric inner membrane protein complex called SecYEG (248). The signal sequence and this heterotrimeric membrane protein complex are conserved throughout biology (249). The essential ATPase SecA, together with the proton motive force, drives this translocation reaction (250). Periplasmic and OM proteins are generally translocated in posttranslational fashion (i.e. synthesis and translocation are not coupled). Proteins must be secreted in linear fashion from the amino to the carboxy terminus like spaghetti through a hole as SecYEG cannot handle folded molecules. The cytoplasmic SecB chaperone maintains these secreted proteins in unfolded form until they can be secreted (251). During the secretion process the signal sequence is proteolytically removed by Signal Peptidase I called LepB (211). Other components of the Sec translocon, such as SecD, SecF, and YajC, perform important but nonessential function(s) during translocation, perhaps facilitating release of secreted proteins into the periplasm. Once released, periplasmic proteins are "holded" by chaperones function to prevent misfolding and aggregation before final OM insertion and assembly (see 3.1.3. Porin biogenesis). The other translocation system in the inner membrane is called Tat and translocates folded proteins (252). *E. coli* uses the Tat system for proteins which have prosthetic groups that must be added in the cytoplasm,

and this constitutes a small fraction of the secreted proteins. Other bacteria, such as thermophiles, use the Tat system extensively; presumably because it is easier to fold proteins in the cytoplasm than it is in the hostile environments they live in. In terms of components, the Tat system is remarkable simple; three components. TatB and TatC function to target proteins for translocation by TatA, but how this system recognizes that the substrate is folded, and how it accomplishes the translocation reaction are not yet understood.

3. Porin channels

3.1. Functional and structural properties of classical porins

3.1.1. Structure

As mentioned earlier, classical or general diffusion porins OmpF, OmpC and PhoE of *E. coli* are trimers of 16-stranded β-barrels (220, 221) (**Figure 19**). The large number and configuration of the β-strands allow for the formation of a central hydrophilic pore in each β-barrel. However, the pore is constricted by the inwardly folded extracellular loop L3 (shown in orange in **Figure 19**). This loop, together with the opposite barrel wall, form the so-called eyelet or constriction zone, which determines the size exclusion limit and other permeation properties of the barrel. At this level, the pore size of OmpF is 7 × 10 Å (220). A conserved set of charged residues decorates the eyelet: negatively charged residues (in red in **Figure 19**) are found on the L3 loop itself, and positive charges (in blue in **Figure 19**) form a cluster on the opposite barrel wall, creating a strong transversal electric field. These residues have been shown to play an important role in ion movement and ion selectivity. The β-strands are connected to each other by short turns on the periplasmic side and long loops on the extracellular side. This protruding extracellular domain provides a site for interactions with specific colicins and phages that use porins as surface receptors (208).

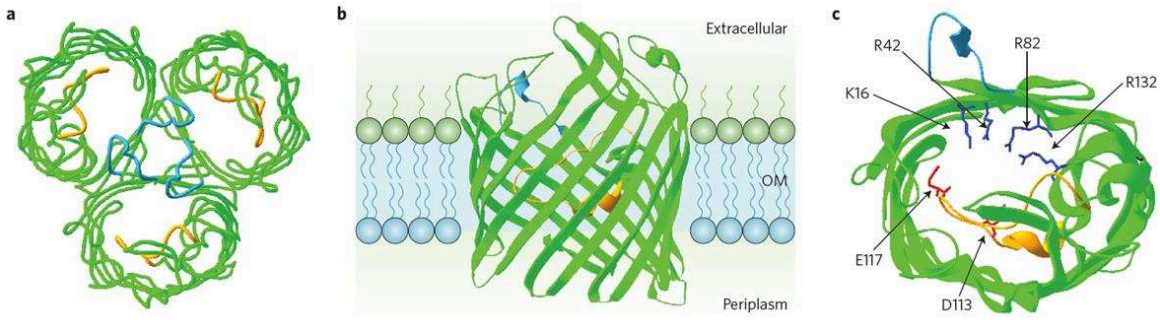

Figure 19: Structure of the OmpF porin of *E. coli*: a) View of the trimer from the top, perpendicular to the plane of the OM. Loop 2 (L2; blue) plays a role in interacting with the neighboring monomer. L3 (yellow) narrows the channel. b) View of the monomer

from the side. L2 and L3 are colored as in a. c) View of the monomer from the top, showing the constricted region of the channel, which is formed by E117 and D113 from the L3 loop, as well as four basic residues from the opposing barrel wall, K16, R42, R82 and R132 (figure acquired from Masi *et al.*, 2017).

3.1.2. Pore properties and permeation assays

The functional properties of porins have been the subject of investigation for over 30 years. Initial work established the size exclusion cutoffs of porins by measuring the transport of various size sugars using liposome swelling assays (252). A value of about 600 Daltons was determined for OmpF (254), which implies that ions, amino acids, and small sugars use general diffusion porins for gaining access to the periplasm. Disaccharides, larger sugars and other molecules need to use dedicated pathways for OM transport (208). These early studies established the molecular sieving properties of porins, and provided an explanation for the high diffusion rates of these compounds through the OM (255). The application of electrophysiology to the study of porins, along with computational studies, has permitted a better understanding of porin permeation at the molecular level (see 4.1. Porin mediated uptake across the outer membrane).

The traditional electrophysiological approach is the study of porin-mediated ion currents in planar lipid bilayers (also known as "black lipid membranes" or "BLM"). A lipid bilayer is formed over an aperture pierced through a Teflon film separating two chambers. Each chamber contains a buffered ionic solution and an electrode used to measure electric current due to the flow of ions across the bilayer and to clamp the transmembrane potential required to promote ion movement. Purified detergent-solubilized channel proteins or proteoliposomes are added to one chamber (the so-called *cis* side), and spontaneously insert in the bilayer over time. The sequential insertions of open channels in the membrane lead to discrete current jumps due to ion movement through the open channels. The conductance (i.e. the amount of current per unit voltage) of a channel can be obtained from measuring the size of these current jumps. In the case of porins, this would represent the trimeric conductance, since porins typically purify and insert in the bilayer as trimers. By manipulating the protein concentration, it is possible to ensure that either many or only one porin trimer inserts, and investigations can be performed on single channels or on populations of channels. After insertion, the channel activity can be studied in various conditions (*e.g.* in the absence or in the presence of putative permeating molecules) and membrane potentials.

The patch-clamp technique has also been applied to the study of purified porins reconstituted in artificial liposomes. Here, a small patch of liposome membrane is drawn at the tip of a 1 μM-diameter glass pipette, and the current flowing through this patch is recorded at a fixed membrane potential. Because of the small area of membrane under investigation, the patch clamp technique typically offers a better signal-to-noise ratio than BLM. This technique permitted the discovery that porins flicker between multiple states, whose kinetics and conductance can be affected in mutants and in the presence of

modulators. Studies performed by the Benz and the Rosenbusch groups in the 70's and 80's established some of the hallmark properties of the general diffusion porins, such as high ionic current due to the relatively large pore size, low ionic selectivity (although some porins show preference for cations (OmpC) or anions (PhoE)), and high open probability, in standard bilayer electrophysiology conditions of low voltage, neutral pH and high ionic strength (256-259). Computational modeling studies have suggested that the paths taken by anions and cations are divergent at the eyelet, as cations are drawn close to the negative charges of the L3 loop, and anions flow near the positively charged cluster of the opposite barrel wall (260). This type of work emphasizes the notion that the permeating ions interact with the wall of the channel and that ion movement does not follow simple diffusion. This was demonstrated experimentally by measuring the conductance and selectivity of various general diffusion porins in solutions of varying ionic strength or pH, and in variants with mutations at specific pore exposed residues (261-264). Bezrukov's group showed that the selectivity of OmpF for cations relative to anions increases sharply in solutions of low ionic strength (261). The channel reaches nearly ideal cation selectivity in solutions of < 100 mM KCl. Furthermore, at pH's < 4, the channel reverses its selectivity from preferring cations to preferring anions. The authors combined these experimental observations with calculations of the distribution of charged residues in the pore lumen and concluded that electrostatic interactions exist between the permeating ions and the charges of ionizable residues over the entire channel length. However, shifts in selectivity are detected upon mutations of single residues. Substitution at the pore-exposed D113 residue in OmpF (262) and its homolog in OmpC (264) decrease cation-selectivity. Opposite effects are seen upon charge removal at arginines of the constriction zone (264).

Some of these techniques as well as their applications for studying translocation of antibiotics are shown below (**Figure 20**).

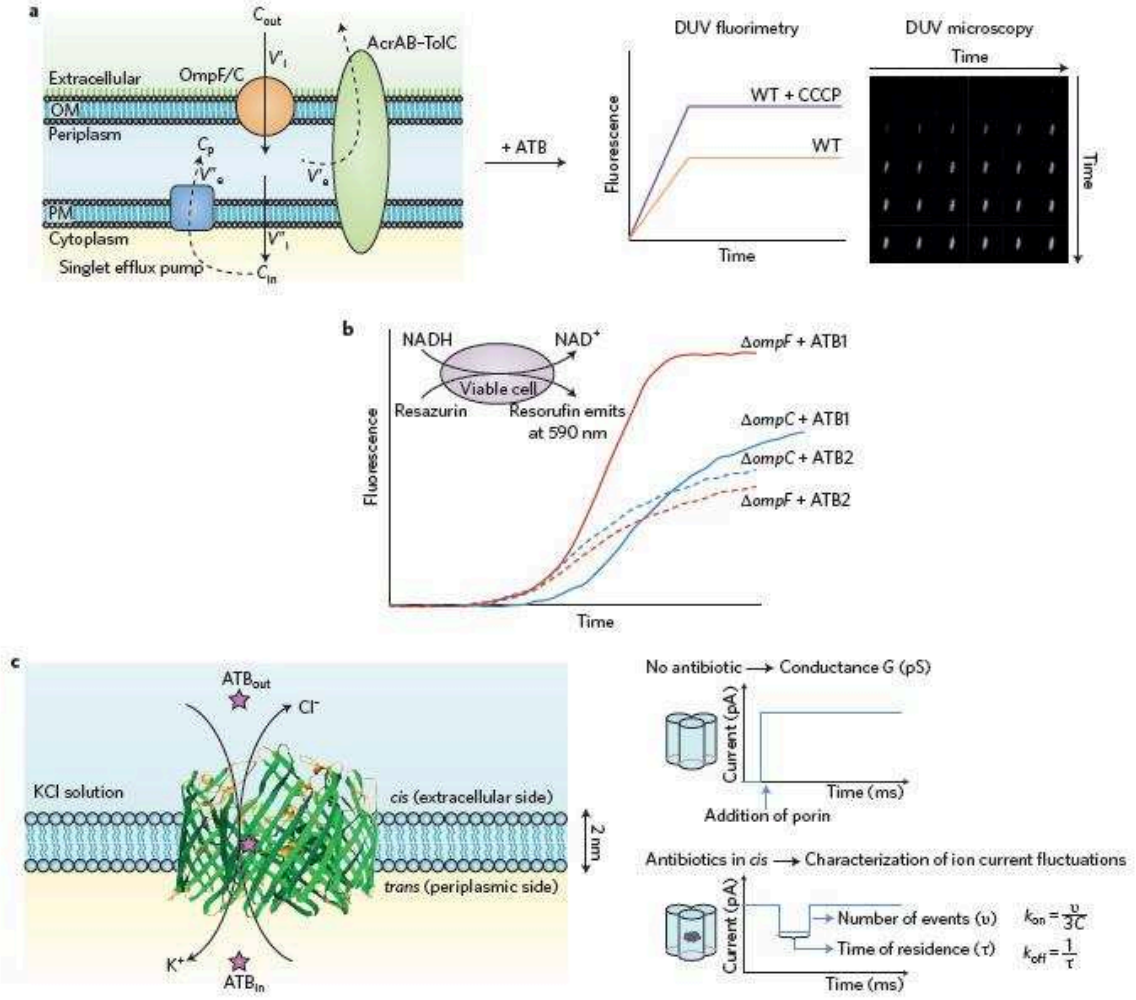

Figure 20: Methods for measuring intracellular accumulation and porin-mediated uptake of antibiotics. (a) DUV methods. Deep Ultraviolet (DUV) methods were developed for studying translocation of fluoroquinolones, which are intrinsically fluorescent, across the envelope of Gram-negative bacteria. Fluoroquinolone accumulation can be investigated in whole cells after cell lysis, or in intact cells by DUV microspectrofluorimetry or DUV fluorescent imaging (265). Fluoroquinolone (ATB, antibiotic) is added to intact cells at a concentration of C_{out}. The drug crosses the two membranes at the net rate V_i, which is a combination of V'_i and V''_i corresponding to the influx rates across OM through porins and IM, respectively. This influx is also balanced by two processes: (1) efflux into the external medium through the major trans-membrane AcrAB–TolC pump at the rate V'_e; and (2) efflux into the periplasm by several minor inner membrane 'singlet' pumps at the rate V''_e. In this model, C_{in} can be measured from the maximum fluorescence intensity. Using mutants and addition of CCCP (cyanide m-chlorophenyl hydrazone, an inhibitor of proton transport), the contribution of efflux by *acrB* or *tolC* has been determined (266, 267, 268). Similar approaches can be used to evaluate the role of specific OM porins on fluoroquinolone influx, assuming that drug diffusion across the inner membrane is not a limiting factor to reach maximal C_{in}. These approaches have shown that the drug resistance characteristics of *E. aerogenes* clinical

isolates probably rely on efflux, as CCCP treatment restores drug accumulation to susceptible strains. *C*p represents the periplasmic concentration. (b) Resazurin-based bacterial viability assay. Actively metabolizing bacterial cells reduce the viability dye resazurin to resofurin, which emits fluorescence at 590 nm. Kinetic assays provide a population-based readout for drug accumulation, which can be used to dissect the impact of different chemical structures and bacterial factors on net influx. In the example below, the use of this assay with different porin mutants can help differentiate their preference for different antibiotics (ATB1, ATB2). (c) Electrophysiology. The planar lipid bilayer technique is one of the electrophysiological approaches used to study the biophysical properties of bacterial pore-forming proteins. In an asymmetrical setup, a single porin is inserted in an artificial membrane which is added to the *cis* side. Addition of porins will produce conductance, while addition of antibiotics will produce ion current fluctuations that can be used to calculate drug on and off rates. Note that these calculations do not reflect specificity of transport (picture taken from Masi *et al*., 2017).

3.1.3. Porin biogenesis

As described earlier, nascent OMPs are first synthesized in the cytoplasm with an N-terminal signal sequence, which targets them to the Sec translocon for transport across the inner membrane to the periplasm (269).

Periplasmic chaperones function to protect OMPs during their transit through the periplasm. Three such proteins have been well characterized and shown have general chaperone activity: SurA, which also functions as a peptidyl-proline isomerase (270271), Skp (273, 274), and DegP (275, 276). Genetic analysis indicates that these three proteins function in parallel pathways for OMP assembly; SurA functions in one pathway; DegP/Skp function in the other. Mutants lacking either one of these pathways are viable, but cells cannot tolerate loss of both (277). Mutants lacking SurA and Skp, or SurA and DegP are not viable and they show massive defects in OMP assembly. Therefore, these chaperone pathways seem redundant. However, this redundancy does not reflect equal roles in OMP assembly. The major OMPs, which account for most of the protein mass of the OM, show preference for the SurA pathway (278). At present no OMP that prefers the DegP/Skp pathway has been identified. However, the primary role of the DegP/Skp pathway may be to rescue OMPs that have fallen off the normal assembly pathway, particularly under stressful conditions. The periplasmic chaperones deliver OMPs to a multicomponent complex called the β-barrel assembly machinery (BAM) complex, which has been shown to be responsible for folding and inserting OMPs into the OM (279, 280) (**Figure 21**). In *E. coli*, the BAM complex consists of five components called BamA (aka YaeT/Omp85), BamB (YfgL), BamC (NlpB), BamD (YfiO), and BamE (SmpA) (281-284). BamA, a 16-stranded OMP itself, is the central and essential component of the complex; BamB, BamC, BamD, and BamE are all lipoproteins which are anchored to the OM via lipidation of the N-terminal cysteine residue. BamA and BamD are essential

for viability; however, all components are required for efficient OMP folding/insertion (282, 1). Studies have shown that both BamB and BamD interact directly with BamA via non-overlapping binding sites while BamC and BamE interact directly with BamD to stabilize the complex (282, 283). Structures of all the Bam components have now been reported including partial complexes of BamAB and BamCD (284-295). The full-length structure of BamA from *Neisseria gonorrhoeae* revealed a large periplasmic domain consisting of five polypeptide transport associated (POTRA) domains and a C-terminal 16-stranded β-barrel domain. Subsequent studies showed that lateral opening of the barrel domain was required for function in BamA, strengthening an existing hypothesis that the barrel domain must open laterally in the membrane to allow insertion of the substrate OMPs into the OM (289, 291, 296, 297). It has been proposed that BamB might serve as a scaffold, assisting in the handoff of nascent OMPs by SurA/Skp to BamA, while BamC, BamD, and BamE may serve support roles in regulating the function of BamA (287, 290, 298). The structures have offered clues to how each component may function within the complex; however, the lack of structural information regarding the fully assembled complex has hindered progress towards exploring the mechanism further. To address this, Backelar *et al.* have solved the structure of the BAM complex from *E. coli* and showed that the periplasmic domain of BamA in a closed state that prevents access to the barrel lumen from the periplasm. Furthermore, binding of BamCDE to BamA causes an unprecedented conformational change, leading to opening of the top of the barrel domain along the exit pore and structural rearrangement of the lateral opening site. These structural changes suggest that the role of BamCDE may be to modulate the conformational states of BamA, thereby serving as a regulatory step in the function of the BAM complex (299).

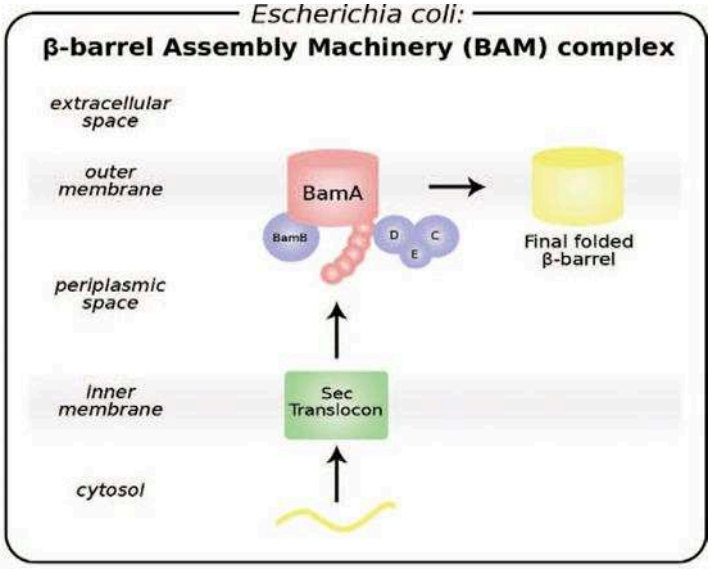

Figure 21 : The *E. coli* BAM complex and homologous systems. In Gram-negative bacteria, OM β-barrel proteins are first synthesized in the cytosol of the cell and then targeted to the IM. This diagram compares the three pathways as the unfolded substrate protein (yellow curve) is directed by associated translocons (green) to the assembly complex consisting of the core BamA homologue (pink) and accessory proteins (purple), to form the final folded β-barrel (yellow cylinder). For simplicity, other proteins and chaperones involved in the pathways are not shown. A: The *E. coli* β-barrel assembly machinery (BAM) complex consists of membrane embedded BamA, and four accessory lipoproteins: BamB, C, D, and E. Substrate proteins cross the inner membrane via the Sec translocon and travel through the periplasmic space before being assembled by the BAM complex at the OM (picture acquired from Kim *et. al.* 2012).

3.1.4. Regulation of porin expression

The regulation of expression of classical porins in *E. coli* is here summarized and will be further integrated in pathways for remodeling the OM (section 5). PhoE is expressed only under phosphate starvation, since the *phoE* gene is a member of the phosphate regulon (301). The expression of the two major porins, OmpF and OmpC, is exquisitely regulated. The apparent purpose of this regulation became clear when it was discovered that OmpF produces a slightly larger channel than OmpC (302, 179). Thus, noxious agents such as antibiotics and bile acids diffuse far better through the larger OmpF channel, as seen clearly from the observation that low concentrations of antibiotics select for *ompF* mutants but never for *ompC* mutants (303). In its natural habitat, the intestinal tract, *E. coli* encounters 4 to 16 mM bile salts (304), and it is most important to minimize their influx. The conditions prevailing in the intestinal tract, high osmotic strength and high temperature, both favor the production of OmpC (with its narrower channel) and repress the production of OmpF. On the other hand, the increased production of OmpF under low-temperature, low-osmolarity conditions (for example, in lake water) will benefit *E. coli* by facilitating the influx of scarce nutrients. Although it is now admitted that the electronegativity of the pore interior rather than the pore size determines the permeability of OmpF and OmpC channels (221, 305, 306), this model is still valid. The molecular mechanism of this OmpF/C regulation has been studied extensively (307). Environmental osmotic activity is sensed by the sensor component EnvZ of the archetypal two-component system, EnvZ-OmpR, and high osmolarity results in the phosphorylation of OmpR. The *ompF* gene, with its high affinity OmpR-binding sites, is transcribed even when the phosphorylated OmpR (OmpR~P) is scarce. However, as the concentration of OmpR~P increases, additional binding of these molecules results in increased transcription of *ompC* and repression of *ompF.* High temperature, on the other hand, increases the transcription of an antisense RNA, *micF* (308, 309). This RNA binds to the 5' untranslated region (UTR) of the *ompF* mRNA and inhibits its translation (310). Finally, oxidative stress and the presence of salicylate also

increase *micF* transcription and prevent the production of OmpF porin post-transcriptionally. The intestinal tract, the normal environment of *E. coli*, is thought to be mostly anaerobic. Interestingly, anaerobiosis was found to modify the osmoregulation of OmpF and OmpC (311). Thus, under anaerobiosis, OmpC is expressed at a rather high level even in fairly low-osmolarity media, and the repression of OmpF by osmotic activity occurs more strongly than under aerobic conditions. This modification of the regulatory response, which is expected to favor the survival of *E. coli* in the intestinal tract, occurs through the cross-talk activation of OmpR by the ArcB sensor, which senses the anaerobic condition (311).

Regulation of porin expression also occurs in response to the presence of chemicals in the environment. It was found in 1991 that salicylate in the medium decreased OmpF synthesis (312). This is now known to be a part of the global regulation of porins mediated by three XylS-AraC family regulatory proteins, MarA, SoxS, and Rob (313, 314). Thus, the increased production of MarA (caused by some environmental chemicals, such as salicylate, inactivating its cognate repressor, MarR) or SoxS (caused by the inactivation of its repressor SoxR via its oxidation) or the binding of coregulators such as dipyridyl (315) or some bile salts (316) to Rob activates the transcription of *micF*, thereby decreasing OmpF synthesis. Interestingly, all these environmental signals also result in the increased production of the main multidrug efflux pump, AcrAB. Together, these responses prevent the entry of toxic molecules through porins, a reasonable response for *E. coli.* The benefit is clear from the observation that resistance to several antibiotics is moderately increased in the presence of bile salts (316), a normal component of the environment of *E. coli.*

3.2. Other porins

3.2.1. Other porins in *E. coli* and *Salmonella spp.*

The genome of *E. coli* K12 encodes several general diffusion porins other than OmpF and OmpC. PhoE is another major OMP that is often included with OmpF and OmpC as classical or general diffusion porins but transports inorganic phosphate and is selective for anions (223). The NmpC porin, which belongs to the OmpF-OmpC-PhoE cluster and which is not expressed in K-12 because of the insertion of the IS *5* element close to the distal end of the gene, is similar to the lc porin coded by the genome of a lambdoid phage, PA-2 (317). The transcription of *nmpC* is apparently up-regulated by growth in a slightly alkaline media (318). A survey of the K-12 genome using a program detecting β-barrel proteins indicated that the product of gene *b1377* is a homolog of OmpC that belongs to the classical porin family (319). This trimeric porin, called OmpN, was originally found in *E. coli* B, then in K-12, and was expressed and purified (320). Its channel property was also reported to be very similar to that of OmpC. This protein is

not expressed wild-type strains; only studies have reported the effect of environmental conditions on the production of this porin (321, 322). Mutants expressing the OmpG porin were isolated in *E. coli* K-12 by Misra and Benson (323) by using a selection procedure that favors mutant cells capable of taking up large nutrients. OmpG is a porin with unusual properties (324). First, it appears to lack, on the basis of its sequence, the large loop 3 that is ubiquitous in classical trimeric porins. Second, it produces an unusually large channel, as expected from its 3D-structure. Third, it appears to exist as a monomer, unlike members of the classical porin family. The large channel size and the monomeric nature of OmpG were confirmed by single-channel conductance and folding studies in another laboratory (325, 326). The protein was made into a two-dimensional crystal, and its study also confirms the monomeric nature of this porin (327). The *ompG* gene appears to be the last gene in a putative 11-gene operon, which contains genes needed for the ATP-binding cassette (ABC) transporter-catalyzed uptake of oligosaccharides, as well as various genes presumably involved in the degradation of such compounds (324). Therefore, it seems likely that it is a large-channel porin needed for the uptake of larger oligosaccharides. OmpG is expressed, at a low level, in *Salmonella* and *Shigella*, but only trace levels are seen in wild-type *E. coli* K-12. *E. coli* OmpW is a receptor for Colicin S4 (328). It is part of a family of proteins by the same name. It shows high similarity to the OmpW protein in *Salmonella typhimurium*. The crystal structure of OmpW has been determined to resolutions of 3.5 Å (329) and 2.7 Å, revealing an 8-stranded β-barrel structure with a narrow hydrophobic channel (330). This structure suggests OmpW functions in transport of small, hydrophobic molecules across the OM (330). *In vitro* studies have investigated the strategies used by a number of OMPs, including OmpW, to efficiently fold into the membrane (331). Expression of OmpW is upregulated in response to tetracycline and ampicillin (332) but downregulated upon induction of the small noncoding RNA, RybB, which is a member of the σ^E regulon (333). Xiao et al. also showed that the maximal expression of *ompW* is during the aerobic-to-anaerobic transition, and that seems to be related to cell survival under microaerobic conditions. In these conditions, the repression of *ompW* is mediated by the global anaerobic transcription factor FNR (334). Mutational experiments and gene expression analysis suggest that FNR is an antagonist of the *ompW* repression mediated by the histone-like nucleoid structuring protein H-NS under aerobic conditions (334).

Like *E. coli* K12, *Salmonella enterica* serovars Typhy and Typhimurium produce OmpF and OmpC as abundant proteins in their OMs. In *E. coli* and serovar Typhimurium OmpF and OmpC porins are regulated reciproqually by the osmolarity of the growth medium. The *ompD* gene, which is absent in the genome of serovar Typhi, encodes the most abundant protein of the OM of serovar Typhimurium, a porin similar in primary amino acid sequence to major porins OmpF, OmpC and PhoE (335, 336). It has channel properties comparable to those of OmpF-OmpC as judged from single-channel conductance data (337). Liposome-swelling studies apparently have not been carried out with this porin. The production pattern of this porin on complex agar media and in

synthetic liquid media seems to fit with the idea that OmpD is synthesized in a cyclic AMP-dependent manner (336). Above all, *Salmonella* uses many small regulatory RNAs in order to fine-tune the production of OmpD at the post-transcriptional level (see 5.4. sRNA regulation) (339, 340). Besides, serovars Typhi and Typhimurium also encode porins OmpS1 and OmpS2, which are expressed at low levels under in vitro culture conditions. In particular, these two porins are potent protective immunogens with adjuvant properties (341).

3.2.2. Porins in other *Enterobacteriaceae*

General diffusion porins are well conserved among *Enterobacteriaceae*. Clinically relevant species such as *E. aerogenes*, *E. cloacae* and *K. pneumoniae* also produce homologs of OmpF and OmpC — namely, Omp35 and Omp36; OmpE35 and OmpE36; OmpK35 and OmpK36. Of note, an especially large fraction of clinical isolates of *E. aerogenes* were found to have alterations in porins (342, 343, 344); together with the induction of the chromosomal cephalosporinase, this might explain high level of resistance towards β-lactams in this species. *K. pneumoniae* is unusual among the *Enterobacteriaceae* in lacking the ability to produce a chromosomally encoded, inducible class C β-lactamase and instead produces a chromosomally encoded, weak class A enzyme (345). This situation suggests that the loss of porin might play a larger role in the resistance of *K. pneumoniae* to β-lactams. As mentioned above, the crystal structure of all the OmpF and OmpC orthologs are now known (306, 346). Biochemical studies including liposome swelling assays showed pore sizes similar to that of *E. coli* porins and a preference for the more hydrophilic members among cephalosporins (306)

Bacteria	Characterized porins	3D structure of porins	Porin alteration in clinical isolates
E. aerogenes ***E. cloacae***	Omp36*, Omp35‡	Omp36, Omp35, OmpE36, OmpE35	Omp35§‖, Omp36§‖
E. coli	OmpC, OmpF, OmpN¶, PhoE	OmpC, OmpF, PhoE	OmpC§‖, OmpF§‖,
K. pneumoniae	OmpK36*, OmpK35‡, OmpK37¶	OmpK36, OmpK35	OmpK35§, OmpK36§, OmpK37§
S. enterica **serovars** ***Typhimurium*** **and** ***Enteritidis***	OmpC*, OmpF‡, OmpD, OmpS2¶	None	Major porins (OmpC§, OmpF§, OmpD§)

Table 1: Main features of porins. *OmpC family. ‡OmpF family. §Identification of porin loss in resistant isolate. ||Identification of porin mutations in resistant isolate. ¶Quiescent porin family.

4. Antibiotic transport across the cell envelope of Gram-negative bacteria

4.1. Porin-mediated uptake across the outer membrane

The permeability of porins to β-lactam antibiotics has been demonstrated by various means. Evidence for a direct role of porins in mediating the diffusion of β-lactams was provided by purifying and reconstituting porins into liposomes and using either a liposome swelling assay (252), or measuring the antibiotic degradation rate by an entrapped β-lactamase (347). Measurement of antibiotic flux in whole cells was originally developed by Zimmermann and Rosselet (348) and then extensively used by Nikaido's group to characterize the permeability of cephaloridine and other cephalosporins in various cells types (wildtype and porin mutants), by taking advantage of the fast rate of cephalosporin degradation by periplasmic β-lactamase (252). Rates of the order of $\sim 10–50\times10^{-5}$ cm/s were found for the permeation of zwitterionic drugs through OmpF, but were much reduced for anionic compounds. A molecular explanation for these findings has recently emerged from a more detailed view of the interactions of the permeating drugs with the porin channels, obtained from the combination of electrophysiology and computational studies. Bezrukov and colleagues demonstrated that ampicillin acts as a transient open channel blocker of the OmpF porin in a pH dependent manner, with a maximum block in a pH range where the ampicillin molecule is zwitterionic (349). Molecular dynamics calculations explain this pH dependence, as they reveal that the drug molecule perfectly occludes the pore in the zwitterionic form, as it interacts simultaneously with negatively charged residues of L3 and positively charged residues of the barrel wall (**Figure 19**). Such complementation between the charge distributions on the drug molecule and the narrowest region of the OmpF pore has also been found for another zwitterionic β-lactam, amoxicillin (350). On the contrary, poor interactions were delineated for the di-anionic carbenicillin and the mono-anionic β-lactams azlocillin and piperacillin. This negligible binding correlates with the poor diffusion rates measured from such compounds from liposome swelling assays (351). On the other hand, high diffusion rates were obtained for ampicillin and amoxicillin. Thus, it appears that interactions at the OmpF constriction zone facilitate the drug translocation, and that the nature and position of specific charges on the antibiotic molecule and on OmpF play a major role in these interactions. Experimentally, site-directed mutations of many key charged residues of the porin constriction zone affect β-lactam flux and sensitivity (352-355). The involvement of specific OmpF residues as anchorage points for several cephalosporins has been suggested from computational studies as well (352). Some mutations also involved uncharged residues. For example, the diffusion of radiolabeled cefepime was drastically decreased in the

G119D and G119E mutants (356). The X-ray structure of the G119D mutant OmpF shows that the introduced aspartate residue protrudes in the eyelet and constricts the diameter the pore (357). Consequently, the channel conductance, diffusion rate of various sugars and sensitivity to cephalosporins are greatly reduced (356, 357). On the other hand, mutations at the R132 residues lead to improved growth on maltodextrins relative to wildtype (264) and increased cefepime diffusion (356), possibly due to an increase in pore diameter (358).

Quinolones are believed to use a dual pathway for entry into bacterial cells, because drug flux and susceptibility are both sensitive to the presence of porins (in particular of OmpF) and to modifications of the LPS barrier (359, 360). The relative contribution of the two pathways correlates with the hydrophobicity and the protonation state of the quinolones, in the manners described below. Hydrophobic quinolones are more effective in LPS mutants (361). There is a report that the quinolone fleroxacin induces the same perturbations of the OM as does gentamycin or EDTA, supporting the contention that quinolones might act as chelating agents and use a self-promoted pathway as aminoglycosides and cationic peptides do (359). However, the sensitivity of cells to less hydrophobic quinolones, such as norfloxacin and ciprofloxacin and other drugs with similar hydrophobicity coefficient of less than 0.1, was not much affected in mutants in LPS structure (360), suggesting that they might use porins for access through the OM. Indeed, a reduced accumulation of radiolabeled norfloxacin was observed in *E. coli* strains lacking OmpF (362). Moreover, the flux of norfloxacin in *E. cloacae* was inhibited in the presence of spermine or cefepime, both known to use porins for permeation through the OM, thus confirming that norfloxacin diffuses through the porin lumen (363). Nikaido and Thanassi have proposed that quinolones exist in equilibrium with charged and uncharged species depending on the solution pH (364). For example, they calculated that about 10% of norfloxacin exists as an uncharged species at pH 7.4, and this ratio is even higher (~ 40% at pH 6.5) for amifloxacin. These authors have argued that the uncharged quinolone molecules cross the OM through the lipid bilayer, while the negatively charged molecules are likely to pass through porin channels as magnesium chelates. Thus, the relative contributions of the porin-mediated and lipid-mediated pathways are likely to depend on the protonation-deprotonation states of the drug, which will themselves be influenced by external pH. In addition, the charged species are proposed to accumulate in the periplasm due to the interior-negative Donnan potential across the OM (364). This accumulation leads to high cytoplasmic levels as well, as the cytoplasm equilibrates very rapidly with the periplasm, even for drugs with oil/water partition coefficient less than 0.1. In porin-deficient mutants, quinolones still permeate through the OM bilayer itself in their uncharged form, but do not accumulate in the periplasm because they are not sensitive to the Donnan potential, thus leading to decreased cytoplasmic concentrations and efficacy.

The uptake of tetracycline by *E. coli* cells was shown to be reduced in a mutant lacking OmpF (365), confirming the suggestion that it uses this pathway based on increased resistance in mutants with decreased *ompF* expression (362). This accumulation, however, is not null in the absence of OmpF, and it positively correlates with pH, i.e. there is less influx of tetracycline at lower pH (pH 6.0) relative to neutral pH, or even 7.8 (365). Tetracycline has a pKa of 7.7, and therefore exists mostly in a protonated form at a pH's under the pKa. In this uncharged form, tetracycline is believed to enter cells by diffusion through the OM lipid barrier (364). Thus, tetracycline, like fluoroquinolones, uses both a porin- and a lipid-mediated pathway, depending on its protonated status.

4.2. Porins and antibiotic resistance

As described in the sections above, porins provide a favored path through the OM to small hydrophilic antibiotics, such as β-lactams, as well as tetracycline, chloramphenicol and fluoroquinolones (208). Any decrease in the ability or rate of entry of these compounds can lead to resistance. There is an abundance of reports of antibiotic resistance acquired through loss or functional changes of porins in a large number of *Enterobacteriaceae*, such as *E. coli*, *E. aerogenes* and *K. pneumoniae* (112, 208, 366-370). Although much of the mechanistic studies described above have focused on OmpF because of its well understood structural and functional properties relative to any other major porins, many of the reports of changes in porin expression often implicated both OmpF and OmpC. The role of minor porins (such as NmpC), or those expressed in specific conditions (such as PhoE), perhaps should not be underestimated, but there are far fewer reports on the involvement of these porins in antibiotic resistance. Still it appears that PhoE can serve as a conduit for entry of β-lactams (and be an even better one than OmpF and OmpC if the drug bears a negative charge) (302), as well as for chloramphenicol and tetracycline (371). It would be impractical in this manuscript to cite all or even most of studies linking antibiotic resistance to general diffusion porins, but we can highlight some of the generally found common themes with specific examples. There are two major porin-based mechanisms for antibiotic resistance that have been reported in clinical isolates: 1) alterations of OM profiles, including either loss/severe reduction of porins or replacement of one or two major porins by another (342, 344, 372, 373); 2) altered function due to specific mutations reducing permeability (374-376). This section provides selected examples from the literature that describe porin modifications and antibiotic resistance. These examples are discussed in light of recent structural and functional studies.

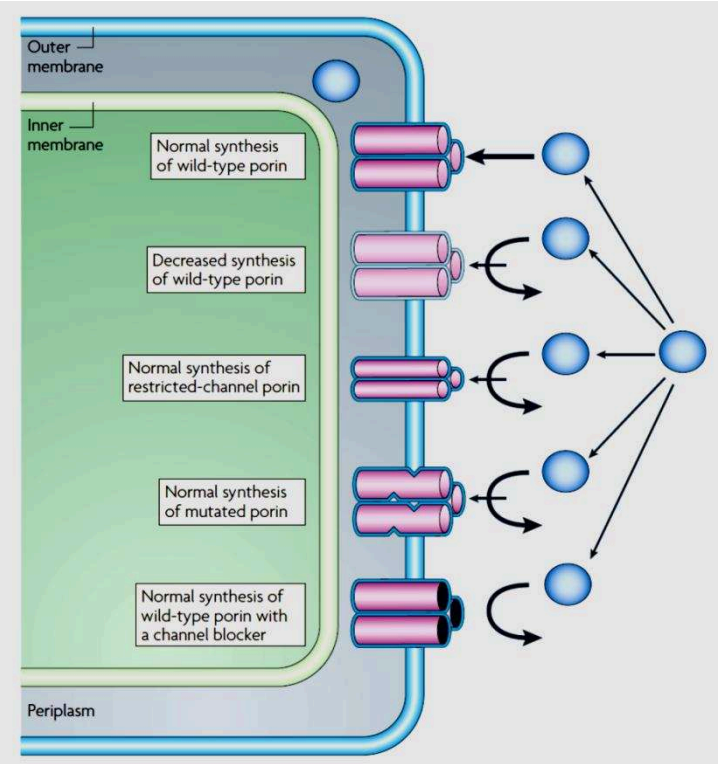

Figure 22: Multidrug resistance mechanisms associated with porin modification. This figure shows the various resistance mechanisms that are associated with porin modification. The β-lactam molecules and porin trimers are represented by blue circles and pink cylinders, respectively. The thickness of the straight arrows reflects the level of β-lactam penetration through porin channels. The curved arrows illustrate the uptake failure that occurs with: a change (decrease) in the level of porin expression; an exchange in the type of porin that is expressed (restricted-channel porin); and mutation or modification that impairs the functional properties of a porin channel (mutated porin). The effect of pore-blocking molecules (black circles) is shown at the bottom of the figure (this diagram was taken from Pagès *et al.*, 2008).

As antibiotic resistance poses a daunting problem in hospital-acquired infections, Pagès and colleagues analyzed the porin content of 45 β-lactam resistant clinical isolates of *E. aerogenes* obtained from French hospitals (342). Of those, 44% were shown to lack porins, as determined by immunodetection. The MIC of four antibiotics (cefepime, imipenem, cefotaxime and moxalactam) was drastically increased. Additionally, many strains displayed high constitutive or inducible β-lactamase activity, but some strains did not, and thus antibiotic resistance appears to originate essentially from the lack of porins. The increase in MIC for those porin-deficient strains was similar to those with robust β-lactamase activity, indicating that a reduction of porin-mediated permeability can be an efficient strategy for antibiotic resistance on its own.

Here, it is worth to note that clinical strains of *E. coli*, *K. pneumoniae* and *Enterobacter* spp. preferentially express OmpC- than OmpF-type porins (377). Therefore, it is important to understand the molecular basis of antibiotic transport through OmpC. In two documented cases, β-lactam-resistant clinical isolates of *E. aerogenes* contained the OmpC orthologue Omp36, which carried the mutation G112D in L3 (375, 376). The homologous mutation G119D in OmpF of *E. coli* narrows the size of the channel as the large side chain of Asp protrudes into the channel lumen and confers a drastic reduction in β-lactam susceptibility (356). Recent studies also found a series of OmpC mutants that were isolated from a patient with chronic *E. coli* infections and additive mutations that conferred increased resistance to a variety of antibiotics, including cefotaxime, ceftazidime, imipenem, meropenem and ciprofloxacin (374). Crystal structures of these mutant proteins showed no major changes in the channel size or ion conductivity, and the authors hypothesized that changes in the electric field at the constriction zone played a role in the channel permeability (378). Molecular dynamics simulations predicted that the mutations changed the vector of the electric field inside the channel, thus trapping antibiotics in an unfavorable orientation above the constriction zone. However, these data are difficult to reconcile with electrophysiology data, which showed that the presumed flux (as measured by ion current fluctuations) of imipenem decreased by 15-fold in the mutant while no significant differences were observed for meropenem (379). This highlights the need for robust assays to evaluate porin-mediated translocation of clinically relevant antibiotics into intact cells. To date, only a few studies have combined cell-based and cell-free assays to analyze the translocation of β-lactams across porins (306, 380). First, James *et al.* characterized translocation of β-lactams through *E. aerogenes* Omp36 by using electrophysiology and rate killing assays and found that high affinity constant (k_{on}) values for ertapenem and cefepime binding to Omp36 correlated well with the efficacy of killing of cells expressing only Omp36. Conversely, ampicillin and ceftazidime were shown to have low k_{on} values. Although the rate of killing was not reported, other studies have demonstrated that these two antibiotics show a preference for OmpF-type channels (380-384). More recently, Acosta-Gutierez *et al.* determined the X-ray crystal structures of the principal general porins from three species of *Enterobacteriaceae*, including that of *E. aerogenes* (Omp35 and Omp36), *E. cloacae* (OmpE35 and OmpE36) and *K. pneumoniae* (OmpK35 and OmpK36) and determined their antibiotic permeabilities as well as those of the orthologues from *E. coli* (OmpF and OmpC). Starting from the structures of the porins and a set of β-lactam antibiotics, the authors propose a physical mechanism underlying antibiotic translocation and condense it in a computationally efficient scoring function. The scoring function showed good agreement with data from *in vitro* liposome swelling assays and *in vivo* rate killing assays (306). This approach offers a new perspective for predicting permeability through OM porins of Gram-negative bacteria: instead of searching for an energy minimum or a binding site obtained by docking procedures, it assesses how a molecule can compensate the inherent entropic barrier of pores. This approach provides a set of molecular determinants that are easily calculated: size, dipole

moment and net charge. Because the molecular mechanism of antibiotic translocation depends on the porin/antibiotic structural features, the derived scoring function can be readily extended to porin mutants found in resistant clinical strains. Furthermore, this approach is computationally efficient and can be used to screen large virtual libraries of compounds to identify new scaffolds with good permeation through porins as a starting point for developing new antibacterial compounds.

Antibiotic resistance often occurs upon exposing sensitive *E. coli* cells to progressively increasing concentration of the antibiotic. The treatment, in fact, leads to a chromosome-mediated multiple antibiotic resistance (Mar phenotype), where the cells become insensitive to a variety of hydrophilic and lipophilic antibiotics (385, 386). The response involves the coordinated change in the levels of multiple proteins including porins and drug efflux pumps, through mechanisms involving transcriptional and posttranscriptional regulation (387). In particular, the upregulation of *marA* leads to increased levels of the small RNA *micF*, which inhibits translation of *ompF* RNA. Decreased OmpF levels are also postulated to originate from the periplasmic accumulation of other OM proteins, such as TolC and OmpX, which might titrate away the chaperones and assembly proteins required for membrane insertion of OMPs (387). Another example of upregulation of OmpX in coordination with a strong repression of general diffusion porins has also been documented for acquired resistance to a large number of antibiotics of a strain of *S. enterica* serovar Typhimurium after exposure to nalidixic acid (388). In this case, repression also included other porins, besides OmpF, such as NmpC, LamB and Tsx. The substitution of a narrower porin in lieu of the constitutively expressed large general diffusion porins is another strategy for acquiring antibiotic resistance. For example, some clinical isolates from *K. pneumoniae* lack the large diffusion channels OmpK35 and OmpK36, but express a normally quiescent porin, OmpK37, which appears to form a smaller pore on the basis of sugar permeability (389). This porin is similar to OmpN of *E. coli* and OmpS2 of *S. typhi*, two porin types which are normally strongly down-regulated in laboratory media conditions. The presence of OmpK37 combined with the absence of OmpK35 and OmpK36 lead to a drastic increase in the MIC's of cefotaxime and cefoxitin, but not of carbapenems, indicating that these compounds might still be able to flux through OmpK37. This provides an explanation for the fact that *K. pneumoniae* infections resistant to most β-lactams can still be treated by carbapenems.

Altered porin function leading to reduced permeation rate is another strategy found in antibiotic resistant bacteria. A hot spot for single or multiple mutations leading to such phenotype is the L3 loop, which delineates the constriction zone of general diffusion porins. A clinical isolate of *E. aerogenes* was found to have a glycine to aspartate substitution on the L3 loop of its major porin (375), which might lead to a distortion of the loop or further narrowing of the pore lumen, as in G119D of OmpF (356). This mutant is characterized by a 3-fold decrease in porin conductance and a

drastic reduction in cephalosporin sensitivity. It was found later on that this porin is Omp36, which is highly similar to *E. coli* OmpC (376). This clinical isolate and two others from *E. aerogenes*, in fact, present multiple mutations in the porin gene, and are also highly resistant to cefepime, cefpirome and imipenem.

4.3. Non-porin pathways

4.3.1. Self-promoted uptake:

The concept of self-promoted uptake (SPU) of compounds across the OM of Gram-negative was proposed by Hancock, based on his extensive studies on the uptake of polycationic antibiotics such as the aminoglycosides and polymyxins (390). SPU is a process by which cationic molecules displace the divalent cations (Ca^{2+} or Mg^{2+}), which stabilize the phosphate groups of Lipid A and the phosphorylated core sugars (**Figure 2**). This destroys the LPS cross bridging and resultantly destabilizes the OM. It is similar to treatment with cationic chelators like EDTA that disaggregates the entire OM, enhancing the uptake of lysozyme and β- lactams (391). While in case of SPU, polycations cause localized disruption of LPS. SPU enables polycations such as polymyxin B nonapeptide to sensitize *E. coli* and *S typhimurium* to hydrophobic antibiotics (392). Also, a variety of polycations including gentamicin and poly-L-lysine, could permeabilize *P. aeruginosa* LPS sufficiently to allow passage of nitrocefin into the periplasm. It is noteworthy that SPU is reversible by Mg^{2+} addition (393). Besides polymyxins and aminoglycosides, there are other antibacterial compounds that appear to use the SPU pathway. Work by Hancock *et al* proposed that Mg^{2+} supplementation of azithromycin increased the MIC of the antibiotic against *E. coli* by 8-fold. MIC is the central concept in antibiotic dosing which is defined as the lowest concentration of the drug that inhibits the visible growth of target bacterial population. So, this improved efficacy of azithromycin over chemically similar erythromycin can be attributed to its better access to the SPU pathway due to the additional positive charges (394). Another study by the same group observed the same phenomenon when the sugar moiety of an active glycopeptide teicoplanin was removed and lipophilic cationic moieties were added (395).

4.3.2. Hydrophobic uptake pathway:

The OM of Gram-negative bacteria does not allow the passage of amphiphilic compounds (not even remotely hydrophobic). The non-fluid continuum formed by the LPS molecules is a very effective barrier for hydrophobic molecules. However, permeabilization to hydrophobic substances can be achieved by addition of compounds that either remove (*e.g.* EDTA by chelation), or completely displace (*e.g.* polycations) divalent cations from their LPS binding sites at the cell surface. Thus, these two factors of stabilizing influence of divalent cations and the LPS at the cell surface are the primary

factor in exclusion of moderately hydrophobic substances. As most of the antibiotics are water soluble at therapeutically relevant concentrations, this mode of uptake has less clinical significance. However, some antibiotics can be considered moderately hydrophobic in that they will partition into organic solvents in two phase partitioning experiments. The high MICs of bacterial species for such antibiotics are indicative of the barrier effect of the OM. In agreement with this, alteration of this barrier by treatment with permeabilizers or by specific OM mutations affecting LPS will decrease MICs for these antibiotics. In some bacterial species such as *Neisseria* and *Haemophilus*, MICs for moderately hydrophobic antibiotics are substantially decreased and it can be assumed that these bacteria present outer surfaces to the environment that are less effectively stabilized (370).

4.4. Transport across the periplasmic space and the inner membrane

The double membrane system of Gram-negative bacteria is designed to be selective for the uptake of "desirable" solutes while discriminating against foreign substances that might be toxic. Lipophilic molecules, which could penetrate the inner membrane are prevented from doing so by OM exclusion and may be substrates for efflux pumps (see 4.4. Multidrug efflux pumps). Small hydrophilic molecules can enter the periplasm through OM porins, and are possibly less subject to efflux, but their entry through the inner membrane is likely to be hindered.

The inner membrane of Gram-negative bacteria can be said as a standard phospholipid bilayer (396) greatly discriminating against polar and highly charged molecules, which diffuse through the bilayer much more slowly than neutral lipophilic solutes. In order for the bacterium to take up the hydrophilic and often strongly charged small molecules required for its metabolism, the inner membrane contains a large number of solute-specific energy-dependent transporters to promote their passage and concentration inside the cell. Despite the preference of the inner membrane for neutral compounds, weakly charged, protonatable molecules with sufficient lipophilicity can penetrate the inner membrane with the aid of the proton motive force (PMF) (365, 397, 398, 399). The proton gradient of the PMF, ΔpH, can promote diffusion of weak acids by neutralization of charge and the membrane potential, $\Delta\Psi$, attracts weak bases.

Antibiotics that are destined for the cytoplasm are required to penetrate the inner membrane and this question is still under debate. This is particularly true as there has been an increase in the studying the mechanism of entry across the envelope of Gram-negative bacteria with the goal for aiding in antibacterial discovery. Translocation across the inner membrane is apparently not challenging for lipophilic antibiotics since the phospholipid bilayer of the inner membrane is largely permeable to them (400). Relatively hydrophobic antibiotics such as the macrolides, lincosamides, oxazolidinones and rifamycins appear to cross the inner membrane by simple diffusion (111). In contrast, the inner membrane is largely impermeable to large, uncharged polar

molecules and all charged molecules including ions (401). The antibiotics belonging to these categories require specific uptake systems to cross the inner membrane. For example, D-cycloserine, an antibiotic for treatment of tuberculosis is transported across the inner membrane via the D-alanine transport system and coupled to the proton motive force (402). Another example is fosfomycin which is transported by using the glycerol-3-phosphate or the hexose phosphate transporters. Finally, transport of aminoglycosides requires both the electrochemical gradient across the inner membrane and the electron flow through the respiratory chain, but whether it requires a specific IM transport system is still unknown.

If rules for translocation across the OM and efflux avoidance could be derived and applied to modification of, for example, compounds directed Gram-positive bacteria, the compounds would likely arrive in the periplasm, but they might be unlikely to retain their ability to cross the inner membrane. Similarly, such rules may be unhelpful in optimization of in vitro inhibitors of cytoplasmic enzymes (obtained as HTS hits, for example) that require properties enabling passage through both membranes while avoiding efflux pumps. As noted above, it seems that chemical characteristics that allow passage through OM porins (hydrophilic and charged) and perhaps avoidance of efflux pumps may prevent diffusion through the cytoplasmic membrane which favors neutral lipophilic compounds (111). Clearly, there are exceptions, compounds which do reach the cytoplasm of Gram-negative bacteria. What are the properties of these successful exceptions and do they represent a compromise or consensus set of physicochemical characteristics that could be emulated? With this in mind, it is crucial to develop methods that allow the quantification of intracellularly accumulated compounds (265-268, 403, 404).

4.5. Multidrug efflux pumps

The susceptibility of Gram-negative bacteria to antibiotics is defined by two opposing fluxes across the two membranes of these species (**Figure 13**) (400, 405, 112). As described in the above sections, the influx of antibiotics is significantly slowed by the elaborate OM (208, 367). The LPS-containing bilayers are more rigid than normal phospholipid bilayers, slowing passive diffusion of hydrophobic compounds, whereas narrow pores limit by size the penetration of hydrophilic drugs. The slow influx of drugs across the OM is further opposed by active efflux mediated by multidrug efflux transporters. Multidrug efflux transporters are structurally and functionally diverse, with some transporters pumping antibiotics across the inner membrane and reducing concentration of antibiotics in the cytoplasm, whereas others expel antibiotics from the periplasm into the external medium. The latter transporters confer resistance to multiple classes of antibiotics by associating with periplasmic and OM accessory proteins to form trans-envelope complexes (**Figure 13**) (366, 405). The clinical relevance of efflux of multiple antibiotics has also been clearly established. For example,

in clinical isolates of *E. aerogenes and K. pneumoniae*, MDR is linked to overproduction of the AcrAB-TolC efflux pump (363, 366, 406, 407, 408). The interplay between influx and efflux mechanisms, together acting in synergy, defines the steady-state accumulation level of antibiotics at targets (409, 410).

Multidrug efflux pumps encoded on the bacterial genomes commonly belong to the ATP- binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), small multidrug resistance (SMR), proteobacterial antimicrobial compound efflux (PACE) and resistance- nodulation-cell division (RND) superfamilies (**Figure 23**). Apart from the ABC transporters, which utilize the energy of ATP binding and hydrolysis to transport the drugs across the membrane, all known multidrug efflux pumps are H^+ (or Na^+)–drug antiporters (411, 412). Most efflux pumps are active as single-component membrane proteins residing in the inner bacterial membrane, and are either functional as monomers (MFS, PACE) or as dimers (ABC, SMR). The drug is collected from either the cytoplasm or the inner leaflet of the inner membrane for transportation into the periplasm. The drug re-entry is prevented back from the periplasm with the help of a tripartite modular system. This tripartite modular system consists of an inner membrane transporter (ABC, MFS or RND), a periplasmic adaptor protein and an OM channel, to fully translocate the drugs from the periplasm across the OM. Whereas drug access into the binding pocket(s) of the tripartite ABC and MFS transporters is considered to be from the cytoplasmic side (or inner membrane inner leaflet), the RND-type multidrug efflux complexes are involved in clearing a multitude of structurally diverse compounds from the periplasm that have either just entered via the OM, or have previously been extruded from the cytoplasm by the single-component transporters (400). Thus, while the RND-type tripartite efflux systems are considered major participants in intrinsic multidrug resistance by Gram-negative bacteria, their resistance capacity is dependent on the single component inner membrane transporters. Examples of structurally and functionally well-studied efflux systems are the *E. coli* AcrAB–TolC and *P. aeruginosa* MexAB–OprM systems (413). In AcrAB-TolC, AcrB is the inner membrane drug transporter; AcrA is the periplasmic adaptor and TolC is the OM protein channel.

One of the most intriguing aspects of the RND transporters is their substrate polyspecificity (366). They confer resistance towards multiple antibiotics, detergents, bile acids, solvents and dyes, with a wide combination of physicochemical properties; including a diversity of charges, hydrophobicity, and size (with masses ranging from 86 Da (hexane) to 1415 Da (bleomycin)). In addition, many of the paralogues encoded on the bacterial genome display overlapping specificity. AcrB from *E. coli* is one of the most versatile pumps and transports almost the entire palette of harmful substances mentioned in this manuscript, but does not transport aminoglycosides and some of the more hydrophilic β-lactams, which in *E. coli* are transported by the AcrAD–TolC system instead (414, 415). X-ray structures of AcrB (*E. coli*), MexB (*P. aeruginosa*) and MtrD (*N. gonorrhoeae*) RND multidrug efflux components have been solved and have shed some

light on the substrate specificity, as well as on the general mechanism of transport and drug–H^+ antiport coupling (416-421).

AcrB from *E. coli* displays a homotrimeric setup arranged into a porter and a funnel domain, with each protomer comprising 12 transmembrane helices and two separate periplasmic loops (416, 422, 423). Each protomer adopts one of three different conformational states, designated loose, tight and open, in analogy with the three states of the F1Fo-ATP synthase, and all are part of a rotational cycle that includes the accessing, binding, and extrusion of the drug towards the TolC channel and thus to the exterior of the cell (412). According to our current understanding, AcrB drug entry pathways can be located at the level of both the outer leaflet of the inner membrane and the periplasm. Crystallographic analysis revealed a rather large access (proximal) pocket where larger drugs such as erythromycin, rifampicin and doxorubicin (as a dimer) bind. Within the same trimer, in the tight protomer, smaller drugs like minocycline, doxorubicin (monomer), rhodamine and a set of pyridopyrimidine/pyranopyridine inhibitors of the pump bind in the deep (distal) binding pocket (422-1). Between these drug-binding pockets, a flexible glycine-rich stretch of 11 amino acids, the switch loop, has been shown as essential for effective transport of drugs, that is, from the access pocket to the deep binding pocket (422, 424). The flexibility of this loop is important for its function, and substitution of particular glycines alter the substrate preference or stall transport completely.

True understanding of the molecular basis of polyspecificity still requires complete sets of co-crystallization data. Alternative structural methods, such as single-particle cryo-electron microscopy (cryo- EM) analysis could also open up a new avenue for obtaining atomic structures for the RND components or for the entire tripartite systems, including bound drugs. To date, cryo-EM structures have been published for the MexAB–OprM and AcrAB–TolC tripartite systems, both as detergent-solubilized genetically fused complexes and as unmodified proteins in lipid nanodiscs (242, 426, 427). A further challenge will be to reconstitute the entire tripartite setup in two-batch proteoliposomal systems that better model the endogenous two-membrane setup of these systems (428). The recent report of a functional reconstitution of the MexAB–OprM system using this setup made it possible to trace the transport of a drug (a fluorescent dye) and protons (via a pH-sensitive fluorescent reporter) in parallel. Optimization of this system promises insight into the largely unanswered question of H^+ and drug stoichiometry. Additionally, kinetic studies on the isolated RND systems will also allow more accurate determination of substrate preference and inhibitor properties without pleiotropic and off-target effects, which could complement measurements in whole cells (429-431).

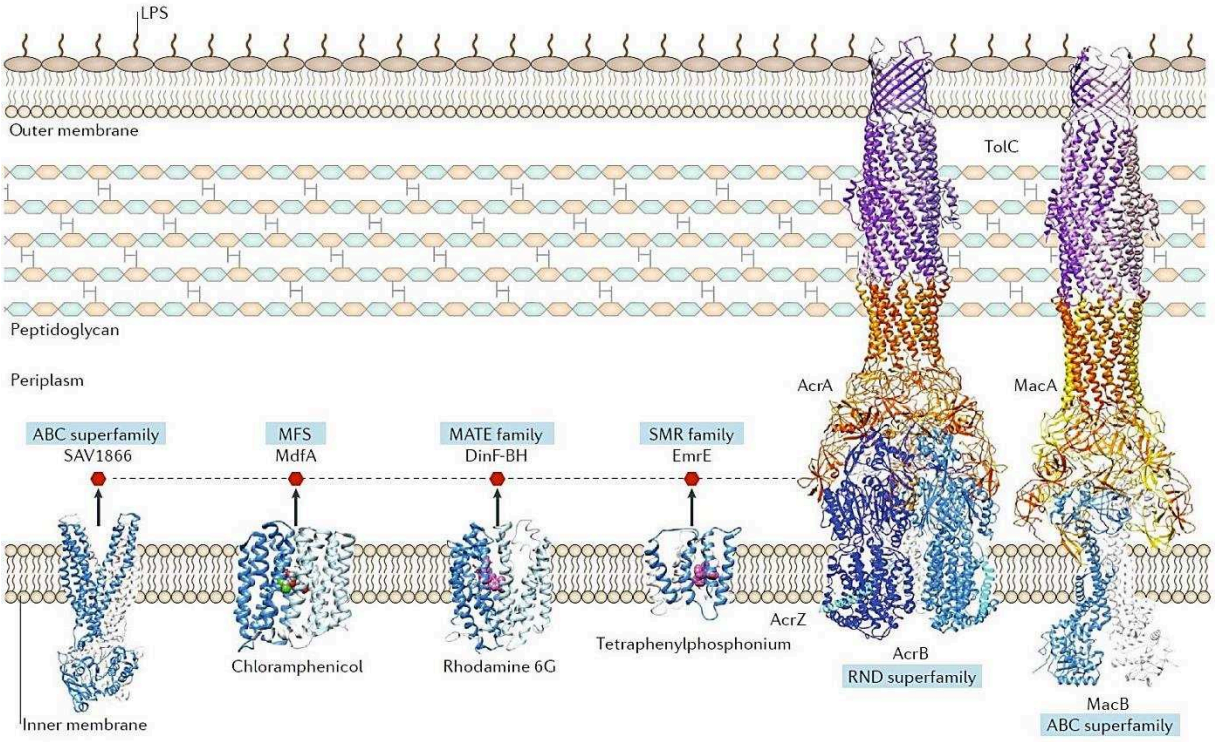

Figure 23 : Schematic of representative structures of multidrug transporters and tripartite assemblies. The envelope of Gram- negative bacteria has three principal layers: the OM, the inner membrane and the peptidoglycan cell wall in the interstitial periplasm between the two membranes. In Gram- negative bacteria, all the drug transporters are located in the inner membrane. Structures of representatives of each of the transporter families are presented, including the ATP- binding cassette (ABC), major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), small multidrug resistance (SMR) and resistance- nodulation-cell division (RND) families. The ABC transporters use ATP to drive transport processes, and the other families depicted use electrochemical gradients for an energy source. The groups differ in architectural features, as described in more detail in the main text. The ligands for the MFS, MATE and SMR representatives are indicated. The RND superfamily drug transporters mostly assemble with their partner proteins to form tripartite pumps, and these bind substrates at the outer leaflet of the inner membrane and periplasm and efflux them to the cell exterior. By contrast, members of the other families of drug transporters usually function as independent units in the inner membrane to translocate substrates across the membrane bilayer. It is likely that these transporter systems cooperate with RND-type tripartite efflux pumps to deliver substrates across the entire cell envelope as part of a larger drug efflux superorganization. The structures of the proteobacterial antimicrobial compound efflux (PACE) class of transporters have not yet been experimentally elucidated and are therefore not included here. Some ABC superfamily and 14-transmembrane helix MFS transporters can also form tripartite pumps and directly transport substrate from the inner leaflet of the inner membrane and cytoplasm to the exterior. The interactions of the tripartite pumps with the peptidoglycan layer are

not well characterized presently and are depicted speculatively. AcrAB–TolC and MacAB–TolC are RND- based and ABC- based tripartite multidrug efflux pumps, respectively. Sav1866 Protein Databank (PDB) identifier: 2HYD; MdfA PDB: 4ZOW; DinF- BH PDB: 4LZ9; EmrE PDB: 3B5D; AcrA/B/TolC EM Data Bank (EMDB) entry: EMD-8640; MacA-TolC EMDB: EMD-3652; MacB EMDB: EMD-3653. LPS, lipopolysaccharide (picture obtained from Du *et al*., 2018).

4.5.1. Efflux pump inhibitors

Lack of new antibacterial compounds in the pipeline has pushed for research efforts to rejuvenate and revitalize the old antibiotics for the treatment of Gram-negative infections (432, 433). Old drugs can be used in combination with 'adjuvants or chemosensitizers' to enhance/recover their activities by halting various resistance mechanisms active in clinical pathogens (434, 435, 436, 437). In this context, the drug efflux pumps are of particular interest. A new group of antibacterial molecules called efflux pump inhibitors (EPIs) has been developed to bypass the bacterial antibiotic resistance by blocking the efflux activity in order to restore the normal intracellular concentration for the antibiotic used for clinical treatment (438, 408). The effect of various EPIs on the activity of specific classes of antibiotics against different clinical isolates expressing drug efflux pumps *(e.g. E. coli, E. aerogenes, K. pneumoniae, P. aeruginosa, etc.*) has been evaluated in different studies. The effectivesness of EPIs depends on their intracellular (or periplasmic) concentration, and thus their uptake through the OM is a key step (408). Studies have shown that, depending on the antibiotic class and the type of EPI used, various discrepancies on the final restored level of antibiotic activity or/and on the amount of EPI needed for restoration can be observed. Different comparative studies have been performed using quinoline derivatives and also PAβN as a reference (439, 440, 441). It has been reported that with the same bacterial strain, for instance a selected *E. aerogenes*, *K. pneumoniae* or *E. coli* isolate, the activity spectrum of a defined EPI is different regarding the antibiotic tested as a competitive substrate for pump activity. This phenomenon was known since the first EPI was reported: thus, PAβN, which decreases drastically the levofloxacin MIC in MexAB-OprM-overproducing *P. aeruginosa*, showed very little effect on the MIC of carbenicillin (442). In a recent study, Chevalier and colleagues demonstrated that PAβN and quinazoline derivatives do not have the same enhancer effect on ciprofloxacin, sparfloxacin and erythromycin activity evaluated in an *E. aerogenes* strain overproducing AcrB; a similar difference is also noted with *K. pneumoniae* and *P. aeruginosa* strains for other antibiotics (443, 444). This could be associated with the respective affinity of the ligands, for example EPI or antibiotic molecule, for the pump site, but also with the level of expression of the acting pump under the tested conditions. Moreover, some EPIs can be more active on a specific efflux pump: for example, there are EPIs that inhibit only the MexAB-OprM system among several RND pumps that confer drug resistance to *P.*

aeruginosa (445; also for a review, see 408). Regarding this point, it is important to mention that the original screening protocols used to develop and select EPIs, in terms of bacterial efflux target and antibiotic used as a substrate, play an important role in the affinity and activity spectrum of EPI (408). This is illustrated in the case of the two EPI families (peptidomimetics and quinoline derivatives) developed recently (440). Similar comparison studies have been performed between PAβN and 1-(1-naphthylmethyl)-piperazine (NMP), an original EPI (446). The activity of NMP was different from that of PAβN on a collection of clinical isolates of *E. coli*, in particular regarding the macrolide resistance reversal (447). Moreover, NMP displays a moderate activity in reversing MDR in *Citrobacter freundii, E. aerogenes, Serratia marcescens* and *K. pneumoniae* clinical isolates. Its effects on the reversal of resistance depend on bacterial species and drug and are different from those seen with PAβN (448). Thus, the selectivity/efficacy of efflux pump and the activity of the respective EPIs on the degree of altered resistance are strongly interconnected. The question remains about the development of molecules that mimic the structure of a specific antibiotic molecule (via the use of appropriate pharmacophoric groups) to favor a directed improvement of the activity on a single antibiotic class. This may be a key question for the development and selection of future 'adjuvants or chemosensitizers' able to restore a significant antibiotic concentration inside the bacterium.

5. Remodeling of the cell envelope

The Gram-negative cell envelope and cytoplasm differ significantly, and separate responses have evolved to combat stress in each compartment. An array of envelope stress responses (ESRs) exists, each of which is focused on different parts of the envelope. In this section, I will provide a detailed overview of the two major ESR pathways, the alternative sigma factor σ^E and the two component system CpxA-CpxR, with respect to envelope permeability control and antibiotic resistance. Briefly, the σ^E response is conserved in many enterobacteria and is tuned to monitor pathways for the maturation and delivery of OM porins, lipoproteins, and lipopolysaccharide to the OM. It detects perturbations through interactions between either the exposed C-terminus of misfolded OMPs and DegS periplasmic protease, or between the anti-anti-sigma factor RseB and periplasmic LPS molecules, respectively. Both initiate a regulated intramembrane proteolysis cascade ultimately leading to the liberation of σ^E from a membrane-bound anti-sigma factor RseA and the upregulation of adaptive factors, including chaperones, proteases, membrane biogenesis proteins, and a set of small RNAs (sRNAs) that downregulate OMP production (339, 340, 426, 449-451). The Cpx ESR comprises the CpxA sensor kinase and response regulator CpxR. Envelope stresses causing protein misfolding, and adhesion, inactivate the periplasmic inhibitory protein CpxP, trigger CpxA-mediated phosphorylation of CpxR, and altered expression of periplasmic foldases and proteases, respiratory complexes, transporters, and cell wall

biogenesis enzymes that impact resistance to a number of antibiotics, particularly aminoglycosides (452, 453). In this section, I will also emphasize on the role of specific antibiotic resistance regulatory pathways such as Mar, Ram and Rob. All these regulatory networks are complex and often interconnected. Nonetheless, their understanding both at the transcriptional and post-transcriptional levels is important for interpreting resistant phenotypes.

5.1. Antibiotic stress responses

Long term and widespread usage of antibiotics has led to the development of ubiquitous antibiotic concentration gradients. Not just in humans but these gradients also exist in livestock and various environmental outlets like sewage water, sludge, rivers and lakes. As a consequence, bacteria are frequently exposed to lethal or sub-lethal concentration of drugs. However, the resultant effects of exposure to this antibiotic concentration gradient on the bacterial physiology are not well known. Overall, known effects include genetic (mutational) or phenotypic variability, which generate activation of bacterial resistance or tolerance in bacterial populations.

During a clinical treatment, the basic rationale of antibiotic dosing is to maintain a drug concentration higher than the MIC to clear the infection. Because of the wildly help assumption that most of the clinically relevant resistance emerges as a result of bacterial exposure to antibiotics dosage much higher than their MIC, the potential for sub-lethal antibiotic concentrations to select for resistant mutants has mostly been ignored. Studies have shown that low antibiotic concentrations exert their effects on multiple levels (i) as selectors of resistance, by enriching for pre-existing resistant bacteria and selecting for de novo resistance (ii) as generators of genetic and phenotypic variability, by increasing the rate of adaptive evolution, including resistance development (iii) as signaling molecules, by influencing various physiological activities, including virulence, biofilm formation and gene expression. Enrichment of pre-existing resistant mutants: In a classical work by Gulberg et al., *E. coli* and *S. typhi* were competed at a range of antibiotic concentration for determining the minimal selective concentration (MSC) of each antibiotic (454). Interestingly, several of the resistant mutants had MSC values 10-fold lower than the MIC. The strains carrying a transposon *Tn10* encoding tetracycline resistance or S83C mutation in *gyrA* associated with fluoroquinolone resistance had an MSC of 100-fold or 230-fold lower than MIC. This provided the evidence that the bacterial exposure to low antibiotic concentrations in vivo leads to the preferential expansion of resistant subpopulations.

De novo selection of resistance: In the same study, multiple independent lineages of wild-type *E. coli* and *S. typhi* were created under constant exposure of sub MIC levels of ciprofloxacin and streptomycin (454). Not surprisingly in all the evolved lineages the number of resistant sub populations progressively increased throughout the course of the experiment.

Generators of genetic and phenotypic variability: In the 1960s, it was found that sub-MIC concentrations of the aminoglycoside streptomycin causes misreading errors during translation, which result in phenotypic changes, such as reduced growth rate. It was further proposed that these errors in protein synthesis might be propagated to the genome, owing to the production of defective DNA polymerases by error-prone translation. Because of this fact, a growing body of evidence today suggests that several antibiotics at sub-inhibitory concentrations — and not just those that induce translational errors — can increase the rate of resistance. This involves an increase in the rates and frequency of various genetic processes, including horizontal gene transfer (HGT), recombination and mutagenesis (455-457).

Increased mutagenesis: Sub-MIC antibiotic concentrations have been shown to increase mutagenesis, which is also associated with the induction of the SOS response. SOS response refers to a set of co-regulated genes that are induced in response to DNA damage. The system is widespread in bacteria and promotes cell survival by repairing damaged genomes. The key proteins that are involved are RecA and LexA. Inactivation of either RecA or the presence of a non-cleavable LexA repressor -both results in inhibition of the of the SOS response activation which ends this mutagenic effect. A recent study elucidated the mechanism by which sub-inhibitory concentrations of the β-lactam antibiotic ampicillin increases mutagenesis in *E. coli* (457). Mutagenesis induced by sub-MIC levels of ampicillin was caused by the combined activities of both the normal replicative DNA polymerase (in the absence of adequate mismatch repair, owing to MutS depletion because of RpoS induction by antibiotics) and of the error-prone DNA polymerase IV, which is part of the RpoS regulon itself.

Phenotypic variability: The effects of sub-MIC antibiotic concentrations are not only limited to genetic alterations but also affect bacterial phenotypes. This is clearly evident from several studies that show that sublethal antibiotic levels increase the frequency of persisters. The slowly growing nature of bacterial persisters, as a consequence of reduced metabolic activity, is thought to be responsible for their ability to survive exposure to antibiotics (458). Two major differences distinguish persistence from resistance: first, antibiotic tolerance is not heritable as it is not caused by genetic mutation and, second, persistence is a transient state that is reversed following the removal of the antibiotic. Persistence is suggested to have important clinical implications as it is thought to reduce the rate of clearance of bacterial infections and to potentially promote the emergence of genetically resistant mutants.

Low antibiotic levels as intercellular signals: Antibiotics at low, non-inhibitory concentrations can function as signaling molecules between cells of the same species or between cells of different species. Such signaling has a range of functional consequences, including the induction of conjugative transfer, gene expression, quorum sensing, biofilm formation and bacterial virulence (460-462).

5.2. Global MDR regulators

Overall, MDR in *Enterobacteriaceae* is under the positive regulation of global transcriptional regulators and targets are negatively regulated by local repressors. External factors have been shown to trigger one or more of these regulatory pathways, thereby altering the cell envelope permeability and antibiotic susceptibility (**Figure 23**).

Major global regulators include MarA, RamA (absent in *E. coli*) and Rob belong to the Ara/XylS superfamily and coordinate the expression of porin and efflux genes. This family also includes the oxidative stress regulon SoxSR (463). 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 (464). The Mar (multiple antibiotic resistance) operon is well conserved in *Enterobacteriaceae* with two divergent transcriptional units comprising *marC* and *marRAB* separated by the *marO* operator (465). The *marC* gene encodes a putative integral protein of the inner membrane with a function uninvolved in MDR. *marR* encodes a repressor that binds to *marO* upstream of an activator encodes by *marA*. De-repression of *marA* in response to several chemical and antibiotic stresses triggers a cascade of events that results in global control of membrane permeability by the downregulation of porin synthesis and overexpression of efflux pump components (466, 467). 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 (468).

Another member of the AraC/XylS family, Rob regulates genes involved in resistance to antibiotics, organic solvents and heavy metals. Over-expression of Rob in *E. coli* produces both increased organic solvent tolerance and low-level resistance to multiple antimicrobial agents, due to the increased expression of the AcrAB efflux pump (469). Rob is constitutively expressed, thus MDR phenotype is observed when its expression is suppressed.

Oxidative stress regulon SoxSR is another positive MDR global transcriptional activator where SoxS is the effector of the regulon. Once SoxR is oxidized by agents like H_2O_2, NO, paraquat, it triggers the the transcription of the gene *soxS* (470). SoxS is involved in activation of the MDR phenotype in *E. coli* and *S. typhi* and can induce the transcription of *micF* and *acrAB*. The Mar-boxes are also target sequences for the binding of SoxS and the phenotype induced by SoxS is similar to that induced by MarA. SoxS is also able to activate MarA expression and together they activate many of the same genes (471).

Another global regulator of OM 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. This protein regulates the expression of porins and several efflux pumps in *E.*

coli and *E. aerogenes* in response to osmotic stress (472). There is evidence that this H-NS also controls expression of OmpX (473).

Negative regulation by repressors of porins and efflux pump also impacts MDR phenotype. OmpX is a small OMP, of which overexpression is associated with a decreased expression of Omp36 (the OmpC ortholog in *E. aerogenes*) and a decreased susceptibility to β-lactams. Studies have indicated that expression of OmpX itself is controlled by number of environmental factors, including salicylate via MarA and paraquat via SoxS (473). A very rapid MarA-dependent response pathway for upregulation of o*mpX* has been shown to occur within 60–120 min upon cell exposure to salicylate. This work by Dupont *et al*. identified a dramatic decrease in OmpF levels, as a first line of defense together with the development of resistance to β-lactams and fluoroquinolones by altering OM permeability (473).

5.3. Envelope stress responses

Gram-negative bacteria possess multiple ESRs to counteract external stress on cell envelope, which includes both cell membranes and cell wall. As mentioned earlier, the major ESRs are in the form of two-component systems such as CpxRA, Rcs, PhoPQ, EnvZ-OmpR and BaeSR. Additionally the stress-responsive alternative sigma factor σ^E and the phage shock response also have a key role in *E. coli* and closely related *Enterobacteriaceae* (453). Each of these ESRs is activated following the perturbation of particular components of the envelope and/or by exposure to particular environmental stresses to remodel the cell envelope and maintain cellular homeostasis (474, 475). This is particularly true for the σ^E-dependent stress response in *E. coli*, as the *rpoE* gene, which encodes σ^E, is essential for viability. Here, I will essentially focus on ESRs that impact on MDR by regulating porin expression together with many other targets (regulons) — namely Cpx and σ^E (**Figure 24**). These ESRs are often interconnected, regulate and are regulated by sRNAs in order to control target genes both at the transcriptional and post-transcriptional levels.

Accumulation of misfolded OMPs in the periplasm, presumably reflecting problems in protein assembly or transport across the IM, can be detected by regulatory sensors that activate either the Cpx TCS or the alternative sigma factor σ^E. σ^E and Cpx are the two major pathways that control the envelop integrity with overlapping regulon members (476, 477), but respond to different inducing cues (449).

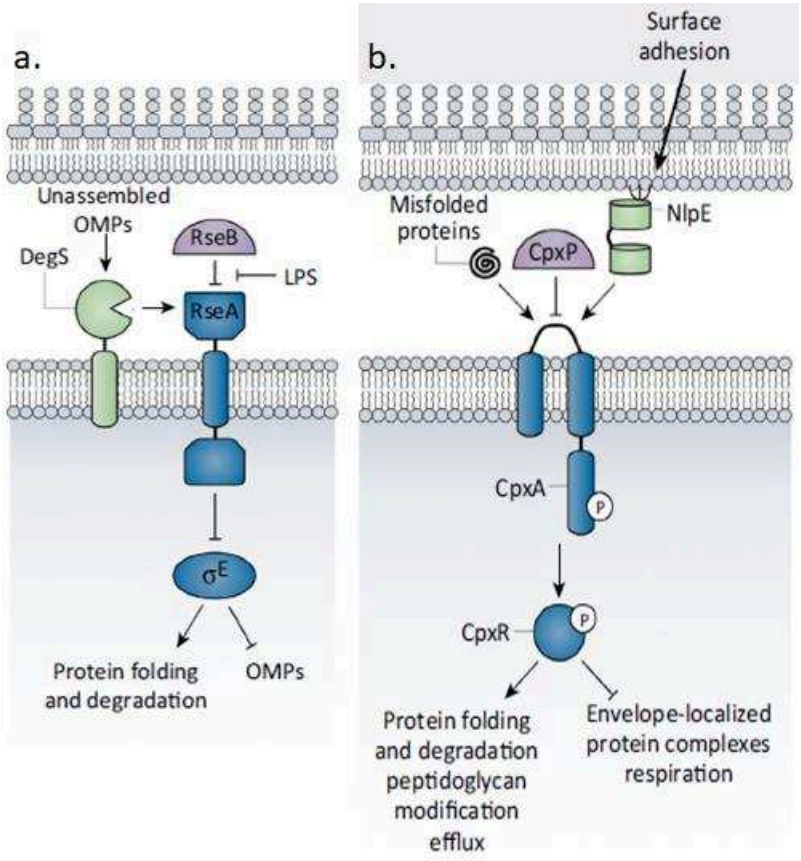

Figure 24: Schematic representation of the two main Gram-negative ESRs σ^E (a) and Cpx (b). They are induced by a variety of envelope stresses including antibiotics. In response, they alter the expression of adaptive functions that modify the cell envelope, rid the cell of the toxic compounds, and/or repair the damages caused (figure accquired from Guest & Raivio, 2016).

The σ^E pathway of *E. coli* was the first ESR to be identified. In the absence of inducing signals, σ^E is held at cytoplasmic side of the inner membrane by the anti-sigma factor RseA, a single-pass membrane protein (478, 479). A periplasmic protein, RseB, binds to the periplasmic domain of RseA and enhances the inhibition of σ^E (
Figure 24a) (478479). Upon cell envelope stress, σ^E is released from RseA by a proteolytic cascade whose end result is the complete degradation of RseA and the release of σ^E to direct transcription (480). The so called regulated intramembrane proteolysis (RIP) proteases of the cascade- DegS and RseP (formerly known as YaeL) act sequentially cleaving RseA first in the periplasmic and then in the transmembrane region (481, 482). The cytoplasmic domain of RseA (RseAcyto) bound to σ^E is then released and degraded by cytoplasmic proteases, primarily ClpXP (483-485). The proteolytic cascade is induced by a conserved YxF peptide (where x is any amino acid) found at the C-terminus of OMPs (486). This peptide is normally buried and inaccessible

in folded porin trimers. When porin folding is disrupted, the peptide is exposed and binds to DegS, activating DegS to cleave RseA and initiate the response (486). Proper porin folding and transit to the outer membrane involves a series of steps, and disruption of this pathway at any point may lead to improperly folded porins with exposed C-termini. Therefore, it has been proposed that porins provide a sensitive measure of cell envelope homeostasis (487).

The regulatory pathway is not only designed to have a sensitive trigger specifically tuned to the inducing signal, but also includes a homeostatic mechanism providing a quick and efficient method to reset the switch and deactivate the response. Once σ^E is activated it transcribes the genes in its regulon (488). Although the σ^E regulon includes genes that affect many aspects of the cell, a significant fraction of its known regulon members encodes chaperones required for the delivery and assembly of porins in the outer membrane, chaperones required for the delivery and assembly of LPS in the outer membrane, proteases to degrade terminally misfolded porins, and at least two small RNAs, RybB and MicA, that target mRNAs encoding porins for degradation (333, 489-491) and one, MicL, that target mRNA of the major outer membrane lipoprotein Lpp (492). Therefore, the activation of the σ^E pathway increases the capacity of the cell to deliver proteins to the outer membrane, facilitates the removal of misfolded porins, and reduces new porin synthesis reducing the load on the system. Each of these systems helps to lower the level of unfolded porins, thereby reducing the inducing signal and returning DegS to the inactivated state.

The Cpx two-component system comprises the CpxA sensor kinase and response regulator CpxR (**Figure 24b**). Envelope stresses including alkaline pH, periplasmic protein misfolding, inner membrane abnormalities such as misfolded transporters or accumulation of the lipid II precursor, induce the dissociation of the accessory protein CpxP from CpxA, trigger CpxA-mediated phosphorylation of CpxR (CpxR~P), and alter the expression of protein foldases and proteases, respiratory complexes, inner membrane transporters and cell wall biogenesis enzymes, all of which materializes resistance to a number of antibiotics, particularly aminoglycosides and β-lactams (493, 452, 453). The Cpx-mediated regulation of porins occurs at several levels. At the transcriptional level, CpxR~P has been shown to bind directly the *ompF* and *ompC* promoters (494). More recently, it has been found that the small IM protein MzrA connects Cpx and EnvZ/OmpR (495). In this pathway and upon the activation of Cpx, MzrA interacts directly with EnvZ, which in turn, stabilizes OmpR~P (496). In sensing different signals, the interconnection between Cpx and EnvZ/OmpR allows cells to adapt to diverse environmental stresses. Finally, although Cpx contributes to antimicrobial resistance by regulating a number of genes, its precise role and that of other two component systems in the development of MDR in clinical isolates is still poorly documented (231, 344).

5.4. **sRNA regulation**

Bacterial sRNAs are ubiquitous multipurpose regulators present in all domains of life. They act modulating transcription, translation, mRNA stability, and DNA maintenance or silencing, and achieve these diverse outcomes through a variety of mechanisms, including changes in RNA conformation, protein binding, base pairing with other RNAs, and interactions with DNA. In bacteria, transcript size of sRNAs varies from 50 to 300 nucleotides. Base-pairing sRNAs can be either *cis*-acting or *trans*-acting, depending on their genomic location and with respect to their target mRNAs (497, 498) (). Many of these sRNAs function by imperfect base pairing with multiple mRNA targets and often require the help of the RNA chaperone Hfq (497). Base-pairing sRNAs act as major post-transcriptional regulators that can either enhance or repress mRNA decay and/or translation by binding to the 5' untranslated rtegion (UTR) of mRNA targets (497, 498). However, some sRNAs function by the interaction with regulatory proteins, often acting by sequestration. Initially, sRNAs were identified by the analysis of transcription from intergenic regions (499). Extensive RNA-seq analysis of transcriptome of various regulators, identification of RNAs by ligating Hfq-bound sRNAs and sequencing sRNA–RNA interactions (RIL-seq) or by UV-cross-linking sRNA–target RNA duplexes to RNase E (CLASH) or immunoprecipitations with Hfq and ProQ have vastly increased the repertoire of sRNAs and their targets (500-503). These studies have revealed that sRNAs can originate from various genomic regions, including antisense to coding regions (501-503), 3' UTRs (492-506), 5' UTRs (507) and even coding regions (501) (). Specific sRNAs control the expression of sigma factors like the stationary-phase sigma factor RpoS, the envelope stress-responsive sigma factor RpoE and the activity of house-keeping sigma factor RpoD (497, 498, 508). The sRNA-mediated control has also been described for several regulatory factors like FlhDC (motility/flagellar expression), Lrp (amino acid biosynthesis), CRP (catabolite repression) and SoxS (oxidative stress) (509- 511). Thus, sRNAs are deployed by each of the major stress-responsive pathways, controlling the expression of crucial regulatory molecules that can rapidly alter gene expression profiles, allowing fast adjustment to different growth conditions. Transcription of most trans-acting sRNAs is controlled by transcriptional factors that include global regulators, two-component systems and specific stress-responsive sigma factors (511, 505). One of the well-studied cases are regulations of ESR by the sigma factor σ^E, the two-component system CpxAR and regulatory sRNAs that are members of their respective regulons (505, 506). As previously described in this manuscript, σ^E is specifically induced in response to OMP misfolding, defects in the LPS composition and an imbalance between phospholipids and LPS molecules (508). On one hand, RpoS, RpoN, RpoD, RpoE sigma factors and phosphor-relay regulatory systems, like Rcs and QseEF, positively regulate transcription of the *rpoE* gene in response to specific stress conditions (507). On the other hand, availability/activity of σ^E is also subjected to a negative regulation by the anti-sigma factor RseA and a feedback control by σ^E-regulated

sRNAs (479, 508, 512). As several sRNAs, belonging to stress-responsive regulons, also act in a negative feedback mechanism, this suggests functional overlaps and co-integration of signals to achieve homeostasis. Consistent with integration of diverse signals, the expression of many stress-responsive regulators and sigma factors are regulated by sRNAs (505, 511). In this section, I will address how major stress-responsive regulatory systems engage sRNAs to regulate the envelope stress response and co-ordinate the gene expression upon other specific stresses, specifically upon antibiotic stress.

5.4.1. Mode of action of sRNA regulation

5.4.1.1. *Trans*-encoded sRNAs

Most of the identified bacterial sRNAs so far are encoded in different genomic loci than their target mRNAs and share only limited complementarity with their targets. Base pairing between the sRNA and its target mRNA can promote or inhibit translation, and can decrease or increase mRNA stability. Base pairing between the sRNA and its target mRNA usually leads to repression of protein levels through translational inhibition, mRNA degradation, or both (513, 514). Most of the *trans*-encoded sRNAs have been shown to bind the 5' UTR of mRNAs, thereby inhibiting translation by base pairing with the ribosome-binding site (RBS) and/or upstream of the AUG codon of the mRNA (515) (lower panel A).The sRNA-mRNA duplex is then frequently subject to degradation by RNase E, or RNase III, the two major bacterial endoribonucleases (516) (lower panel B). Activation of the translation of the target mRNAs can also be a common occurrence when base pairing of the sRNA disrupts an inhibitory secondary structure that sequesters the RBS (514, 517) (lower panel C). Few examples are *trans*-encoded sRNAs which increase the stability of mRNAs by inducing cleavage by endonucleases that generates an mRNA with a different 5' structure that either increases stability (518) or by protecting the 5' end of mRNAs from nucleases (519). For example, base pairing between the sRNA and its target mRNA might be able to block an RNase E recognition site, leading to increased stability of the target RNA. Theoretically, base pairing between a *trans*-encoded sRNA and its target could also promote transcription termination or anti-termination, as has been found for some *cis*-encoded sRNAs.

There is little correlation between the genomic location of *trans*-encoded sRNAs and their target mRNA genes. In fact, several *trans*-encoded sRNAs are known to base pair with multiple mRNAs. This results from the fact that they have limited complementarity with their target mRNAs, usually in discontinuous patches, rather than extended regions of perfect complementarity, as for *cis*-encoded sRNAs. The region of base pairing between *trans*-encoded sRNAs and target mRNAs is typically 10–25 nucleotides, but only some of the nucleotides seem to be critical for regulation. For

example, although the SgrS sRNA can form 23 base pairs with the *ptsG* mRNA, only changes in 4 of these nucleotides in SgrS affects downregulation of *ptsG* (520).

5.4.1.2. *Cis*-encoded sRNAs

A significant number of protein-encoding genes are transcribed from the reverse complementary strand in a wide range of bacterial species (521). In general, overlapping transcription results in the generation of *cis*-encoded anti-sense RNAs (asRNAs) whose size usually varies between tens to hundreds of nucleotides. By 2007, only about 30 bacterial asRNAs had been identified. Indeed, antisense transcription detected in initial tiling array experiments was suspected to be an experimental artifact generated during cDNA synthesis. This problem was overcome by the development of strand-specific cDNA synthesis protocols (522). The number of reported asRNAs has lately exploded due to the use of RNA-seq. However, characterization of the physiological function of individual asRNAs is growing at a much slower speed.

AsRNAs are known to affect the expression of the target gene by different mechanisms (upper panel) (523). (i) One is transcription interference, in which transcription from one promoter is suppressed by a second promoter located in the opposite strand. Transcription interference does not involve base-pairing and does not occur when the asRNA is provided in *trans*. This effect was first detected in two convergent bacteriophage promoters that produce transcripts which have an overlap of 62 bp at their 5' ends (524). It was observed that the stronger promoter significantly reduced the activity of the weaker promoter. When the promoters were oriented divergently, the transcription from both promoters was not affected despite the transcripts still maintained their regions of complementarity. Moreover, the introduction of a terminator before the convergent weak promoter resulted in reduced interference. These results led to the conclusion that the convergent orientation of the promoters was the source of the interference rather than base pairing. (ii) asRNAs also can alter induce mRNA transcription attenuation, in which base pairing of the asRNA to the target mRNA causes premature transcription termination. In some cases, base pairing of the asRNA to the mRNA has been shown to induce the formation of a terminator structure in the target mRNA. One example of this type of regulation is the asRNA RNAβ, encoded opposite to the *fatDCBA-angRT* iron transport-biosynthesis operon in the fish pathogen *Vibrio anguillarum* (525). Premature transcription termination results in increased levels of the *fatDCBA* fraction of the mRNA compared to the down-stream *angRT*, providing a mechanism for miscoordinated expression within an operon. (iii) asRNAs can promote changes in the target RNA stability by the same mechanism as intergenic sRNAs, where the asRNA either promotes or blocks degradation or cleavage of the mRNA by ribonucleases. As example, RNase III is known to be responsible for the of the plasmid-encoded *copT-copA* and *hok-sok* asRNA pairs (526, 527). The AmgR RNA of *Salmonella enterica* induces degradation of the *mgtC* mRNA in a manner that requires RNase E but not RNase III (413). (iv) Just as trans-

encoded sRNAs, asRNAs whose complementarity extends into the 5' UTR of their target RNA can impact on ribosome binding to the target mRNA (either positively or negatively) by affecting the target RNA structure. In addition, regulating the expression of the opposite gene is not the only function of certain asRNAs. As intergenic sRNAs, some asRNAs encode small proteins, and some have the potential to act on multiple targets in trans. The number of existing asRNAs is far from complete, and other mechanisms of action will probably be found. Some possibilities are that antisense transcription serves to stabilize certain regions of the chromosome or as a defense against plasmids containing complementary regions.

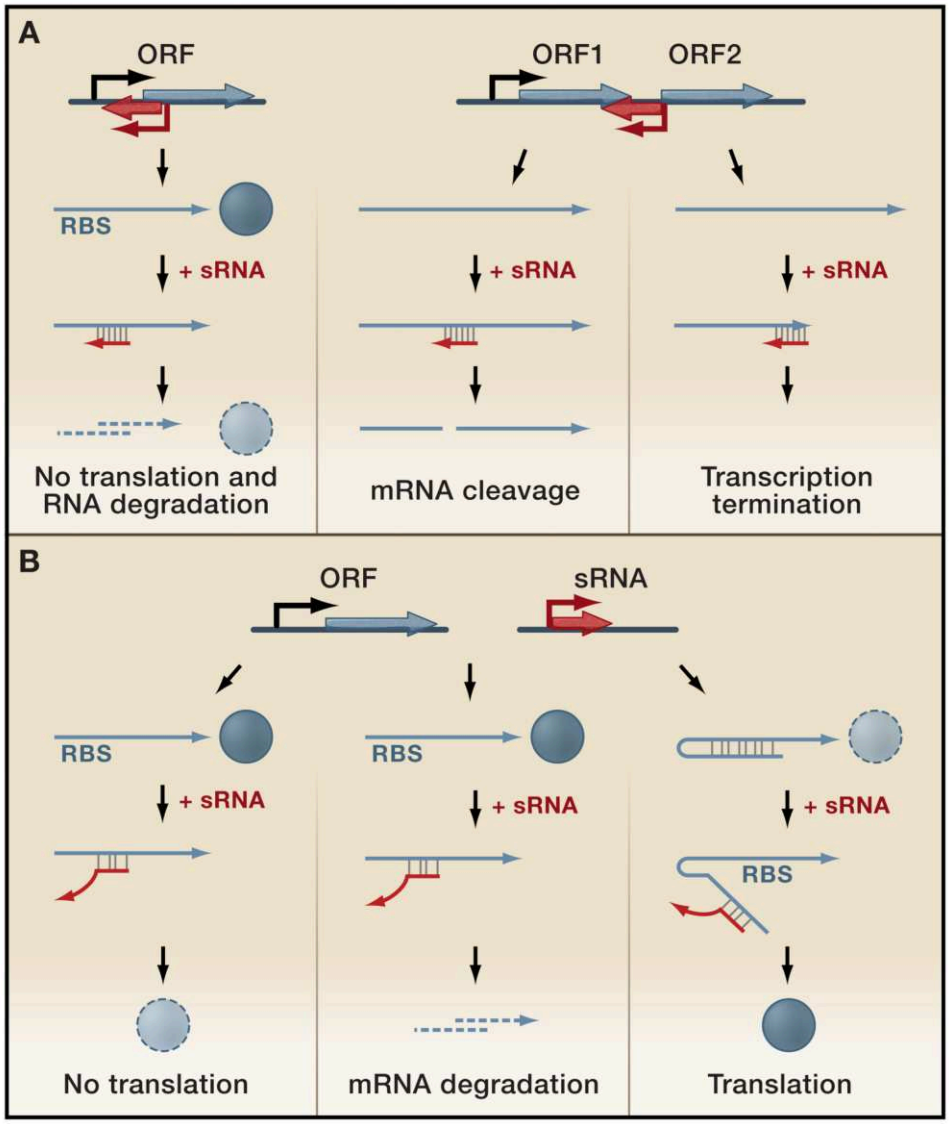

Figure 25: Gene arrangement and regulatory functions of base pairing regulatory RNAs. (A) Two possible configurations of *cis*-encoded antisense sRNAs (red) and their target RNAs (blue) which share extensive complementarity. (Left panel) An sRNA encoded opposite to the 5' UTR of its target mRNA. Base pairing inhibits ribosome binding and often leads to target mRNA degradation. (Right panels) An sRNA encoded opposite to the sequence separating two genes in an operon. Base pairing of the sRNA can target RNases to the region and cause mRNA cleavage, with various regulatory effects, or the sRNA can cause transcriptional termination, leading to reduced levels of downstream genes. (B) Genes encoding *trans*-encoded antisense sRNAs (red) are located separate from the genes encoding their target RNAs (blue) and only have limited complementarity. *Trans*-encoded sRNA can act positively by preventing the formation of an inhibitory structure, which sequesters the ribosome binding site (RBS) (right panel) (igure acquired from Waters & Storz, 2009).

5.4.1.3. Protein-binding sRNAs

The number of sRNAs that directly regulate the activity of proteins is much lower than the number of intergenic sRNAs and asRNAs. However, their regulatory roles are by no means less powerful. sRNAs can regulate RNA binding proteins by containing the protein recognition sequence (molecular mimicry), often in multiple copies. This is the case of *E. coli* CsrB RNA, which has 18 binding sites for the RNA binding protein CsrA, which regulates mRNA translation and stability. The *P. aeruginosa* CrcZ RNA is another example of this kind of regulation. CrcZ contains 5 CA-rich motifs which can bind up to 5 copies of the translation repressor protein Crc (527). Other sRNAs bind enzymes, and can inhibit, activate or modify protein activity. The most studied example is 6S RNA, which binds to the house-keeping form of RNA polymerase (σ^{70}-RNAP) by mimicking the secondary structure of DNA during transcription initiation (reviewed in 529). The changes on transcription after binding 6S RNA are very complex, which indicates that σ^{70}-RNAP activity is modified rather than simply inhibited (530). It is expected that the number of sRNAs that bind to proteins will increase, and that more modes of regulation will be discovered. For example, it is proposed that sRNA binding to proteins might also modulate proteins by allosteric regulation or by tethering proteins close to each other (**Figure 26**).

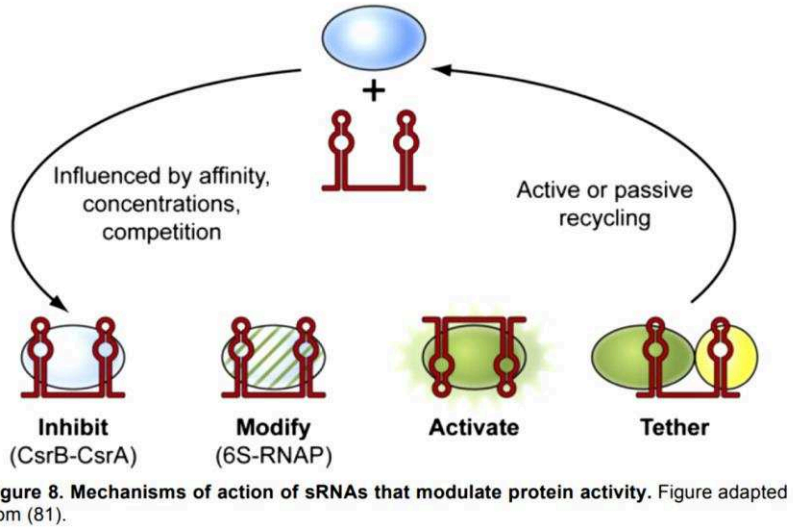

Figure 8. Mechanisms of action of sRNAs that modulate protein activity. Figure adapted from (81).

Figure 26: Mechanisms of action of sRNAs that modulate protein activity (Picture acquired from Storz et. al, 2011).

5.4.2. sRNAs regulating the ESR

5.4.2.1. MicF and MicC regulate the two major classical porins, OmpF and OmpC

As previously described, OmpF and OmpC are amongst the most abundant OMPs. Of the two, OmpC forms the smaller pore, and plays the predominant role under conditions where nutrients, as well as toxins are abundant, whereas the wider OmpF pore is thought to be important under conditions of limiting nutrients and of low toxin levels. The differential expression of these two classical porins underlies a complex regulation at the transcriptional level (307). The post-transcriptional repression of OmpF, by the MicF sRNA, has been matched for OmpC through the discovery of MicC (531) (**Figure 28**).

The 93-nucleotide MicF sRNA is located upstream the *ompC* promoter and was first shown to inhibit OmpF production through the decrease of the *ompF* mRNA (510). The discovery of the post-transcriptional repression of OmpF by MicF revealed a direct base-pairing between MicF and a fragment of the *ompF* mRNA encompassing both the RBS and the start codon (532, 533) (**Figure 27**). The expression of MicF itself is subjected to multiple signals and regulatory pathways (534). Positive regulation occurs via the EnvZ-OmpR two-component system under high osmolarity conditions (535), via SoxS in response to oxidative stress (510) and via MarA in response to antibiotic stress

(536). At high osmolarity, expression of MicF is induced upon the activation of the response regulator OmpR and high level of OmpR~P, which, at the same time negatively regulates the *ompF* transcription but stimulates that of *ompC*.

The 109-nucleotide MicC sRNA has been identified more recently in a computational screen as a sRNA encoded in the *ompN-ydbK* intergenic region, divergent to the *ompN* gene, which encodes a quiescent porin (529). This study first showed that MicC represses OmpC at the post-transcriptional level by direct base-pairing to a 5' UTR of the *ompC* mRNA, thus preventing the formation of a functional translation initiation complex (**Figure 27**). In addition, Northern blot analysis of MicC and MicF expression profiles from a variety of growth conditions showed the two RNAs to accumulate in almost a mutually exclusive fashion and that these two sRNAs could act in conjunction with the Env-OmpR two-component system to control the OmpF/C porin ration in response to a variety of stresses, including not only the osmolarity but also the growth temperature and medium starvation. Our work has further shown that *ompN* and *micC* are subjected to dual regulation upon exposure to certain antimicrobials such as β-lactams in a σ^E-dependent manner (299). This is consistent with the fact that *ompN-micC* and *ompC-micF* share similar genetic organization and previous research have shown that *ompC* and *micF* are co-induced under specific conditions too (i.e. high osmolarity via EnvZ- OmpR). The *micC* gene is well-conserved among enterobacteria and its expression is highly regulated. The physiological of MicC is still unclear, although down-regulating OmpC at the post-transcriptional level during an antibiotic could be required for bacterial adaptation. Therefore, the identification of additional MicC targets as well as information of MicC expression in MDR clinical strains could help addressing this question.

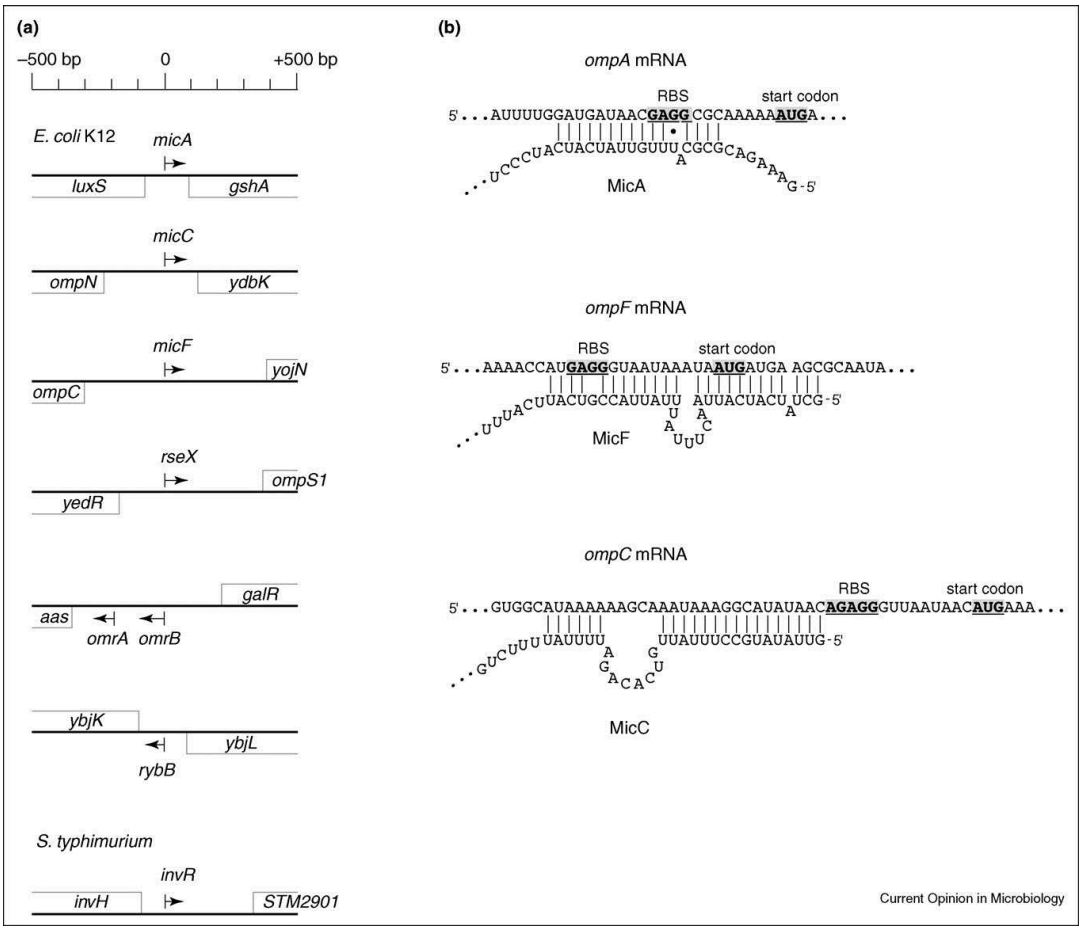

Figure 27: Major OMP-regulatory sRNAs in enterobacteria. (a) Genomic location of *E. coli* sRNA genes (*micA*, *micC*, *micF*, *omrAB*, *rseX* and *rybB*), and of the pathogenicity island borne *invR* gene in *Salmonella enterica* serovar Typhimurium (+ strand genes above line; – strand genes below line). (b) Inhibitory RNA duplexes formed by Mic sRNAs with the 5' UTRs of their respective target mRNAs. The interactions shown were biochemically mapped by in vitro structure-probing of MicA–*ompA* and MicF–*ompF* complexes (310, 537, 538), or are supported by the successful introduction of compensatory base-pair changes in the case of MicC–*ompC* mRNA (531). The AUG start codon and the ribosome binding site (RBS) of the *omp* mRNAs are highlighted (this diagram was taken from Vogel & Papenfort, 2006).

5.4.2.2. Regulation of the RpoE-dependent ESR by sRNAs

Enterobacteria such as *E. coli* and *Salmonella* are now known to encode at least twelve OMP-regulating sRNAs (InvR, SsdR, MicA, MicL, MicC, MicF, IpeX, OmrAB, RseX, RybB and CyaR) (**Figure 28**). These sRNAs exert their functions under a variety of growth and stress conditions, including the σ^E-mediated ESR. An sRNA–OMP network is emerging in which some sRNAs act specifically on a single *omp* mRNA (i.e. *E. coli* MicC and MicF), whereas others control multiple *omp* and non-*omp* mRNA targets (i.e. *E. coli*

RybB). Importantly, these sRNAs serve to provide a rapid response and can either amplify the signal or act by a negative feedback.

5.4.2.2.1. RpoE-regulated MicA and RybB control of OMP synthesis

OmpA is highly conserved among enterobacteria. OmpA is abundant OMP as it occurs at approximately 100 000 copies/cell and has no pore but a structural function by anchoring the OM to the murein layer of the periplasmic space. The *ompA* mRNA is abundant and long-lived. However, it was early noted that *ompA* mRNA stability varied greatly depending on the growth rate: specifically, it becomes destabilized at the onset of stationary phase (541). Over the years, several models were invoked to explain this regulation, including a growth-dependent abundance of two factors, RNase E and Hfq, which were shown to affect *ompA* mRNA decay by degradation and competition with the 30S ribosomal subunit, respectively. However, none of these models was fully consistent, and was often in conflict with reports by others, as summarized in (537, 538, 542). The discovery of the sRNA MicA shed new light on this issue. MicA was first identified in a global *E. coli* sRNA screen, and observed to accumulate as a 70-nucleotide transcript when cells ceased growth (499). Two groups have now demonstrated that MicA accounts for much of the stationary phase specific instability of *ompA* mRNA (537, 538). In wild type cells, MicA levels inversely correlate with *ompA* mRNA levels during growth and the stationary phase specific decrease of *ompA* mRNA levels is abrogated upon *micA* deletion. Furthermore, overexpression of MicA results in reduction of OmpA protein levels. Overall, the underlying molecular mechanism of MicA is similar to that of MicC and MicF (537, 538). The MicA function does not contradict the previously observed roles of Hfq and RNase E in *ompA* mRNA decay. Indeed, as MicA strongly requires Hfq, both for its own intracellular stability and for annealing to *ompA* mRNA (537, 538). Furthermore, it is probable that by masking the *ompA* ribosome binding site, MicA accelerates the RNase E-dependent decay of this mRNA, similar to what has recently been demonstrated for other Hfq-dependent *E. coli* sRNAs (539, 540). Therefore, MicA provides a growth rate-specific factor for *ompA* mRNA decay.

In *E. coli*, RybB is a 80-nucleotide sRNA that is also synthesized in a σ^E-dependent fashion (333, 543, 489). Transient induction of RybB was first shown to decrease the levels of the mRNAs encoding OmpC and OmpW. Like most *trans*-encoded sRNAs, RybB binds to Hfq and act by base-pairing with targets mRNAs (333, 544, 545). Several groups working with *E. coli* and *Salmonella* reported that both RybB and MicA require σ^E for their transcription and down-regulate σ^E activity, thereby creating autoregulatory loop (333, 489-491). Interestingly, these studies questioned the primary simplistic model in which the specialized function of σ^E-dependent sRNAs is to stop de novo synthesis of abundant OMPs upon σ^E induction. In fact, RybB and MicA together target >30 mRNAs of *E. coli*: MicA represses the synthesis of major OMPs by targeting mRNAs of *ompA*, *ompX* and *lamB* and non-OMP targets like *phoP*, *lpxT* and *htrG* (512, 546, 547); RybB has even more *omp* mRNA targets and non-*omp* targets that include *waaR*, *htrG*, *fadL* and *rbsK/B*

(512, 491). Overall, the main function of MicA and RybB sRNAs seems to protect the cell from the loss of viability when σ^E activity is inadequate (512).

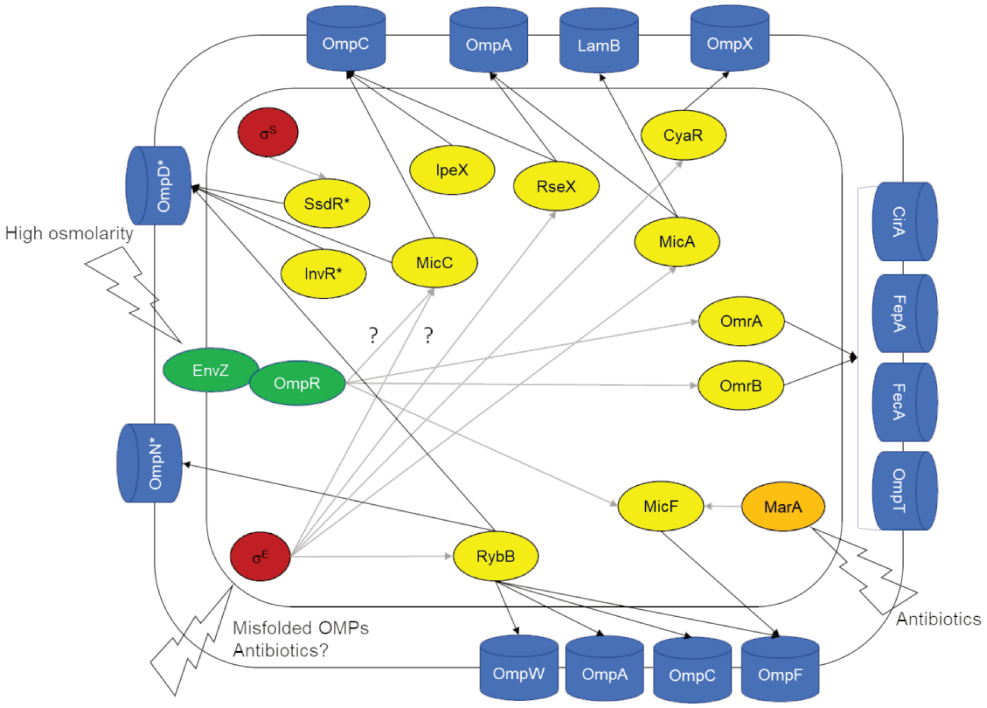

Figure 28: An emerging network of sRNAs that control OMP expression in enterobacteria. Regulatory sRNAs are shown in yellow circles within a schematic drawing of an *E. coli* cell. InvR and SsdR sRNAs are *Salmonella*-specific and are shown with a star symbol (*). The black lines indicate negative regulations of sRNAs on OMPs; the gray lines represent the impact of ESRs on sRNA expression (figure adapted from Papenfort & Vogel, 2006). See text for more details on the OMP targets, and the input signals of the network.

5.4.2.2.2. RpoE-regulated MicL (SlrA) and repression of Lpp synthesis

MicL was identified independently by two groups, during characterization of suppressors of Δ*lapB* mutants that overproduce LPS (508), and during examination of σ^E-regulated sRNAs (492). The *lapB* gene is essential and its absence leads to increased synthesis of LPS at the expense of phospholipids, aggregation of proteins involved in LPS biosynthesis and decoupling of LPS synthesis with LPS transport (508). Consequently, Δ*lapB* exhibits hyper-elevated levels of σ^E and Cpx regulons. The main function of MicL was shown to be the Hfq-dependent inhibition of *lpp* mRNA and repression of the RpoE

activity. In support of these results, overproduction of MicL mimics Δ*lpp* phenotype (508). Since Lpp contains three acyl chains, reduction in Lpp synthesis by MicL can liberate pools of phospholipids that can overcome toxicity due to the excess of LPS and depletion of the common precursor for phospholipids. Interestingly, MicL is encoded within the *cutC* open reading frame and is transcribed from its own σ^E-regulated promoter (492, 508). This sRNA is synthesized as a precursor of 308 nucleotides and is processed to a mature 80-nucleotide sRNA located within the 3' UTR of *cutC*. Importantly, overexpression of MicL strongly represses elevated toxic levels of the σ^E regulon in Δ*lapB* and hence overcomes Δ*lapB* defects (508). This negative feedback control of the σ^E expression by increased synthesis of MicL is an additional robust mechanism of maintaining cellular homeostasis by the sRNA arm of σ^E. Indeed, recent global studies have revealed mRNAs of *rpoE*, *acpP*, *fabF*, *mazF* and *fixA* as additional base-pairing targets of MicL (501). Taken together, three σ^E-regulated sRNAs (RybB, MicA and MicL) provide a negative feedback arm.

5.4.2.2.3. Remodeling of LPS and integrated control by RpoE and PhoPQ

Under σ^E-inducing conditions, *E. coli* remodels its LPS synthesis that is controlled by σ^E-regulated sRNAs, particularly RybB. Upon the σ^E induction, the RybB sRNA represses the synthesis of WaaR glycosyltransferase, causing truncation in the outer core of LPS and concomitant incorporation of a third Kdo (WaaZ-dependent) and rhamnose in the inner core (547). Interestingly, under such conditions, the *waaZ* mRNA accumulation also increases, contributing to the synthesis of LPS with a third Kdo (547). This mode of LPS remodeling could be adaptive in function, as the non-essential *waaZ* gene becomes essential for the bacterial growth when the LPS is tetraacylated and for interaction with the host, since such an LPS cannot ligate O-antigen due to loss of the attachment site (547). This drastic shift causing the accumulation of a rare form of LPS is accompanied by a nonstoichiometric modification of the second Kdo by phosphoethanolamine (P-EtN). The transfer of P-EtN to the second Kdo is important for resistance to cationic antibiotics like polymyxin B and is mediated by the product of the RpoE-regulated *eptB* gene (548). The *eptB* mRNA synthesis is usually repressed by the PhoPQ-regulated MgrR and by the ArcZ sRNAs (548). However, this silencing by MgrR and ArcZ is overcome when σ^E is induced. MicA contributes to remodeling of LPS by inhibiting PhoP and LpxT at the post-transcriptional level by direct base pairing with these target mRNAs (512, 546). The PhoPQ two-component system regulates some LPS alterations and is required for transcription *mgrR*. The MicA-directed repression of LpxT is interesting, since LpxT mediates the phosphorylation of lipid A that causes an increase in the negative charge of LPS. The GcvB sRNA also negatively regulates the *phoPQ* expression that is independent of MicA (549). Interestingly, SoxS, a global regulator of the oxidative stress response, is a positive regulator of transcription of the waaY gene, whose product phosphorylates heptose II and is itself subjected to a negative regulation by the MgrR sRNA (510). Another sRNA that regulates LPS modification is MicF, since

base pairing of MicF within the coding sequence of the *lpxR* mRNA decreases its stability by rendering it susceptible to degradation by RNase E (550). LpxR is a lipid A deacylase and this modification occurs after LPS translocation. Hence, the regulation of LpxR by MicF contributes to the LPS modification event that occurs in the OM, expanding the role of sRNAs at various steps of the LPS biosynthesis (505). Such controls exerted by sRNAs on regulators of the stress response that at the same time regulate LPS structural alterations demonstrate rewiring of networks to cope with envelope stress.

5.4.3. Bacterial adaptation to antibiotics through sRNAs

As described in the sections above, an increasing number of sRNAs has recently been discovered and shown to possess regulatory functions. sRNAs are synthetized under specific environmental conditions (*e.g.* those causing envelope stresses) and play a major role in the regulation of various physiological processes (*e.g.* the maintenance of envelope homeostasis). Most of them act via an imperfect anti-sense base-pairing with their target mRNAs, and duplex formation usually results in the inhibition of mRNA translation. Compared to protein-based regulatory mechanisms, sRNAs requires less energy, act faster, and allow a coordinated response on multiple targets. Owing these characteristics, sRNAs allow efficient bacterial adaptation to changing environments. Therefore, one can argue a possible link between sRNA expression and adaptation to antibiotic exposure. In particular, exposure to sub-inhibitory antibiotic concentrations is not lethal but induces protective stress response(s) to reduce antibiotic activity and impact antimicrobial susceptibility.

Accumulating evidence illustrates that *trans*-encoded sRNAs are key players in regulatory circuits controlling antibiotic resistance (reviewed in 551, 552, 553) in both Gram-positive and Gram-negative bacteria. Typically, antibiotic resistance circuits govern various processes, including functions required for antibiotic uptake (554, 555, 556, 557), modifications of the cell envelope (558, 559), drug efflux pumps expelling antibiotics (560, 561), metabolic enzymes conferring resistance (562), production of biofilms protecting from antibiotics (563) and DNA mutagenesis mechanisms facilitating evolution of novel resistances (564). In this section, I will essentially focus on sRNA controlling the Gram-negative envelope permeability.

5.4.3.1. sRNAs modulating drug uptake

As discussed earlier in this manuscript, antibiotics must first cross the OM of Gram-negative bacteria to reach their intracellular targets through a lipid-mediated pathway (for hydrophobic antibiotics) or via water-filled porins (for hydrophilic antibiotics). To become resistant, bacteria can alter permeation of antibiotics through the OM by modifying these uptake pathways. Interestingly, the expression of some of these macromolecules can be regulated by sRNAs and therefore impacts resistance. In *E. coli*, GcvB sRNA regulates the *sstT*, *oppA*, and *dppA* genes involved in amino acid,

dipeptide, and oligopeptide transport (565). GcvB also negatively regulates *cycA* mRNA, which encodes a permease for glycine, D-alanine, D-serine, and D-cycloserine transport into the bacteria (554). As a consequence, a Δ*gcvB* mutant is more susceptible to D-cycloserine than the parental strain, due to increased CycA levels and increased transport of the antibiotic (554). GcvB also negatively regulates the PhoPQ two-component system by translational repression of PhoP and could be involved, through *eptB*, in LPS modifications and resistance to CAMPs (see below).

In *E. coli*, MicF sRNA regulates *ompF* expression by pairing with *ompF* mRNA, inducing translation inhibition and mRNA degradation and in turn reducing permeability with respect to several antibiotics (308). Overexpression of MicF in *E. coli* increases cephalosporin, norfloxacin, and minocycline MICs, whereas depletion of this sRNA reverses those phenotypes, except for minocycline (556).

5.4.3.2. sRNAs modulating drug efflux

DsrA sRNA is a key regulator of essential pathways in *E. coli*, including general stress response (σ^S), genome compaction (H-NS), cell wall biosynthesis (MreB), and ribose metabolism (RbsD) (566). DsrA is also involved in antimicrobial resistance by regulating the expression of the MdtEF efflux pump (560). Indeed, when overexpressed in an efflux-defective Δ*acrB* mutant, the presence of DsrA results in significantly increased oxacillin MICs (×8), erythromycin MICs (×4), and novobiocin MICs (×4) via an RpoS-dependent pathway.

In *E. coli*, overexpression of RyeB increases susceptibility to quinolones (556). Overexpressing RyeB results in a decrease in the expression level of *tolC* mRNA, whereas *tolC* mRNA expression is upregulated in a Δ*ryeB* mutant. Named SdsR in *Salmonella* spp., RyeB is an abundant stationary-phase Hfq-dependent sRNA whose transcription depends on σ^S (511). SdsR represses *tolC* mRNA levels by pairing with its 5' UTR, 33 nucleotides upstream of the target mRNA RBS (561). SdsR overexpression also increases susceptibility to other antibiotics such as novobiocin and, to a lesser extent, erythromycin and rifampin. SdsR represses biofilm formation independently of pairing with *tolC* mRNA, suggesting additional targets. SdsR is a conserved sRNA from enterobacteria, and its activity in *tolC* mRNA repression was also found in *Salmonella* (567).

5.4.3.3. sRNAs modulating LPS and cell wall synthesis

In Gram-negative bacteria, LPS and cell wall are the respective targets of CAMPs including polymyxins and β-lactams. A common mechanism of resistance to CAMPs is LPS modifications (568). MgrR, a Hfq-dependent sRNA expressed in *E. coli* and other *Enterobacteriaceae*, is part of the PhoPQ regulon, that has a PhoP- PhoQ two-component system activated under conditions of low Mg^{2+} levels or by CAMPs. MgrR actually

downregulates *eptB* mRNA, which encodes a phosphoethanolamine transferase involved in LPS modifications (558). As mentioned earlier, EptB modifies the core oligosaccharide of the LPS, which reduces the net anion charges and electrostatic repulsion between LPS molecules, leading to polymyxin resistance. An *mgrR*-deleted mutant is 10 times more resistant to polymyxin B than the parental strain, whereas complementation of the *mgrR* mutation restores polymyxin susceptibility. It is noteworthy that the reduction of *eptB* mRNA levels by MgrR was also previously demonstrated in *Salmonella* (559). Additionally, in *E. coli*, the expression of *phoPQ* is directly repressed by the MicA sRNA (546). This may influence CAMP resistance since it downregulates MgrR, via its action on PhoP, which itself represses *eptB* mRNA expression.

Although less documented than LPS modifications, structural modifications of the Gram-negative cell wall contribute to antibiotic resistance. Peptidoglycan synthesis involves an array of enzymes across all cellular compartments (cytoplasm, inner membrane, and periplasm), and the expression of some of these enzymes can be regulated by sRNAs. GlmS catalyzes synthesis of glucosamine-6-phosphate (GlcN6P) from fructose-6-phosphate and glutamine, a key metabolite in cell wall biosynthesis. GlcN6P is further converted by GlmM and GlmU enzymes to UDP-*N*-acetylglucosamine (UDP-GlcNAc), a common precursor for peptidoglycan and LPS synthesis. Bacilysin (tetaine) and Nva-FMDP [L-lysyl-L-norvalyl-N(3)-(4-methoxyfumaroyl)-L-2,3-diaminopropanoic acid] are dipeptide antibiotics that impair cell envelope synthesis by GlmS inhibition through covalent modification (569). In *E. coli* and, presumably, in most *Enterobacteriaceae* species, *glmS* expression is controlled by GlmY and GlmZ sRNAs (570). GlmZ pairs with and activates *glmS* mRNA translation. Although GlmY is similar to GlmZ with respect to sequence and predicted structure, GlmY lacks a region complementary to *glmS* mRNA and does not directly activate *glmS* translation. Instead, GlmY expression inhibits a GlmZ processing event, disallowing *glmS* translation activation. Thus, GlmY functions by titration of an RNA processing factor away from homologous GlmZ sRNA. The GlmY/GlmZ pair provides resistance to bacilysin. Both *E. coli* and *Salmonella* respond to these antibiotics by increasing *glmS* expression to compensate for GlmS activity inhibition (562). GlmS inhibition by antibiotics leads to GlcN6P deprivation, sensed by GlmY sRNA, triggering its accumulation. Cells adjust GlmS expression levels to overcome growth inhibition by the GlmS inhibitor.

5.4.4. RNA-Seq based transcriptomics

The transcriptome is the complete set of transcripts in a cell, and their quantity, for a specific developmental stage or physiological condition. Understanding the transcriptome is essential for interpreting the functional elements of the genome and revealing the molecular constituents of cells and tissues, and also for understanding development and disease. The key aims of transcriptomics are: to catalogue all species of transcript, including mRNAs, non-coding RNAs and small RNAs; to determine the transcriptional structure of genes, in terms of their start sites, 5′ and 3′ ends, splicing

patterns and other post-transcriptional modifications; and to quantify the changing expression levels of each transcript during development and under different conditions.
RNA-Seq uses recently developed deep-sequencing technologies. In general, a population of RNA (total or fractionated, such as poly (A)+) is converted to a library of cDNA fragments with adaptors attached to one or both ends (see fig 20). Each molecule, with or without amplification, is then sequenced in a high-throughput manner to obtain short sequences from one end (single-end sequencing) or both ends (pair-end sequencing). The reads are typically 30–400 bp, depending on the DNA-sequencing technology used. In principle, any high-throughput sequencing technology (571) can be used for RNA-Seq, and the Illumina IG (572), Applied Biosystems SOLiD (573) and Roche 454 Life Science (574) systems have already been applied for this purpose. The Helicos Biosciences tSMS system has not yet been used for published RNA-Seq studies, but is also appropriate and has the added advantage of avoiding amplification of target cDNA. Following sequencing, the resulting reads are either aligned to a reference genome or reference transcripts, or assembled de novo without the genomic sequence to produce a genome-scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene.

Although RNA-Seq is still a technology under active development, it offers several key advantages over existing technologies. First, unlike hybridization-based approaches, RNA-Seq is not limited to detecting transcripts that correspond to existing genomic sequence. For example, 454-based RNA-Seq has been used to sequence the transcriptome of the Glanville fritillary butterfly. This makes RNA-Seq particularly attractive for non-model organisms with genomic sequences that are yet to be determined. RNA-Seq can reveal the precise location of transcription boundaries, to a single-base resolution. Furthermore, 30-bp short reads from RNA-Seq give information about how two exons are connected, whereas longer reads or pair-end short reads should reveal connectivity between multiple exons. These factors make RNA-Seq useful for studying complex transcriptomes. In addition, RNA-Seq can also reveal sequence variations (for example, SNPs) in the transcribed regions. A second advantage of RNA-Seq relative to DNA microarrays is that RNA-Seq has very low, if any, background signal because DNA sequences can been unambiguously mapped to unique regions of the genome. RNA-Seq does not have an upper limit for quantification, which correlates with the number of sequences obtained. Consequently, it has a large dynamic range of expression levels over which transcripts can be detected: a greater than 9,000-fold range was estimated in a study that analyzed 16 million mapped reads in Saccharomyces cerevisiae18, and a range spanning five orders of magnitude was estimated for 40 million mouse sequence reads (575). By contrast, DNA microarrays lack sensitivity for genes expressed either at low or very high levels and therefore have a much smaller dynamic range (one-hundredfold to a few-hundredfold). RNA-Seq has also been shown to be highly accurate for quantifying expression levels, as determined using quantitative PCR (qPCR) and spike-in RNA controls of known concentration (575). The results of RNA-Seq also show high levels of reproducibility, for both technical and

biological replicates (572). Finally, because there are no cloning steps, and with the Helicos technology there is no amplification step, RNA-Seq requires less RNA sample.

Taking all of these advantages into account, RNA-Seq is the first sequencing-based method that allows the entire transcriptome to be surveyed in a very high-throughput and quantitative manner. This method offers both single-base resolution for annotation and 'digital' gene expression levels at the genome scale, often at a much lower cost than either tiling arrays or large-scale Sanger EST sequencing

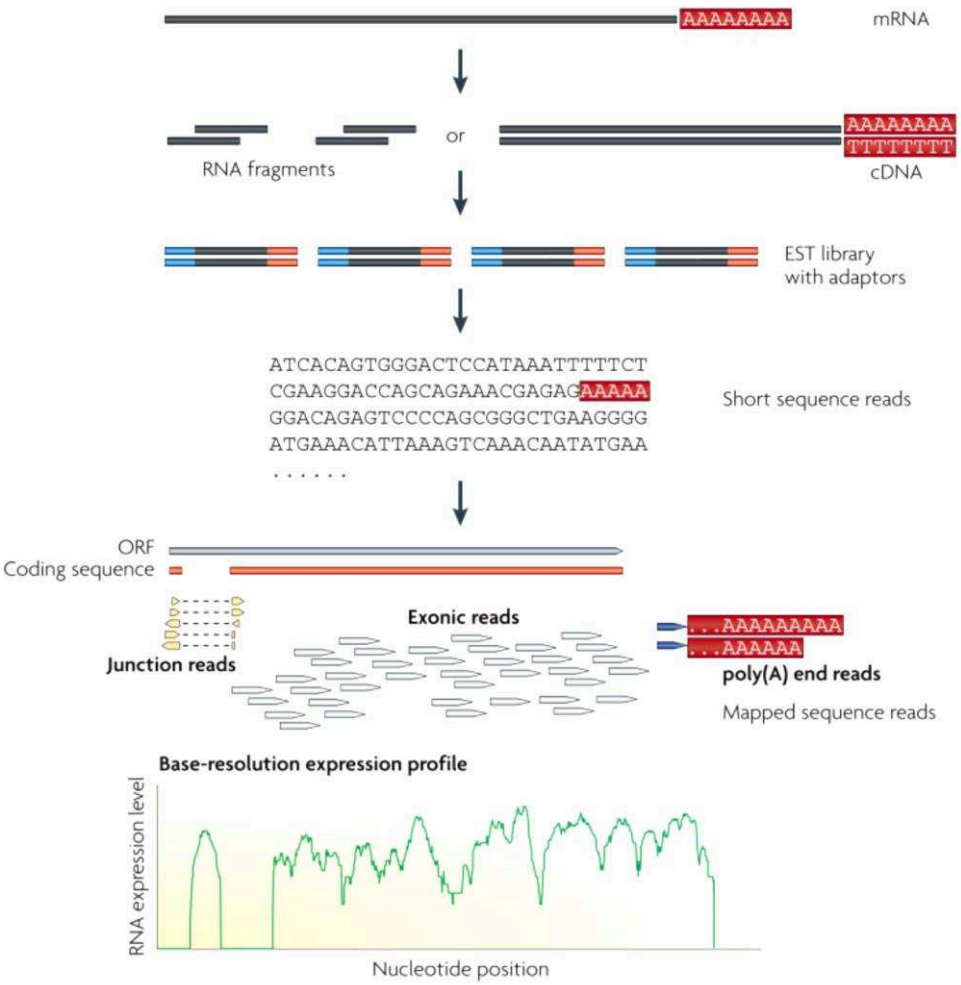

Figure 29: A typical RNA-Seq experiment- Briefly, long RNAs are first converted into a library of cDNA fragments through either RNA fragmentation or DNA fragmentation (see main text). Sequencing adaptors (blue) are subsequently added to each cDNA fragment and a short sequence is obtained from each cDNA using high-throughput sequencing technology. The resulting sequence reads are aligned with the reference genome or transcriptome, and classified as three types: exonic reads, junction reads and poly (A) end-reads. These three types are used to generate a base-resolution expression profile for each gene, as illustrated at the bottom; a yeast ORF with one intron is shown (figure acquired from Wang *et al*., 2009).

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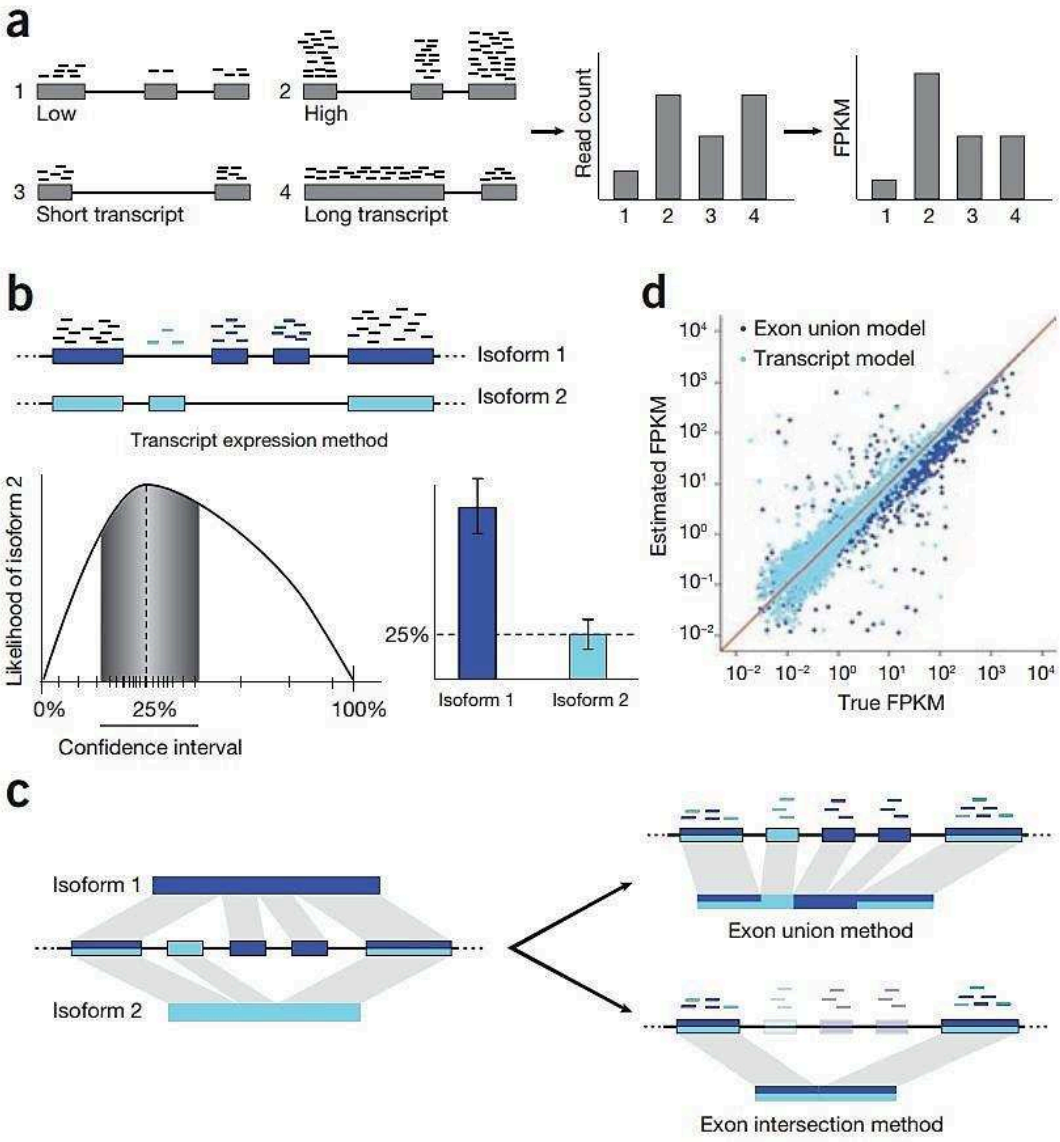

Figure 30: An overview of gene expression quantification with RNA-seq. (a) Illustration of transcripts of different lengths with different read coverage levels (left) as well as total read counts observed for each transcript (middle) and FPKM-normalized read counts (right). (b) Reads from alternatively spliced genes may be attributable to a single isoform or more than one isoform. Reads are color-coded when their isoform of origin is clear. Black reads indicate reads with uncertain origin. 'Isoform expression methods' estimate isoform abundances that best explain the observed read counts under a generative model. Samples near the original maximum likelihood estimate (dashed line) improve the robustness of the estimate and provide a confidence interval around each isoform's abundance. (c) For a gene with two expressed isoforms, exons are colored according to the isoform of origin. Two simplified gene models used for quantification purposes, spliced transcripts from each model and their associated lengths, are shown to the right. The 'exon union model' (top) uses exons from all isoforms. The 'exon intersection model' (bottom) uses only exons common to all gene isoforms. (d) Comparison of true versus estimated FPKM values in simulated RNA-seq data. The x = y line in red is included as a reference (picture taken from Garber *et. al.*, 2011).

Chapter 2 : Results

Aim of our study

Increase in the incidences associated with MDR Gram negative bacteria is alarming. An effective strategy to curb this resistance is by counteracting the bacterial drug impermeability. Gram negative bacteria such as *Escherichia coli* and *Enterobacter spp*. resort to altering their membrane permeability in order to limit the intracellular drug access. In this regard, a specific class of OM proteins (Omps) called porins is among the common targets. As porins function in the formation of water filled channels for movement of small molecules inside the cell, modulating their expression leads to reduced access of the antibiotics. In order to design a better class of drugs with enhanced translocation property across the cell membrane we need to know the molecular regulation of porins. The two classical porins OmpF and OmpC in *E. coli* have been shown to be post transcriptionally regulated by small RNAs *micF* and *micC,* respectively. The various external conditions (stress) responsible for the induction of these small RNAs especially *micC* are essentially unknown.

The objective of our study was to understand the role of MicC on porin including OmpC expression in *E. coli* and how this sRNA is influenced by other factors (external or genetic). One of the other major focuses was to explore the hypothetical regulatory relationship between *micC* and the adjacent *ompN* porin gene. We were further interested in finding out how this regulation impacts the overall antibiotic resistance phenotype of the bacteria.

In this study, we sought to determine the target suite of MicC in *E. coli*. Transcriptomics study was performed to identify previously unknown targets of MicC, which gave us an idea about the far fetching role of this sRNA in cellular physiology, beyond controlling the expression of OmpC. Our work has exhibited the involvement of external stress perpetuated by clinically important class of β- lactam antibiotics and sRNA regulated stress response pathways; those control the composition of porins in outer envelope via *micC* and *ompN*. This work is a stepping stone for understanding the interconnected regulatory mechanisms at play in bacteria when they are exposed to antimicrobials in their environments whether be in nosocomial or *in vivo* settings.

Scientific Article:

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antibiotics

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Article

Dual Regulation of the Small RNA MicC and the Quiescent Porin OmpN in Response to Antibiotic Stress in *Escherichia coli*

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Abstract: Antibiotic resistant Gram-negative bacteria are a serious threat for public health. The permeation of antibiotics through their outer membrane is largely dependent on porin, changes in which cause reduced drug uptake and efficacy. *Escherichia coli* produces two major porins, OmpF and OmpC. MicF and MicC are small non-coding RNAs (sRNAs) that modulate the expression of OmpF and OmpC, respectively. In this work, we investigated factors that lead to increased production of MicC. *micC* promoter region was fused to *lacZ*, and the reporter plasmid was transformed into *E. coli* MC4100 and derivative mutants. The response of *micC–lacZ* to antimicrobials was measured during growth over a 6 h time period. The data showed that the expression of *micC* was increased in the presence of β-lactam antibiotics and in an *rpoE* depleted mutant. Interestingly, the same conditions enhanced the activity of an *ompN–lacZ* fusion, suggesting a dual transcriptional regulation of *micC* and the quiescent adjacent *ompN*. Increased levels of OmpN in the presence of sub-inhibitory concentrations of chemicals could not be confirmed by Western blot analysis, except when analyzed in the absence of the sigma factor σ^E. We suggest that the MicC sRNA acts together with the σ^E envelope stress response pathway to control the OmpC/N levels in response to β-lactam antibiotics.

Keywords: *Escherichia coli*; outer membrane porins; regulatory small RNAs; membrane transport; antibiotic susceptibility

1. Introduction

Antibacterial resistance is broadly recognized as a growing threat for human health [1–3]. As such, increasing antibiotic treatment failures due to multidrug resistant (MDR) bacteria have stirred the urgent need to better understand the underlying molecular mechanisms and promote innovation, with the development of new antibiotics and alternative therapies [4,5]. The efficacy of antibacterial compounds depends on their capacity to reach inhibitory concentrations at the vicinity of their target. This is particularly challenging for drugs directed against Gram-negative bacteria, which exhibit a complex envelope comprising two membranes and transmembrane efflux pumps [6]. The Gram-negative envelope comprises an inner membrane (IM), which is a symmetric phospholipid bilayer; a thin peptidoglycan (PG) layer ensuring the cell shape; and an outer membrane (OM) that is an asymmetric bilayer, composed of an inner phospholipid leaflet and an outer leaflet of lipopolysaccharide (LPS) [7]. First, the OM is a barrier to both hydrophobic and hydrophilic compounds, including necessary nutrients, metabolic substrates and antibiotics, but access is provided by the water filled β-barrel channels called porins [8,9]. In *Escherichia coli*, the channels of the general porins OmpF and OmpC, are size restricted, and show a preference for passage of hydrophilic charged compounds, including antibiotics such as β-lactams and fluoroquinolones. Second, constitutive

tripartite RND (resistance–nodulation–cell division) efflux pumps, such as the AcrAB–TolC pump of *E. coli*, play a major role in removing antibiotics from the periplasm [10]. Importantly, it has been noted that the efflux pumps are synergized by the OM, since, once ejected into the extracellular space, compounds must re-traverse the restricted-permeability OM barrier [10]. Not surprisingly, MDR clinical isolates of *Enterobacteriaceae* generally exhibit porin loss and/or increased efflux, which both contribute to reduce the intracellular accumulation of antibiotics below the threshold that would be efficient for activity [9–11].

Given the importance of the OM in controlling the uptake of beneficial as well as toxic compounds, one can expect that the expression of porins depends on environmental factors, and is well-coordinated at the transcriptional and post-transcriptional levels. Best studied transcriptional regulators are the IM sensor kinase EnvZ and its cognate response regulator OmpR [12]. EnvZ autophosphorylates in response to a specific envelope stress, such as high osmolarity, then transfers its phosphate group to OmpR. OmpR and OmpR-P have different binding affinities to the porin promoters. At low osmolarity, OmpR activates *ompF* transcription, whereas at high osmolarity, OmpR-P represses *ompF* transcription and activates *ompC* transcription. This differential regulation of OmpF and OmpC is consistent with that in high osmolarity environments, such as in a host where nutrients are abundant, the small pore porin OmpC is predominant, thus limiting the uptake of toxic bile salts; whereas in low osmolarity environments where nutrients are scarce, the large pore porin OmpF is expressed [8]. EnvZ–OmpR [12] and CpxA–CpxR [13] are the main two-component systems involved in the transcriptional control of OmpF and OmpC. Interestingly, the two systems are interconnected [14], and mutations have been found in response to antibiotic stresses [15] (Masi M, Pagès J.-M and Kohler T, personal observations).

The post-transcriptional repression of OmpF by the small regulatory RNA (sRNA) MicF has been discovered in 1984 [16–18]. This 93 nucleotide (nt) RNA is divergent to the *ompC* gene, and acts by direct base-pairing to a region that encompasses the ribosome binding site (RBS) and the start codon of the *ompF* mRNA, thus preventing translation initiation [19]. The expression of the MicF sRNA is subjected to multiple signals and regulatory pathways [20]. Positive regulation includes EnvZ–OmpR in high osmolarity conditions [21], SoxS in response to oxidative stress [22], and MarA in response to antibiotic stress [23]. The 109 nt MicC sRNA has been discovered more recently, and shown to repress OmpC by direct base-pairing to a 5′ untranslated region of the *ompC* mRNA [24]. Interestingly, MicC is transcribed clockwise, and is opposite to the adjacent *ompN* gene that encodes a quiescent porin homologous to OmpF and OmpC [25]. Due to the similar genetic organization of *ompN–micC* and *ompC–micF*, and the co-induction of *ompC* and *micF* under specific conditions (i.e., high osmolarity via EnvZ–OmpR), it has been suggested that *ompN* and *micC* could also be subjected to dual regulation [24]. With the recent interest in post-transcriptional regulators, additional sRNAs that modulate expression of abundant OM proteins have been found. As yet, the *ompC* mRNA is targeted by multiple sRNAs MicC [24], RybB [26], RseX [27], and IpeX [28–30]. To date, external growth conditions and regulatory factors that control the expression of MicC and/or OmpN remain largely unknown.

In this work, we first examined the transcription of *micC* and *ompN* in *E. coli* MC4100 cells grown under a series of external conditions by using *lacZ* transcriptional fusions and β-galactosidase assays. We optimized the assay by using 96-well microtiter plates, and screened the entire collection of compounds provided by the Biolog Phenotype MicroArrays^{TM} for bacterial chemical susceptibility, in order to extend the range of putative inducing cues. Results showed that high concentrations of carbapenems and cephalosporins, two clinically relevant classes of β-lactams, induce both *micC* and *ompN*. Then, the impact of carefully chosen inducing conditions on the expression levels of OmpC and OmpN was tested by Western blotting with appropriate antisera. Because the OmpN protein was undetectable in the presence of mild antibiotic stress conditions, we reasoned to investigate the transcription of *micC* and *ompN* in a series of MC4100 derivatives carrying null mutations or multicopy plasmids in order to identify putative transcriptional regulators. Interestingly, we found that OmpN was specifically expressed when the envelope stress sigma factor σ^E was depleted by the overexpression of the anti-sigma RseA, or when the *hns* gene encoding the histone nucleoid structuring

protein, H-NS, was inactivated. Finally, we examined the functional relevance of OmpN as compared to OmpC and OmpF, with respect to drug translocation.

All these data are discussed considering the current knowledge on the Gram-negative envelope stress response pathways.

2. Results

2.1. Screening of MicC and OmpN Inducing Conditions Using LacZ Transcriptional Fusions and Biolog^{TM} Plates

Changes in porin expression play a major role in the development of antibacterial resistance. Because increased levels of MicC are associated with a decreased expression of OmpC, we aimed to examine the expression profile of the MicC sRNA by using a *micC–lacZ* transcriptional fusion in MC4100 cells grown under a series of growth conditions and β-galactosidase assays. First, we selected a number of representative growth conditions, some of which are sensed by known regulatory factors: growth phase (stationary phase accumulates RpoS), exposure to heat shock, high osmolarity (activates EnvZ–OmpR), iron or nitrogen starvation, or exposure to chemicals, such as salicylate (activates MarA), paraquat (activates SoxR/S), or different classes of antibiotics (β-lactams and fluoroquinolones). To determine whether MicC and OmpN are co-regulated, the β-galactosidase activity of an *ompN–lacZ* transcriptional fusion was also tested in MC4100 grown under the same conditions. These preliminary assays showed that growth conditions that are known to induce specific regulatory factors, such as RpoS, EnvZ–OmpR, MarA and SoxR/S, do not significantly affect the activity of the *micC–* and *ompN–lacZ* fusions, suggesting that the expression of MicC and OmpN is not controlled by these regulators. Instead, these assays allowed the identification of β-lactams potent inducers of both the *micC–* and *ompN–lacZ* fusions. As an example, Figure 1a shows that increasing concentrations of the carbapenem biapenem were accompanied with increased β-galactosidase activities. In order to extend the range of putative inducing compounds, we optimized the β-galactosidase assay using preloaded 96-well microtiter plates, and then screened Phenotype MicroArrays^{TM} plates (Biolog PM11 to PM19) for bacterial chemical susceptibility (Supplementary Data 1). A total of 18 compounds were found to increase the activity of the *micC–* and *ompN–lacZ* fusions more than 10 times, and 6 of them were selected for further investigations. Concentrations of compounds for β-galactosidase assays adapted to microtiter plates were determined with respect to their MICs (Supplementary Data 2). The data showed that the activity of the *micC–* and *ompN–lacZ* fusions were strongly increased when cells were exposed to carbapenems (i.e., biapenem and ertapenem) or cephalosporins (i.e., ceftazidime and cefepime) (Figure 1b). Interestingly, these compounds belong to the most potent subclasses of clinically used β-lactams used for treating Gram-negative infections. Other strong inducers include antiseptics (e.g., benzalkonium chloride and benzethonium chloride) and anesthetics (e.g., chlorpromazine HCl), which are also used in the clinics (Figure 1b).

2.2. Effects of MicC and OmpN Inducing Conditions on the Expression Levels of OmpC and OmpN

The effect of MicC overexpression on *ompC* expression was first examined by monitoring OmpC protein levels directly. MC4100 was transformed with the MicC overexpression plasmid (pSD01) and the corresponding empty vector (pDrive). Cultures were induced with IPTG to allow MicC expression, OM extracts were prepared, and levels of OmpC were analyzed by Western blot (WB) with specific anti-peptide antibodies. As shown in Figure 2a, the overexpression of MicC clearly resulted in reduced OmpC levels, confirming that the MicC sRNA represses *ompC* expression. As noted in the section above, high *micC–lacZ* activities were obtained in the presence of high concentrations of compounds, which were detrimental for the cell growth. Therefore, MC4100 was cultured in the presence of sub-inhibitory concentrations of inducing compounds—namely biapenem, imipenem, ertapenem, ceftazidime, cefepime, and chlorpromazine HCl—in order to obtain exponentially grown cells and

examine their effect on OmpC protein levels. As shown in Figure 2a, these conditions only weakly altered OmpC levels.

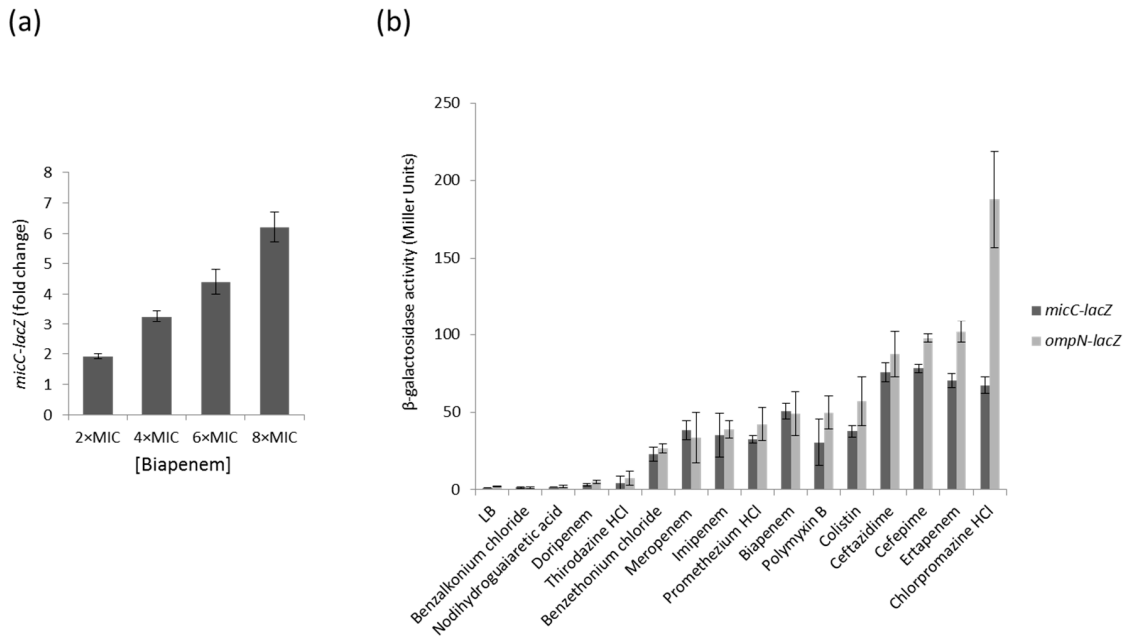

Figure 1. (**a**) Dose dependent *micC–lacZ* activity in presence of increasing concentrations of biapenem (MIC of 0.32 μg/mL); (**b**) β-galactosidase activity of the *micC*- and *ompN-lacZ* fusions in the presence of selected compounds. Values are means from three independent determinations, and standard deviation is represented.

Given the co-induction of *micC* and *ompN*, we also tested whether OmpN expression was increased in the same samples. As a control, MC4100 was transformed with the OmpN overexpression plasmid (pSD04) and the corresponding empty vector (pTrc99A). Cultures were induced with IPTG to allow OmpN expression; OM extracts were prepared and tested for OmpN expression by WB. For this, we generated antibodies against a peptide in loop 7 present in OmpN, but absent in OmpF and OmpC. A single protein of about 39 kDa was detected in the OM extracts of MC4100 (pSD04), but not in that of MC4100 (pTrc99A), suggesting that the detected band is OmpN without cross-reactivity to other porins, and that OmpN production from the chromosome is undetectable (Figure 2b). However, OmpN production was also undetectable in OM extracts prepared from cells grown in the presence of sub-inhibitory concentrations of *micC* inducing compounds (Figure 2b).

These results suggest that transient exposure of the cells to sub-inhibitory concentrations of *micC–lacZ* inducing compounds was not sufficient to yield high levels of MicC and concomitant changes in the porin expression profile. Moreover, it is worth to note that Western blot analysis only provides steady-state levels of OmpC and OmpN. Additional reverse transcription PCR and pulse-chase experiments are needed to conclude the effects of *micC* and *ompN* inducing conditions on the expression of OmpC and OmpN at the transcriptional and post-transcriptional levels, respectively.

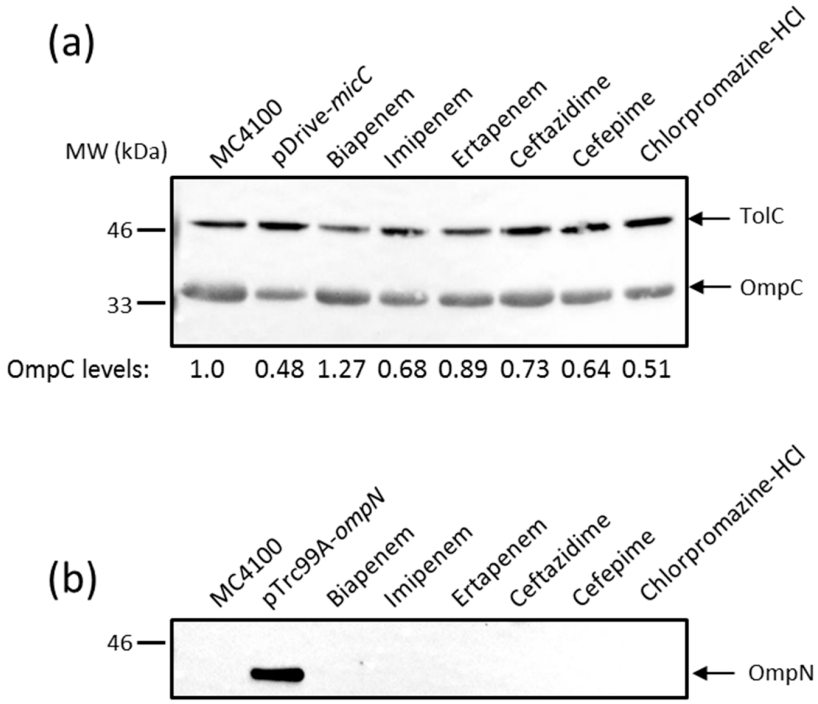

Figure 2. Western blot (WB) analysis of outer membrane (OM) proteins. Cells were grown, and OM extracts were prepared as described in the Materials and Methods. OM proteins equivalent to 0.2 OD_{600} units of cultures were separated by SDS-PAGE, electrotransferred on nitrocellulose membranes, and blotted with the appropriate anti-sera. Data show the production of OmpC (**a**) and OmpN (**b**). Both the positive controls pDrive-*micC* and pTrc99A-*ompN* were induced by 0.4 mM IPTG for 3 h. TolC expression was used for normalizing sample loading, and the expression of normalized OmpC has been expressed in mean values from three independent experiments.

2.3. Identification of Genetic Factors That Impact on MicC and OmpN Expression

micC–lacZ and *ompN–lacZ* transcriptional fusions were transformed into MC4100 derivatives carrying either chromosomal null mutations or overexpression plasmids of several regulatory factors, in order to identify putative repressors or activators, respectively. In *Enterobacteriaceae*, global regulators MarA and RamA have been reported to induce MDR associated with an increase in efflux pump production and a decrease in OmpF expression levels [31,32]. We detected no induction of the reporter fusions, either when these factors were overexpressed from multicopy plasmids or when the corresponding genes were inactivated (data not shown). This observation suggests that the *micC–ompN* operon is not part of the MarA and RamA regulatory pathways, or is strongly silenced by an upstream repressor.

Previous Northern blotting analysis showed that the expression of MicF (repressor of OmpF) was opposite to that of MicC (repressor of OmpC) under most of the tested conditions [24]. Because the osmoregulator OmpR is known to modulate MicF and control the opposite expression of OmpF and OmpC, we tested the impact of an *ompR* mutation on *micC* and *ompN* expression. Here, the activity of the *micC–lacZ*, but not that of the *ompN-lacZ* fusion, was slightly increased in the *ompR* null mutant, thus confirming that OmpR represses MicC (Figure 3a). Whether this regulation is direct or indirect is still unknown.

The last decade has been marked by the identification of several sRNAs. These are differentially expressed, and have been assigned to various important regulons of *E. coli* and *Salmonella*. Examples include the RyhB sRNA as a member of the iron-responsive Fur regulon [33]; MicA and RybB, which are activated by the envelope stress sigma factor, σ^E [26,34,35]; CyaR, whose transcription is governed by the cAMP-CRP complex [36,37]; ArcZ and FnrS, which respond to oxygen availability via the

ArcA/B or Fnr systems [38,39]; MgrR, which is a member of the Mg^{2+}-responsive PhoP/Q regulon [40]; SdsR, which is selectively transcribed by the major stationary phase and stress sigma factor, σ^S [41]; and CpxQ, which responds to the CpxA/R two-component envelope stress system [42,43]. Focusing on envelope stress responses and expression of OM proteins, we examined the impact of CpxA/R and σ^E on *micC* and *ompN* induction. Constitutive activation of the Cpx stress response, by multicopy plasmids expressing an autoactivated CpxA [15] or the signaling lipoprotein NlpE [44], did not increase the activity of the reporter fusion (data not shown). In the opposing scenario, when cells were depleted of σ^E upon the overexpression of its cognate anti-sigma RseA, the activity of both the *micC*– and *ompN–lacZ* fusions resulted in a 3–4-fold increase (Figure 3a). Additionally, OmpN was detected in OM extracts of cells grown under the same conditions (Figure 3b). We suggest this regulation is most likely indirect, as the *micC–ompN* intergenic region does not contain a σ^E core promoter motif [45]. Because RybB is one of the most abundant sRNA, represses OmpC as well as other OM proteins, and is part of the σ^E regulon in *E. coli* [46], we hypothesized that OmpN could be silenced by RybB. However, the activity of the *ompN–lacZ* fusion did not increase in *rybB* and *hfq* mutants, suggesting that the *ompN* mRNA is not targeted by RybB or any other Hfq-dependent sRNA (Figure 3a,b).

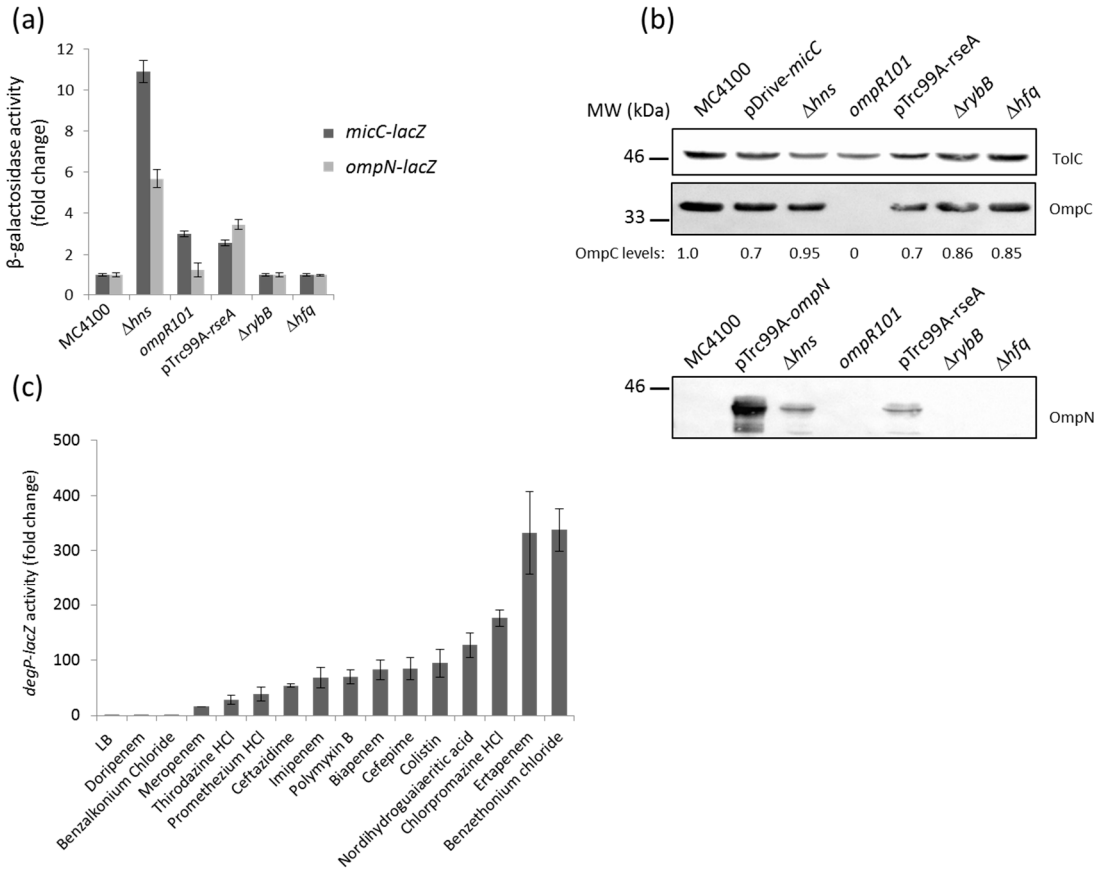

Figure 3. (**a**) β-Galactosidase activity of the *micC*– and *ompN–lacZ* fusions in different genetic backgrounds. Envelope stress sigma factor σ^E is essential in *Escherichia coli*. Therefore, cells were temporarily depleted of σ^E by the overexpression of the anti-sigma factor RseA with 0.4 mM IPTG under heat shock conditions at 42 °C; (**b**) WB analysis of OM proteins. Cells were grown, and OM extracts were prepared as described in the Materials and Methods. OM proteins equivalent to 0.2 ODU of cultures were separated by SDS-PAGE, electrotransferred on nitrocellulose membranes, and blotted with the appropriate anti-sera. Data show the production of OmpC (upper panel) and OmpN (lower panel). TolC expression was evaluated for normalizing sample loading and the expression of normalized OmpC has been expressed in numerical values below the bands; (**c**) β-galactosidase activity of a *degP–lacZ* chromosomal fusion in response to various external stresses.

In order to explore the connection between σ^E and the MicC/OmpN inducing compounds, we examined the effect of the latter on the expression of DegP, a periplasmic protease/chaperone member of the σ^E regulon, by using a *degP–lacZ* fusion [47]. Interestingly, all the compounds that had been identified as inducers of *micC*– and *ompN–lacZ* also activated *degP–lacZ* (Figure 3c). These results suggest a strong link between toxic compounds that target the bacterial envelope, the envelope stress σ^E pathway, and MicC/OmpN expression [48].

Previous studies on porin regulation reported that the H-NS nucleoid protein binds to the *micF–ompC* intergenic region. Expression of the major OM proteins, OmpF and OmpC, is affected by *hns* mutations, such that OmpC expression increases via direct effect at the transcriptional level, while OmpF expression decreases via indirect regulation by the MicF sRNA at the post-transcriptional level [49,50]. Comparative transcriptomic and proteomic studies further confirmed the influence of H-NS on the expression of OmpF and OmpC, but also indicated that *ompN* was upregulated in an *hns* mutant [51]. Here, the activity of both the *micC*– and *ompN–lacZ* fusions was significantly increased (approximately by 11- and 6-fold, respectively) in an *hns* mutant (Figure 3a). The OM profile of this mutant is shown and indicates that the expression level of both OmpC and OmpN is increased by 2–3-fold (Figure 3b). Considering that MicC functions as a repressor of OmpC, negative regulation of non-identified OmpC repressors by H-NS could explain upregulation of OmpC in the *hns* mutant.

2.4. Role of OmpN in Antibiotic Translocation

OmpF and OmpC porins represent the preferred route for the uptake of β-lactam antibiotics across the OM of *E. coli* [6,8,9]. Although OmpN is quiescent porin in *E. coli* [25], the orthologous OmpK37 of *Klebsiella pneumoniae* has been shown to be expressed at low levels under standard laboratory growth conditions, but highly expressed in β-lactam-resistant clinical isolates [52]. As a first step to investigate the role of MicC/OmpN in antibiotic susceptibility profile, we examined the expression levels of OmpF, OmpC, and OmpN in a collection of *E. coli* β-lactam-resistant clinical isolates by WB analysis. None of these isolates produced detectable OmpF, OmpC, or OmpN (Supplementary Data 3). Here, it should be noted that the anti-OmpN antibodies are directed against amino acid residues of the extracellular loop 7, which are specific of *E. coli* OmpN, but also submitted to variability between strains of this species. The impact of MicC in the downregulation of OmpC in these isolates is not known, and should be further investigated by Northern blot analysis. Second, we used a whole cell-based assay to compare the role of OmpN to that of OmpF and OmpC in the uptake of β-lactam antibiotics. To do this, the metabolic activity of *E. coli* W3100Δ*ompF*(pTrc99A) ($OmpF^-$ $OmpC^+$), W3100Δ*ompC*(pTrc99A) ($OmpF^+$ $OmpC^-$), W3100Δ*ompF*Δ*ompC*(pTrc99A) ($OmpF^-$ $OmpC^-$) and W3100Δ*ompF*Δ*ompC*(pSD04) ($OmpF^+$ $OmpC^-$ $OmpN^+$) was monitored in the absence and in presence of representative β-lactams added at inhibitory concentrations, with regards to their capacity to inhibit the reduction of the viability dye resazurin [6]. The results showed that the metabolic activity of *E. coli* expressing either OmpF or OmpC, but not OmpN, was significantly inhibited upon exposure to β-lactams, suggesting that OmpN is not competent for translocation of this class of antibiotics (Figure 4). However, other approaches, such as liposome swelling assays with reconstituted OmpN, are necessary to conclude on this point.

OmpF and OmpC channels are also used for the translocation of various colicins across the OM of *E. coli* [53]. We examined the sensitivity of *E. coli* strains expressing OmpF, OmpC, or OmpN to colicins E2 and E3, by spotting serial 2-fold dilutions onto cell lawns. Interestingly, the expression of any of the three porins yields similar sensitivity (titers of 2×10^{-7}), suggesting that OmpN channels are able to bind and transport porin-dependent group A colicins across the OM of *E. coli* (data not shown). This also points to the different mechanism of antibiotic versus colicin translocation through OM porin channels.

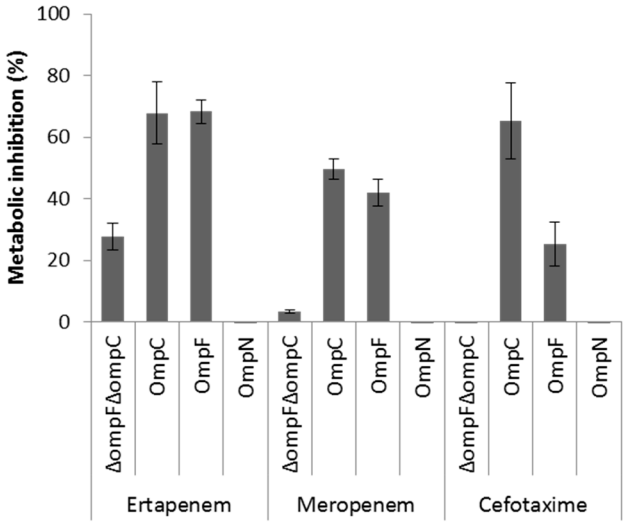

Figure 4. Metabolic inhibition of intact cells expressing OmpF, OmpC, or OmpN in the presence of selected β-lactam antibiotics using a resazurin-reduction-based assay. Actively metabolizing bacterial cells are able to reduce blue resazurin into red resofurin, which emits fluorescence at 590 nm. The experiment was performed in a microtiter plate, and fluorescence was measured every 10 min with an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Inhibition of resazurin reduction in the presence of appropriate concentrations of each antibiotic was translated into % metabolic inhibition.

3. Discussion

sRNAs have become important players in bacterial gene regulation. To date, systematic genome-wide searches have led to the identification of approximately 80 sRNAs in *E. coli*, the majority of which are conserved in *Salmonella* and other closely related species. About one-third of the reported sRNAs repress synthesis of OM proteins. Evidence for important roles of sRNAs in this post-transcriptional regulation was previously established by the fact that the loss of Hfq, the sRNA chaperone [54], results in the overproduction of OM proteins [24,26,27,36,37,41].

In *E. coli*, the conserved Hfq-associated sRNA, MicC, was identified as a repressor of the synthesis of OmpC [24,54]. MicC inhibits the 30S ribosome binding through a conserved 22 bp RNA duplex near the start codon of the *ompC* mRNA [24]. Many parallels have been drawn between the MicC and MicF sRNAs. Both repress the expression of abundant porins by base pairing near the RBS, thereby blocking translation. Both are encoded opposite to another porin gene. Both are also conserved, together with their *omp* target sequences in *Salmonella*, *K. pneumoniae*, and *Enterobacter* spp. However, major questions such as (i) environmental conditions and/or intracellular regulatory pathways that promote maximal expression of MicC; (ii) the co-regulation of MicC and OmpN; (iii) the impact of such regulation on antibiotic susceptibility; and (iv) the prevalence of MicC/OmpN in MDR clinical isolates remain unanswered. In this work, we used *lacZ* transcriptional fusions and β-galactosidase assays to show that the expression of *micC* and *ompN* is co-regulated in response to antibiotic stress. In particular, β-lactam antibiotics are among the most potent inducers of both *micC* and *ompN*. Interestingly, we found that expression of OmpN from a plasmid could not restore the susceptibility of an *E. coli* porin-less strain to β-lactams. In addition, other studies have demonstrated that strains expressing OmpN, but not OmpF or OmpC, were less susceptible to β-lactams [52,55].

Our results also identified that envelop stress sigma factor σ^E and H-NS are two major negative regulators of MicC/OmpN. σ^E is widespread among pathogenic and non-pathogenic bacteria, and becomes activated when bacterial envelope homeostasis is perturbed due to misfolding of OM proteins in the periplasm, or severe OM damage by external stresses [56]. In both cases, the bacteria must decrease the synthesis of major OM proteins. It has been shown that MicA and RybB are the two

most abundant sRNAs responsible for the rapid decay of *omp* mRNAs upon activation of the σ^E envelope stress response [46,57]. Although β-lactams were found to be potent inducers of the σ^E envelope stress response, RybB nor any other Hfq-dependent sRNA could be responsible for *ompN* silencing. This suggests that *ompN* is not subjected to sRNA post-transcriptional regulation. On the other hand, H-NS is a major component of the bacterial nucleoid, and has pleiotropic effects on gene expression, genome stability, and DNA recombination. Previous work has shown that H-NS was required for full expression of OmpF, and that this involves a role for H-NS in repressing the expression of MicF sRNA [48]. Our results also showed that H-NS had a role in repressing the expression of MicC and OmpN.

4. Materials and Methods

4.1. Plasmids and Bacterial Strains

All the *E. coli* strains and plasmids used in this study are listed in Table 1. *E. coli* MC4100 and derivatives were used for *lacZ* reporter gene assays and protein expression analysis. Knockout mutants were generated by P1 transduction from different sources and cured by using the FLP helper plasmid pCP20 to remove the kanamycin resistance cassette [58]. Strains were routinely grown in Luria Bertani (LB) broth (Sigma, Saint Quentin Fallavier, France), supplemented with the following antibiotics when necessary: ampicillin, 100 µg/mL (Amp); kanamycin, 50 µg/mL (Kan); chloramphenicol (Cam), 30 µg/mL; streptomycin 50 µg/mL (Str). *E. coli* W3110 and derivatives were used for translocation assays.

4.2. Plasmid Construction

Genomic DNA was extracted from MC4100 by using the Wizard® Genomic purification kit (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions, and used as a template for all PCR-amplifications. *micC–* and *ompN–lacZ* transcriptional fusions were constructed in the promoter-less *lacZ* containing vector pFus2K [59]. A 184 nt fragment containing the MicC promoter was amplified by using the primer pair SD1 (5′-*TTACGTATC*GGATCC TCGGGGAGTGAAAACATCCT-3′) and SD2 (5′-GCGGATCCCCGCGCAGAATAACGTAT-3′), which contain BamHI restriction sites (underlined) for classic restriction/ligation cloning into BamHI restricted pFus2K (Supplementary data 4) in the orientation of *micC–lacZ* (pSD02). Because the transcription start of *ompN* is only based on promoter prediction, the entire intergenic region between MicC and OmpN was PCR-amplified by using the primer pair SD3 (5′-*GAGCTCGCATGC* GGATCCTGAATAAATCCTTTAGTTATT-3′) and SD4 (5′-*CAGGACTCTAGA*GGATCCCCGCGC AGAATAACGTAT-3′). This generated a 227 nt fragment, which contained BamHI restriction sites (underlined) and extension homologous to BamHI restricted pFus2K for cloning using the In-Fusion ™ cloning kit (Clontech, Saint Germaine n Laye, France), in the orientation of *ompN–lacZ* (pSD03) (Supplementary Data 4). For overexpression of the MicC sRNA, a 410 nt PCR fragment was generated by using the primer pair SD1 and SD5 (5′-*AGGCTCGAG*AAGCTT AGATGCTGCAGCTGAATTTG-3′) inserted into the pDrive vector restricted with BamHI and HindIII under the control of an IPTG inducible promoter by using the In-Fusion ™ cloning kit (pSD01) (Supplementary Data 1). Recombinant plasmids pSD04 and pSD05 were obtained by InFusion cloning of fragments into the pTrc99A vector after digestion with appropriate restriction enzymes. pSD04 contains ompN, which was PCR-amplified by using the primer set SD6 (5′-*CATG*GAATTCATGAAAAGCAAAGTACTGGCAC-3′) and SD7 (5′-*CGACTCAGA*GGATCCTTAGAACTGATAAACCAGACCTAAAGCG-3′) that contain the EcoRI and BamHI restriction sites respectively. pSD05 contains rseA, which was PCR-amplified by using the primer pair SD8 (5′-*GGTATTAG*CCATGGAGAAAG-3′) and SD9 (5′-*CTGTGCCGC* CCCGGGTACTTTCTG-3′) that contain the NcoI and SmaI restriction sites, respectively. All the plasmid constructs were confirmed by sequencing.

Table 1. Strains and plasmids used in this study.

Strain or Plasmid	Description	Source or Reference
E. coli strains		
MC4100	F$^-$ *[araD139]$_{B/r}$ Δ(argF-lac)169 λ$^-$ e14 flhD5301 Δ(fruK-yeiR)725(fruA25) relA1 rpsL150*(Str^R) *rbsR22 Δ(fimB-fimE)632(::IS1) deoC1*	[60]
MH1160	MC4100 *ompR101*	[61]
TR49	MC4100 λRS88[*degP–lacZ*]	[47]
W3110	F$^-$ *λ$^-$ IN(rrnD-rrnE)1 rph-1*	[62]
SR8265	W3110 *rybB*< >*aph*, Kan^R, source for P1 transduction	[63]
PS2209	W3110 Δ*lacZ169*	[64]
PS2652	Δ*lacZ169 zch-506::TnlO hns-1001::*Tn*seq1*, Kan^R, source for P1 transduction	[64]
AG100	F$^-$ *glnX44*(AS) *galK2*(Oc) *rpsL704*(Str^R) *xylA5 mtl-1 argE3*(Oc) *thiE1 tfr-3*	[65]
CH164	AG100 *marA zdd-230*::Tn9, Cam^R, source for P1 transduction	[66]
BW25113	F$^-$ *Δ(araD–araB)567 ΔlacZ4787*(::rrnB-3) *λ$^-$ rph-1 Δ(rhaD–rhaB)568 hsdR514*	[67]
JW4130	BW25113 *hfq::kan*, Kan^R, source for P1 transduction	GE Healthcare
SD01	MC4100 Δ*rybB*	This study
SD02	MC4100 *marA zdd-230*::Tn9, Cam^R,	This study
SD03	MC4100 Δ*hfq*	This study
SD04	MC4100 Δ*hns*	This study
SD05	MC4100 Δ*rpoS*	This study
W3110Δ*ompF*	W3110 *ompF::kan*	M.G. Page
W3110Δ*ompC*	W3110 *ompC::kan*	M.G. Page
W3110Δ*ompF*Δ*ompC*	W3110 Δ*ompF*Δ*ompC*	M.G. Page
Plasmids		
pDrive	PCR cloning vector; Amp^R, Kan^R	Qiagen
pRC1	pDrive containing *Enterobacter aerogenes* MarA	[31]
pRC2	pDrive containing *Enterobacter aerogenes* RamA	[32]
pSD01	pDrive encoding MicC sRNA	This study
pFus2K	Cloning vector with promoter-less *lacZ*, Kan^R	[59]
pSD02	pFus2K containing the *micC–lacZ* fusion	This study
pSD03	pFus2K containing the *ompN–lacZ* fusion	This study
pTrc99A	Expression vector with the inducible P_{TRC} promoter, Amp^R	Pharmacia
pSD04	pTrc99A containing OmpN	This study
pSD05	pTrc99A containing RseA	This study
pBAD24	Expression vector with the inducible P_{BAD} promoter, Amp^R	[68]
pBAD24-NlpE	pBAD24 containing NlpE	M. Masi
pBAD33	Expression vector with the inducible P_{BAD} promoter, Cam^R	[68]
pBAD33-CpxA*	pBAD33 containing an autoactivated (*) CpxA	M. Masi

4.3. β-Galactosidase Assays

β-Galactosidase activity was routinely assayed on log-phase bacterial cultures, as described by Miller [69].

4.4. Determination of Minimal Inhibitory Concentrations (MIC)

MIC values of antibiotics were determined by the microdilution method in Mueller Hinton II broth (MHIIB) (Sigma). Susceptibilities were determined in 96-well microtiter plates with an inoculum of 2×10^5 cfu in 200 µL containing two-fold serial dilutions of each compound. The MIC was defined as the lowest concentration of each compound for which no visible growth was observed after 18 h of incubation at 37 °C. Each assay was systematically performed in triplicate. The average of three independent assays was considered in µg/mL.

4.5. Preparation of the Microtiter Plates for β-Galactosidase Assays

The standard β-galactosidase assay was adapted for compound screening by using 96-well microtiter plates and a SUNRISE™ Tecan for absorbance readings. Briefly, strains were grown to an OD_{600} of 0.6. Cultures were diluted to an OD_{600} of 0.2, and added (200 µL) to the Phenotype MicroArrays ™ test plates (Biolog plates PM11 to PM19) (Supplementary Data 2). After overnight incubation at 37 °C, cells were centrifuged, washed, and treated with ONPG (2-nitrophenyl β-D-galactoside, Sigma) (4 mg/mL). Curves of OD_{420} were plotted over the time (30 min) to identify optimal inducers (Supplementary data 2). Similar experiments were repeated in 96-well microtiter plates preloaded with a chosen concentration range for each compound: each well was loaded with 20 µL of ONPG (4 mg/mL) and 10 µL of compound dilutions (Supplementary Data 3), then cells (170 µL at an OD_{600} of 0.2) were added. The plates were incubated at 37 °C inside the reader, and curves of OD_{420} were plotted over the time (6 h). The obtained readings in presence of ONPG were used to calculate Miller units and for determining the fold change in *lacZ* activity, relatively to standard growth conditions. Experiments were independently repeated at least three times.

4.6. Preparation of OM Extracts

Bacterial cultures (50 mL), grown in the presence or absence of stress, were incubated according to the optimum *micC/ompN* induction conditions determined by the β-galactosidase assay. The cells were washed and concentrated 12.5 fold in 20 mM sodium phosphate buffer (pH 7.4), and lysed by one passage through a cell disruptor (Constant Systems) at 2 kbar. After removal of cell debris by centrifugation (7000× *g*, 20 min, 4 °C) the supernatant was ultracentrifuged (100,000× *g*, 60 min, 4 °C) to collect the whole cell envelopes. These were resuspended in 0.3% *N*-laurylsarcosinate, and incubated for 30 min at room temperature to solubilize the IM. The insoluble OM extracts were pelleted by centrifugation (100,000× *g*, 60 min, 4 °C).

4.7. SDS-PAGE and Western Blot Analysis

OM were prepared as described above, resuspended in 20 mM sodium phosphate buffer (pH 7.4), and kept at −20 °C until use. All samples were diluted in Laemmli buffer (2×: 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue, 125 mM Tris-HCl, pH 6.8) and heated for 5 min at 100 °C before loading. Samples corresponding to 0.2 OD units were separated on 10% SDS-PAGE. To better resolve OmpF and OmpC, 4 M urea was added to the running gel. Proteins were either visualized after straining with Coomassie Brilliant Blue R250 or transferred onto nitrocellulose blotting membranes (GE Healthcare, Aulnay-sous-Bois, France). Primary rabbit antibodies and dilutions were: TolC (1:5000), OmpFd (1:5000), OmpC1 (1:5000), and OmpN (1:1000). Goat anti-rabbit HRP-conjugated secondary antibodies and Clarity Max™ Western ECL Blotting substrates (Bio-Rad, Marnes-la-Coquette, France) were used for detection. Protein bands were visualized with a molecular imager Chemidoc-XRS System (Bio-Rad) and quantified using the Image Lab software (Bio-Rad) by using the TolC band as a standard. Peptide-specific antibodies were used to avoid cross-detection of OmpC and OmpN: OmpC1 antibodies are directed against KNGNPSGEGTSGVTNNG amino acid sequence present in loop 4 [70], and OmpN1 antibodies are directed against the GGADNPAGVDDKDLVKYAD amino acid sequence found in loop 7 (Thermo Scientific Pierce custom antibody service, Villebon-sur-Yvette, France).

4.8. Whole Cell-Based Viability Assay

Resazurin-based CellTiter-Blue® Cell Viability Assay (Promega) was used to determine the metabolic inhibition of cells expressing single porins in the presence of clinically relevant antibiotics as an indicator of porin permeation properties [6]. These assays were performed on W3110 derivatives, i.e., W3110ΔF (expressing OmpC), W3110ΔC (expressing OmpF), and W3110ΔFC transformed with pTrc99A-*ompN* (expressing OmpN). Overnight cultures were diluted to 1:100 and grown until mid-log

phase in MHIIB. Strain containing pTrc99A-*ompN* was grown in the presence of Amp, and OmpN expression was induced with 0.1 mM IPTG for 1 h at 37 °C. When tested for β-lactam permeation, cultures were diluted to 10^7 cells/mL in fresh MHIIB containing 10% of CellTiter Viability Reagent. For strains containing pTrc99A-*ompN*, MHIIB was supplemented with 0.1 mM IPTG, and β-lactamase inhibitors tazobactam and clavulanic acid (4 μg/mL each), to inhibit the activity of the plasmidic AmpC, but not Amp. Microtiter plates (96 well) with black sides and a clear bottom were preloaded with 10 μL of 20× concentrated antibiotic solutions. For each antibiotic, the final concentration in the wells was defined as the maximal concentration that did not alter the metabolism of the porin-less strain, i.e., ertapenem, 0.125 μg/mL; meropenem, 0.125 μg/mL; cefotaxime, 0.0625 μg/mL. Cells (190 μL) were then added to separate wells. Control wells also contained cells with resazurin, but no antibiotic, and resaruzin with antibiotics without cells. Fluorescent signals of resorufin were measured with a TECAN Infinite Pro M200 spectrofluorometer (excitation wavelength 530 nm and emission wavelength 590 nm). Kinetic readings were taken at 37 °C every 10 min for 300 min. The % of metabolic inhibition for each strain exposed to each antibiotic was calculated from the measured difference of relative fluorescence units (RFUs) in the presence (RFU_{ATB}) as compared to in the absence (RFU_{MAX}) of antibiotic. All experiments were performed at least four times.

4.9. Colicin Killing Assays

LB agar plates were overlaid with 4 mL of soft agar (with a final agar concentration of 0.75%) containing 100 μL of *E. coli* overnight cultures. Serial two-fold dilutions of ColE2 or ColE3 (laboratory collection), were spotted in 5 μL drops onto the lawns, and the plates were incubated overnight at 37 °C. Efficiencies of killing were taken as the reciprocal of the highest dilution that gave complete clearing of the lawn.

5. Conclusions

Altogether, these data suggest that exposure to β-lactams induce a complex stress response to reduce the translocation of these antibiotics across the OM in *Enterobacteriaceae*. Further work will analyze how external stresses, such as β-lactams, interact with the σ^E envelope stress response and H-NS in laboratory strains, as well as in MDR clinical isolates.

Supplementary Materials: The following are available online at www.mdpi.com/2079-6382/6/4/33/s1, Supplementary Data 1: Screening of *micC* expression by β-galactosidase assay using preloaded 96-well Phenotype MicroArrays™ plates (Biolog PM11 to PM19) for bacterial chemical susceptibility, Supplementary Data 2: Fifteen compounds were selected to investigate their effects on MicC and OmpN, Supplementary Data 3: The expression of OmpN was evaluated in laboratory and clinical strains of *E. coli* by Western blot analysis, Supplementary Data 4: Partial *pfo*(*ybdK*)-*micC*-*ompN* genetic region.

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Author Contributions: Jean-Marie Pagès and Muriel Masi conceived and designed the experiments; Sushovan Dam performed the experiments; Sushovan Dam, Jean-Marie Pagès and Muriel Masi analyzed the data; Sushovan Dam, Jean-Marie Pagès and Muriel Masi wrote the paper.

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Review article:

Stress responses, OM permeability control and antimicrobial resistance in *Enterobacteriaceae*

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Stress responses, outer membrane permeability control and antimicrobial resistance in *Enterobacteriaceae*

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Abstract

Bacteria have evolved several strategies to survive a myriad of harmful conditions in the environment and in hosts. In Gram-negative bacteria, responses to nutrient limitation, oxidative or nitrosative stress, envelope stress, exposure to antimicrobials and other growth-limiting stresses have been linked to the development of antimicrobial resistance. This results from the activation of protective changes to cell physiology (decreased outer membrane permeability), resistance transporters (drug efflux pumps), resistant lifestyles (biofilms, persistence) and/or resistance mutations (target mutations, production of antibiotic modification/degradation enzymes). In targeting and interfering with essential physiological mechanisms, antimicrobials themselves are considered as stresses to which protective responses have also evolved. In this review, we focus on envelope stress responses that affect the expression of outer membrane porins and their impact on antimicrobial resistance. We also discuss evidences that indicate the role of antimicrobials as signaling molecules in activating envelope stress responses.

INTRODUCTION

Antimicrobial resistance (AMR) is broadly recognized as a growing threat to human health [1–3]. As such, increasing antibiotic treatment failures due to multidrug-resistant (MDR) bacteria have stirred an urgent need to better understand the underlying molecular mechanisms and promote innovation with the development of new antibiotics and alternative therapies [4, 5]. The efficacy of antibacterial compounds depends on their capacity to reach inhibitory concentrations in the vicinity of their target. This is particularly challenging for drugs directed against Gram-negative bacteria, which exhibit a complex envelope comprising two membranes and transmembrane efflux pumps [6, 7]. The Gram-negative envelope comprises an inner membrane (IM), which is a symmetric phospholipid bilayer; a thin peptidoglycan (PG) layer ensuring the cell shape; and an outer membrane (OM) that is an asymmetric bilayer, composed of an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS) [8]. The OM is a barrier to both hydrophobic and hydrophilic compounds, including necessary nutrients, metabolic substrates and antimicrobials, but access is provided by the water filled β-barrel channels called porins [6, 9–12]. In *Escherichia coli*, the channels of the general porins OmpF and OmpC are size restricted and show a preference for passage of hydrophilic charged compounds, including antibiotics such as β-lactams and fluoroquinolones. These porins are conserved throughout the phylum of γ-proteobacteria [13]. Additionally, tripartite RND (Resistance-Nodulation-cell Division) efflux pumps, such as AcrAB-TolC in *E. coli*, play a major role in removing antibiotics from the periplasm [7, 12]. Not surprisingly, MDR clinical isolates of *Enterobacteriaceae* generally exhibit porin loss and/or increased efflux, which act in synergy to reduce the intracellular accumulation of antibiotics below the threshold that would be efficient for activity [10].

Given the importance of the OM in controlling the uptake of beneficial as well as toxic compounds, one can expect that the expression of porins depends on environmental stresses and is well coordinated at the transcriptional and post-transcriptional levels [10, 14–17]. In this review, we will address the porin-mediated influx of antibiotics and give a perspective on the factors, including major regulatory pathways and antibiotic stresses, which control porin expression in *E. coli* and closely relative *Enterobacteriaceae*. Additionally, we will discuss the recent clinical data that illustrate the bacterial strategies using porin modifications to limit antibiotic entry.

ANTIBIOTIC STRESSES

Bacteria are present in a wide range of environments in which they are exposed to diverse toxic compounds or growth-limiting conditions. These include antibiotics used

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Keywords: *Enterobacteriaceae*; envelope stress responses; outer membrane permeability; porins; drug translocation; multidrug resistance.

Abbreviations: AMR, antimicrobial resistance; ESR, envelope stress response; IM, inner membrane; LPS, lipopolysaccharide; MDR, multidrug resistance; OM, outer membrane; PG, peptidoglycan; sRNA, small regulatory RNA; TCS, two-component system.

in the medical environment and agricultural settings. The last few decades have been marked by the constant increase of (multi)drug-resistant clinical isolates to which we have responded by increasing antibiotic dosing. Therefore, antibiotics are present almost everywhere at different concentrations [18]. Although MDR still emerges from bacterial exposure to antibiotic concentrations that are higher than the minimal inhibitory concentrations (MIC, defined as the lowest concentration of a drug that inhibits bacterial growth under defined laboratory conditions), the effects of subinhibitory concentrations on bacterial physiology and AMR have mostly been disregarded. Importantly, studies in this field have shown that low antibiotic concentrations affect bacteria at least at four different levels: (i) as selectors of resistance (by enriching resistant bacteria within populations and selecting for *de novo* resistance mutations) [19]; (ii) as contributors of genetic and phenotypic heterogeneity [20]; (iii) as intercellular signals [21]; and (iv) as inducers of persistence [22]. In this regard, Viveiros and colleagues have demonstrated the induction of high-level resistance to tetracycline (TET) in susceptible *E. coli* K12 obtained by gradual, step-wise increase exposure to subinhibitory concentrations of the antibiotic [23]. Increased expression of the AcrAB efflux pump was found responsible for resistance to TET, which could also be reversed by the use of the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide (PAβN). Interestingly, the TET-resistant strain also exhibited MDR due to repression of OmpF and OmpC expression [24]. Important questions arise from this and other related studies. First is whether the target for signalling resistance is the same as the target that is inhibited by the antibiotic. In the event the antibiotic itself but not a secondary metabolite is the signalling molecule, this could be determined by examining whether the response is alleviated by a target mutation that prevents drug binding. Second is whether and how the antibiotic (or a secondary metabolite) interferes with the ESRs described above. Here, comparative transcriptomics between susceptible and resistant strains would be a valuable tool to answer this question.

GLOBAL REGULATORS

In *Enterobacteriaceae*, the development of MDR is under positive regulation by global transcriptional activators that include members of the Ara/XylS superfamily such as MarA, RamA (absent in *E. coli*) and Rob, as well as the oxidative stress regulon SoxSR [10, 25–29]. Mutations in the corresponding genes are well documented and induce the overproduction of efflux pumps with concomitant repression of porin expression both directly and indirectly [10]. These mechanisms are reviewed in detail in Davin-Regli *et al.* [10]. Negative regulation by repressors of porins also plays a major role. OmpX is a small OM channel [30], of which overexpression is associated with a decreased expression of Omp36 (the OmpC ortholog of *Enterobacter aerogenes*) and a decreased susceptibility to β-lactams [31, 32]. Studies have indicated that expression of OmpX itself is controlled by a number of environmental factors, including salicylate via MarA and paraquat via SoxS [33] A very rapid MarA-dependent response pathway for upregulation of *ompX* has been shown to occur within 60–120 min upon cell exposure to salicylate [32]. This work by Dupont *et al.* identified a dramatic decrease in OmpF levels, as a first line of defence, with simultaneous development of resistance to β-lactams and fluoroquinolones by altering OM permeability.

ENVELOPE STRESS RESPONSES

All living organisms have stress responses that allow them to sense and respond to environmental damaging conditions by remodelling gene expression. As such, Gram-negative bacteria possess stress responses that are uniquely targeted to the cell envelope, including membranes and cell wall. These envelope stress responses (ESRs) are the EnvZ/OmpR, CpxAR (Cpx), BaeRS and Rcs phosphorelays, the stress-responsive alternative sigma factor σ^E and the phage shock response [34–37] in *E. coli* and closely related *Enterobacteriacea*e. Each of these ESRs is activated following the perturbation of particular components of the envelope or exposure to particular environmental stresses. Although ESRs are important in reacting to damaging conditions, stress proteins also play important roles in the maintenance of basic cellular physiology [38, 39]. This is particularly true for the σ^E-dependent stress response in *E. coli*, as the *rpoE* gene, which encodes σ^E, is essential for viability [40]. Here, we will essentially focus on ESRs that impact on AMR by regulating porin expression together with many other targets (regulons) — namely EnvZ/OmpR, Cpx and σ^E (see below and Fig. 1). Additionally, with the recent highlights and advances in RNA-based techniques [41], the repertoire of small regulatory RNAs (sRNAs) has vastly increased and their impact on the OM is continuously emerging [15, 17]. sRNAs alter gene expression, allowing rapid adjustment to different growth conditions [42]. Noteworthy, ESRs are often interconnected, regulate and are regulated by sRNAs in order to control target genes both at the transcriptional and post-transcriptional levels [15–17, 43, 44] (see below and Fig. 1).

Osmolarity was one of the earliest stresses described as influencing OmpF and OmpC expression via the EnvZ/OmpR two-component system (TCS) [45, 46]. EnvZ is a membrane-bound sensor kinase, and OmpR is a cytosolic response regulator which binds to the promoter region of the porin genes. Upon activation, EnvZ autophosphorylates and the high-energy phosphoryl group from EnvZ is subsequently transferred to a conserved Asp residue on OmpR. Phosphorylated OmpR (OmpR ~P) serves as a transcription factor that differentially modulates the expression of the *ompF* and *ompC* porin genes [45]. At low osmolarity, high levels of OmpR ~P activates *ompF* transcription, whereas at high osmolarity, low levels of OmpR~P repress *ompF* transcription and activate *ompC* transcription [47]. This differential production of OmpF and OmpC is consistent with that in high-osmolarity environments, such as in hosts where nutrients are abundant and the least permeable pore

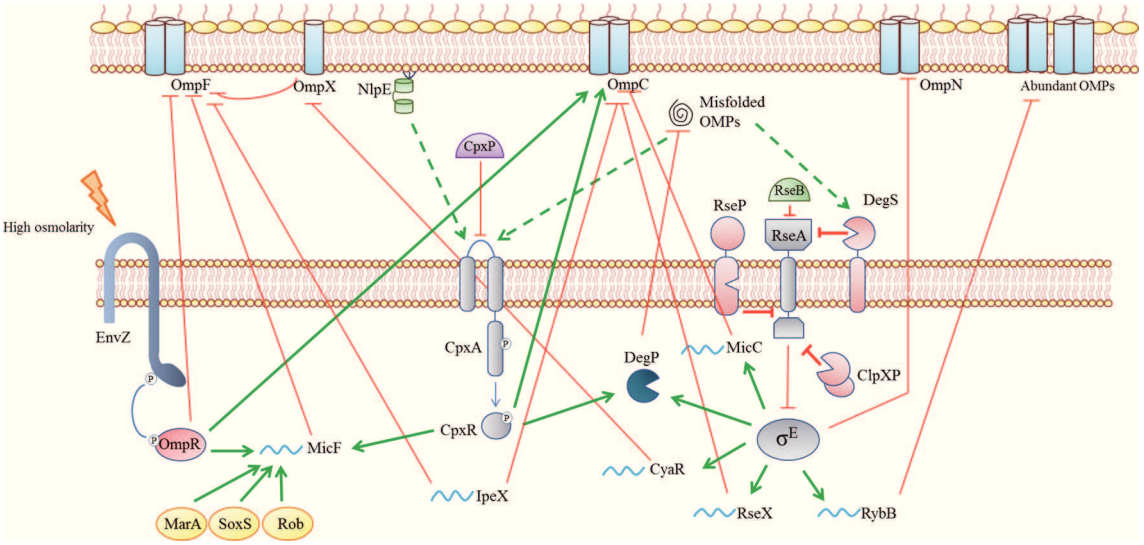

Fig. 1. Major regulatory pathways of porin regulation in *E. coli*: EnvZ/OmpR [46], CpxAR and sigma E (σE) [35] stress response systems are shown, along with known inducing cues and targets relevant to porin regulation. Upregulation is denoted by thick green arrows, while downregulation is denoted by red lines. In the EnvZ/OmpR TCS, activation of the response regulator OmpR results in phosphorylation and OmpR~P downregulates the expression of OmpF both at the transcriptional and post-transcriptional levels, the latter via the MicF sRNA. The mar-sox-rob regulons also downregulate OmpF expression via MicF. Both the CpxAR and σE responses are activated by a variety of envelope stresses. For clarity, only periplasmic misfolded OMPs are represented here. On one hand, CpxR~P alters the expression of multiple genes, including that of micF. On the other hand, the anti-sigma factor RseA is degraded by the successive action of two proteases, DegS and RseP at the periplasmic and the cytoplasmic site. Another protease, ClpXP specifically degrades the cytoplasmic RseA portion bound to σE, leading to its release. A number of σE-regulated sRNAs are indicated: MicC [78] downregulates OmpC and is coupled with *ompN* upregulation [80]; sRNA regulation of porins via CyaR [90], IpeX [111], RseX [86] and RybB [84, 88] are shown accompanied by their activators and porin targets; CyaR negatively regulates the expression of single-channelled porin OmpX [30], which in turn negatively regulates the major porin OmpC. The details of all these interconnected pathways are discussed thoroughly in the text.

channel OmpC is predominant, thus limiting the uptake of toxic bile salts; whereas in low-osmolarity environments where nutrients are scarce, the most permeable pore channel OmpF is expressed [6]. OmpF and OmpC transcriptional regulation by EnvZ/OmpR is also triggered by local anaesthetics, pH and nutrient limitation [46].

Accumulation of misfolded OM proteins in the periplasm, presumably reflecting problems in protein assembly or transport across the IM, can be detected by regulatory sensors that activate either the Cpx TCS or the alternative sigma factor σ^E. σ^E and Cpx are the two major regulation pathways that control the envelop integrity with overlapping regulon members [48–51], but respond to different inducing cues [35]. It is possible that these poorly defined signals (see below) act by causing accumulation of misfolded proteins. However, misfolded proteins are not the inducing signal *per se*, as some induce σ^E but not Cpx and *vice versa*. Recent studies rather suggest that Cpx responds to IM perturbations, while σ^E is activated by signals at the OM. The Cpx system comprises the CpxA sensor kinase and response regulator CpxR. Envelope stresses including alkaline pH, periplasmic protein misfolding, IM abnormalities such as misfolded transporters or accumulation of the lipid II precursor, induce the dissociation of the accessory protein CpxP from CpxA, trigger CpxA-mediated phosphorylation of CpxR, and alter the expression of protein foldases and proteases, respiratory complexes, IM transporters and cell wall biogenesis enzymes [37, 48, 49], all of which affect resistance to a number of antibiotics, particularly aminoglycosides and β-lactams [37, 49, 52–54]. The Cpx-mediated regulation of porins occurs at several levels. At the transcriptional level, CpxR ~P has been shown to bind directly the *ompF* and *ompC* promoters [55]. More recently, it has been found that the small IM protein MzrA connects Cpx and EnvZ/OmpR [56]. In this pathway and upon the activation of Cpx, MzrA interacts directly with EnvZ, which in turn, stabilizes OmpR~P [57]. In sensing different signals, the interconnection between Cpx and EnvZ/OmpR allows cells to adapt to diverse environmental stresses. Finally, although Cpx contributes to AMR by regulating a number of genes [37, 49, 52–54], its precise role and that of other TCSs in the development of MDR in clinical isolates is still poorly documented [58]. On the other hand, the stress-responsive sigma factor σ^E is induced by stresses that disturb the OM, and its regulon members comprise genes that facilitate the biogenesis of OM components, including proteins, lipoproteins and LPS [59–67]. In the absence of inducing signals, σ^E is held at the cytoplasmic side of the

IM by the anti-sigma factor RseA. At the periplasmic side of the IM, RseB binds to RseA, thus enhancing the inhibition of σ^E. Upon activation, σ^E is released from RseA by a proteolytic cascade that starts with the sequential degradation of the periplasmic and transmembrane domains of RseA by DegS and RseP, respectively, followed by the degradation of the cytoplasmic domain of RseA by ClpXP [68]. Interestingly, proteolysis of RseA is triggered by the binding of a conserved peptide found at the C-terminus of OM proteins, which is normally buried in folded porin trimers, to DegS in conjunction with the release of RseB from RseA upon binding of LPS intermediates [69, 70]. Of note, the σ^E-dependent repression of porin synthesis only occurs at the post-transcriptional level, wherein base-paring sRNAs inhibits translation of *omp* mRNAs (see below) in order to maintain the envelope homeostasis under stress conditions, as porins are major abundant proteins under normal growth conditions [6].

The post-transcriptional repression of OmpF by the sRNA MicF was discovered in 1984 [71–73]. This 93-nucleotide (nt) RNA is transcribed in the opposite direction to the *ompC* gene and acts by direct base-pairing to a region that encompasses the ribosome binding site (RBS) and the start codon of the *ompF* mRNA, thus preventing translation initiation [74]. The expression of the MicF sRNA itself is subject to multiple signals and regulatory pathways [75]. Positive regulation includes EnvZ/OmpR under high-osmolarity conditions [76], SoxS in response to oxidative stress [77] and MarA in response to antibiotic stress [25]. The 109-nt MicC sRNA has been identified more recently and was shown to repress OmpC by direct base-pairing to a 5′ untranslated region of the *ompC* mRNA [78]. Interestingly, *micC* is transcribed in the opposite direction to the *ompN* gene that encodes a quiescent porin homologous to OmpF and OmpC [79]. We have recently shown that *ompN* and *micC* are subjected to dual regulation upon exposure to certain antimicrobials such as β-lactams in a σ^E-dependent manner [80]. This is consistent with the fact that *ompN-micC* and *ompC-micF* share similar genetic organization and that *ompC* and *micF* are co-induced under specific conditions (i.e. high osmolarity via EnvZ/OmpR). The last decade has been marked by the identification and characterization of several sRNAs. These are differentially expressed and have been assigned to various important regulatory pathways in *E. coli* and *Salmonella*. Interestingly, many pathways regulate and are regulated by sRNAs [43, 44]. A prime example is EnvZ/OmpR, which activates the expression of MicF (that target *ompF*), OmrA and OmrB (that target *ompT* and mRNA of OM channels for iron-siderophore complexes) [81]; OmrA and OmrB, in turn, repress the translation of the *ompR* mRNA, creating a negative feedback loop [82]. Others examples include the well-conserved σ^E-regulated sRNAs RybB (that target *ompC* and *lamB* in *E. coli*; *ompN* and *ompW* in *Salmonella*), MicA (*ompA*), RseX (*ompC* and *ompA*), CyaR (*ompX*) and MicL (that represses translation of the major OM lipoprotein Lpp) [43, 66, 83–90] (Fig. 1). Of note, all these sRNAs are *trans*-acting, functioning by imperfect base pairing with multiple mRNA targets and require the help of the RNA chaperone Hfq [15–17].

PORIN ALTERATIONS IN CLINICAL ISOLATES

Combined regulations contributed by different stressors lead to hampering of the drug accumulation inside cells under the threshold for bacterial death. In one such study in *K. pneumoniae*, preferential expression of OmpK37 was detected in porin-deficient strains [91]. Amino acid sequencing showed that OmpK37 is highly homologous to quiescent porins OmpS2 from *Salmonella enterica* serovar Typhimurium and OmpN from *E. coli*. Liposome swelling assay with purified porins determined that OmpK37 also has a narrower pore, which was responsible for higher MICs of cefotaxime and cefoxitin antibiotics because of lower drug diffusion. A very recent study identified mutation in the *pho* regulon of an extensively drug-resistant strain of *K. pneumoniae* demonstrating downregulation of *phoE* gene by mutations in *phoR* and *phoB*. Here the PhoE porin, which is normally involved in phosphate transport, promotes restoration of cefoxitin and carbapenem resistance [92]. This is an interesting example of a regulatory mutation that effects porin expression, and clinically favours AMR under antibiotic therapy.

A wide array of chemicals including disinfectants and antibiotics has been shown to modulate OM permeability including expression of porins [93]. In addition, several studies have described the effect of imipenem on porin loss or loss of function mutations in clinical isolates of *Enterobacteriaceae* [58, 94–99].

Porins are trimers of 16-stranded β-barrels, each monomer formed of a central channel constricted by loop 3 that folds inward, thereby restricting the size of the channel. The presence of acidic residues in loop 3 facing a cluster of basic residues on the opposite side of the pore creates a strong transversal electric field [6, 100, 101]. This so-called eyelet or constriction region determines the channel size and ion selectivity, with OmpF being more permeable than OmpC. This latter observation was first attributed to the OmpC pore being slightly more constricted in this porin compared to OmpF [100, 101]. Although the two porins share high sequence similarity, the pore interior is more negative in OmpC than in OmpF [101]. This can also account for the low permeability of OmpC for anionic β-lactams [102, 103]. Moreover, the replacement of all ten titratable residues that differ between OmpC and OmpF in the pore-lining region leads to the exchange of antibiotic permeation properties [104]. Together, these structural and functional data clearly demonstrate that the charge distribution at pore linings, but not pore size, is a critical parameter that physiologically distinguishes OmpC from OmpF.

Functional mutations in porin genes leading to reduced permeability are another strategy found in MDR bacteria. In two documented cases, β-lactam-resistant clinical isolates of *E. aerogenes* contained Omp36 (an OmpC homologue) that

carried the mutation G112D in L3 [96, 105]. The homologous mutation G119D in OmpF of *E. coli* narrows the size of the channel as the large side chain of Asp protrudes into the channel lumen and confers a drastic reduction in β-lactam susceptibility [106]. Consistently, the Omp36 G112D mutant of *E. aerogenes* was characterized by a 3-fold decrease in ion conductance and a significant decrease in cephalosporin sensitivity (e.g. MICs of cefotaxime, cefpirome, cefepime and ceftazidime were 7- to 9-fold higher in the clinical isolate as compared to that in a sensitive reference strain) and cross-resistance to carbapenems [96, 105]. Recent studies also found a series of OmpC mutants that were isolated from a patient with chronic *E. coli* infection and additive mutations that conferred increased resistance to a variety of antibiotics, including cefotaxime, ceftazidime, imipenem, meropenem and ciprofloxacin [107, 108]. Low *et al.* demonstrated that subtle changes in OmpC in clinical isolates of *E. coli* altered antibiotic permeability and thus cell viability [107]. Seven isolates collected over a two-year clinical treatment exhibited increased levels of antibiotic resistance. These isolates exhibited the same two mutations (D18E and S274F) in the OmpC porin with increased levels of antibiotic resistance, thus pointing towards the possible functional role of these mutations in antibiotic influx.

It is worthwhile to note that from our knowledge, porin mutations causing reduced permeability have been described only in OmpC-type porins in *E coli* and *E aerogenes*. Interestingly, this type of porin is expressed under high osmolarity, the same environment the bacteria encounter in hosts. This gives an essential outlook on the host-induced modifications that possibly occur in these pathogens during infection. Using this sort of information can be highly beneficial for designing drugs with an improved diffusion across the bacterial outer membrane.

CONCLUSION

It is noteworthy that the sRNA-mediated stress response mechanism has multiple benefits for bacteria as compared to regulation by protein. Since sRNAs are produced during transcription, the later stages of translation and post-translational modification processes in the cell are completely sidestepped proving to be time and energy efficient for the cell. Not to forget the energy saved in porin assembly and discarding of misfolded proteins, which in itself can induce another stress response mechanism.

Decreased porin expression has been observed as a rapid response to toxic molecules and antibiotics within less than 60 min. Many sRNAs act at the post-transcriptional level, which ensures a rapid response to stressful conditions. In addition, the versatility of sRNAs ensures another level of gene regulation along with protein transcriptional regulators, thus contributing to an additional layer of tighter regulation. Taking into account the major role of the CpxAR and EnvZ/OmpR regulators in response to stressors such as antibiotics, it will be interesting to develop some assays allowing the detection of these kinds of mutations inside clinical isolate. This original diagnostic maybe used for determining the prevalence of these regulation events in clinical strain that have undergone antibiotic stress.

Targeting the early transcriptional step of antibiotic stress response regulatory mechanism is much more logical, especially when we have reports of OMP expression being regulated (both up and downregulation) within 60 min of stress appearance [32]. This will especially promote bypassing of aforementioned mutations in porins in clinical strains that are selected during antibiotic treatment. Targeting of sRNA or sRNA regulators such as MicF or Hfq can rejuvenate failing antimicrobial therapies in regards with membrane impermeability. They can be original targets for increasing the efficiency of existing drugs by providing fitness reduction in bacteria. As of now, a cyclic peptide RI20 has been identified to inhibit Hfq-mediated repression of gene, by binding with proximal binding site of Hfq [109]. Another approach will be to inhibit sRNA interfering with porin expression that is involved in drug translocation. Recently, a small molecule was used to target human microRNA (miR)−525 precursors as an anti-cancer strategy [110]. This promising discovery can be repeated in bacteria for manipulating sRNA levels, which may save the failing antibiotic therapies.

Predictability of an efficient drug based on the SICAR (Structure Intracellular Concentration Activity Relationship) concept, is a step up to efficient drug designing. Briefly, SICAR connects the physicochemical drug properties to the efficacy of translocation through the bacterial membrane and the resulting intracellular accumulation. To achieve this goal, an extensive knowledge of the OM permeability control, including the contribution of sRNAs, is required.

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Conflicts of interest
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Unpublished results:

RNA-Seq profiling of *Escherichia coli* MC4100 for identifying target spectrum of MicC small RNA

RNA-Seq profiling of Escherichia Coli MC4100 for identifying target spectrum of MicC small RNA

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Abstract

Small RNAs are efficient gene regulators. This is because of their rapid synthesis requiring lesser energy for production as compared to proteins, especially under taxing stressful conditions. One of the previously identified sRNA MicC, is conserved in Shigella, Salmonella, and Klebsiella, and is primarily known to inhibit ompC expression at the posttranscriptional level in Escherichia coli. Their existence in multiple enterobacterial genomes suggests the possible presence of multiple sRNA targets. Here we have used whole genome transcriptomics to identify previously unknown candidate targets of MicC. The MicC overexpressing cells were found to differentially express 177 genes, out of which 117 were upregulated and 60 were downregulated. Furthermore, MicC has been discovered to be co regulated with OmpN porin transcription under carbapenem stress. So we also analyzed the bacterial transcriptome under stress by different carbapenems to understand the regulatory mechanism at play leading to the MicC induction.

Keywords: Small RNA, MicC, porins, carbapenems.

Introduction

Antimicrobial resistance (AMR) is a major global public health threat that requires immediate attention effective prevention and treatment (1, 2, 3). A major factor contributing to MDR is the inability of antibiotics to penetrate bacteria and reach their requisite target to be effective (4, 5, 6). In Gram-negative bacteria, such as Escherichia coli, the two porins OmpF and OmpC are among the most abundant outer membrane proteins (>10^5 copies/cell) and form water-filled channels for the diffusion of small hydrophilic molecules including nutrients and antibiotics (6, 8). In resistant clinical isolates, porin defects (i.e. down-regulation of their expression and structural modifications) limit antibiotic uptake, thereby decreasing their intracellular concentration and activity (5, 6, 9, 10). Given the importance of the outer membrane, it is not surprising that the expression of porins is under complex regulation (6, 11). Recent research has shown that enterobacteria use several small regulatory RNAs (sRNAs) to fine-tune porin expression at the post-transcriptional level. Among these, MicF and MicC are the two major sRNAs that negatively regulate OmpF and OmpC, respectively (12, 13, 14). MicF and MicC exhibit similarities in that both repress the expression of porins by base pairing near the ribosome binding site, thereby blocking the translation, and both are encoded opposite to another porin gene (i.e. the micF-ompC and micC-ompN regions). Noteworthy, OmpC the only porin is expressed in clinical isolates — a lack of OmpC is often associated to AMR (15) — and ompN encodes a homolog of the trimeric porins, but the OmpN protein has not yet been characterized (16).

micC is present in almost all enterobacterial genomes, suggesting that conserved targets exist to serve a wide range of cellular functions (12). Our recent work has shown that MicC sRNA acts together with the σE envelope stress response pathway to control the OmpC/N levels in response to carbapenems and cephalosporins (17). To further expand our knowledge of the

MicC target spectrum apart from OmpC expression, we have performed transcriptomics analysis to understand how it impacts the bacterial physiology of E. coli.

We used RNA sequencing in combination to susceptibility assays to determine the impact of the MicC overexpression on the E. coli transcriptome and phenotype regarding antibiotic resistance. This study has shown about 60 previously unknown genes to be MicC targets. They are negatively regulated apart from its original target ompC. These targets were compared with the list of all MicC RNA targets predicted by the 'IntaRNA - RNA-RNA interaction tool' (18, 19). Selected mRNAs with binding property similar to micC-ompC mRNA screened using IntaRNA tool. Identification of the global target spectra of micC is important to understand its impact on the overall bacterial physiology, and more specifically on AMR.

Our work has shown that carbapenems and cephalosporins, two clinically relevant classes of β-lactam antibiotics, are potent inducers of both micC and ompN (17). So we further extended this work by perfoming transcriptome analysis of cells exposed to five clinically relevant carbapenems to understand the common regulatory pathway of micC induction. Are there any common signals or genetic factors activated by this particular class of β-lactams in the cells?

Materials and Methods

Bacterial strains, plasmids, and antibiotic susceptibility testing: E. coli MC4100 was used for analysis in all the studies and was grown in Luria Bertani (LB) broth (Sigma, Saint Quentin Fallavier, France). For MicC sRNA overexpression, MC4100 harboring pSD01 was used, and they were supplemented with 100 µg/mL ampicillin (Amp) for growth (17).
MIC values of antibiotics were determined by the microdilution method in Mueller Hinton II broth (MHIIB) (Sigma) as described in our previous work (20). Susceptibilities were determined in 96-well microtiter plates with an inoculum of $2X10^5$ cfu in 200 µL containing two-fold serial dilutions of each compound. The MIC was defined as the lowest concentration of each compound for which no visible growth was observed after 18 h of incubation at 37^oC. Each assay was systematically performed in triplicates. The average of three independent assays was considered in µg/mL.

RNA isolation and cDNA labeling: Bacterial cultures were centrifuged for 5 min at 2500 g. After removing the supernatant, the pellets were frozen on dry ice and stored at -80 °C. Total RNA was harvested using TRIzol (Invitrogen) and an RNeasy kit (Qiagen) according to the manufacturer's instructions, including a DNase digestion step. The RNA samples were redissolved to produce a final concentration of 300- 500 ng/L. For every RNA sample, 120 L was sent to Shanghai Bio Co., Ltd. and further examined through a quality and quantity test based on electrophoresis before microarray hybridization. Fluorescently labeled cRNA, transcribed from cDNA, was produced using a Quick Amp Kit, PLUS, Two-Color (Agilent p/n 5190-0444) in Agilent's SureHyb Hybridization Chambers. The cRNA was labeled with the fluorescent dyes Cy5 and Cy3-CTP. Double-stranded cDNA was synthesized from 1 g of total RNA using a cDNA synthesis kit according to the manufacturer's protocol (Quick Amp Kit, Agilent). T7 promoter primers were used instead of the poly-T primer provided in the kit.

The Cy3- and Cy5-labeled products were purified using an RNeasy Mini Kit (Qiagen). An aliquot of 1 µL of purified cRNA was used to determine the yield and specific activity with a NanoDrop ND-1000. The amount of Cy3- or Cy5- labeled cRNA was determined by measuring the absorbance at A260 nm, A280 nm, A550 nm (Cy3) and A650 (Cy5). The specific activity (pmol dye per g cRNA) of the cRNA can be obtained from the following calculation: specific activity = (concentration of Cy3/Cy5)/[(concentration of cRNA) * 1000] = pmol Cy3/Cy5 per g cRNA. If the yield is < 825 ng and the specific activity is < 8.0 pmol Cy3/Cy5 per g of cRNA, the experiment does not proceed to the hybridization step. cRNA was repeatedly prepared.

RNA sequencing and analysis: Whole-transcriptome sequencing of the isolates were performed by a custom-made protocol with barcoded RNA libraries to enable pooled sequencing of several samples (10). The bacterial isolates had been grown under standard conditions (LB broth; 37°C) and harvested in RNAprotect (Qiagen) at an optical density at 600 nm (OD_{600}) of 2 before the transcriptome sequencing (RNA-seq) was performed (21, 22). The reads were mapped to the E. coli K-12 wild type reference genome, which is available for download from NCBI. Mapping was performed using stampy, a short-read aligner that allows for gapped alignments (23), and SAMtools (24) was utilized for sequence variation calling. The reads per gene (rpg) values of all genes were calculated from the SAM output files. Testing for differential expression against the MC4100 (three biological replicates) was performed with DESeq (25), an R software package that uses a statistical model based on the negative binomial distribution.

Result and discussion

Impact of MicC overexpression on E. coli transcriptome: E coli cells harboring pSD01 that overexpresses MicC on induction with 0.4 mM for 2hrs were harvested along with its respective control (cells containing empty pDRIVE). The adjusted P value 5 (FDR) of the genes were selected with a cutoff of 0.05, and arranged accordingly. As expected, a substantial reduction in ompC mRNA (~64%) was observed as has been reported in previous studies. Moreover, 59 other targets were identified which an expression profile of less than 50% as had compared to the control cells which are enlisted in Table 1.

Downregulation of all the genes of CysH operon (cysH, cysP, cysB, cysC, cysD, cysE, and cysF) points towards the involvement of MicC in sulphur starvation (26). cysHoperon expression is dependent on the promoter and is independent of the leader region terminator, indicating that the operon is regulated at the level of transcription initiation rather than controlled at the level of premature termination of transcription. In vitro experiments to conclude the binding of MicC to cysH promoter will shed more light in the intricate sulphur starvation stress induced mechanism.

We further ran the REVIGO protocol that uses a simple clustering algorithm that relies on semantic similarity measures to summarize long, unintelligible lists of gene ontology (GO) terms by finding a representative subset of the terms (27, 28). As seen in Figure 1(b), the bubble size indicates the frequency of the GO term in the underlying GOA list of genes downregulated by MicC. Highly similar GO terms are linked by edges in the graph, where the line width indicates the degree of similarity.

The downregulated 60 genes were used to carry out GO enrichment analysis. There are approximately 37 GO terms were assigned, and the fraction percentages for biological process (30), cellular component (2) and molecular function (5) were 81.1%, 5.4% and 13.5%

respectively (Figure 1(b)). Of 30 terms in the biological process, there were terms, such as transmembrane transport, sulphate transport, stress response, protein transport, superoxide metabolism, copper ion homeostasis and response to heat that were directly related to Outer membrane stress response.

Impact of carbapenems on E. coli transcriptome:

Our work with sRNA micC and carbapenems inspired us to look for the E. coli transcriptome under the effect of sub lethal concentrations of five clinically relevant carbapenems namely doripenem, ertapenem, meropenem, imipenem and biapenem. The concentrations of antibiotics were decided with respect to their respective MIC. The cells were treated with half of MIC values for 300 minutes as this was the peak time observed to have highest micC promoter induction in β-galactosidase experiments (17). The objective was to have an understanding of any common sRNA regulatory pathways that is being trigerred by this specific class of β- lactams. An unforeseen clumping of cells was observed repeatedly when the cells were being stored in RNAprotect® Cell Reagent solution. This possibly led to an error prone collection of total RNA and the subsequent transcriptomic analysis was not conclusive. A refined protocol of a balanced antibiotic concentration enough for producing a stress response at the sRNA level and non-reactiveness of RNA stabilizing solution will possibly allow a more successful transcriptomic analysis.

RNA-RNA interaction using IntaRNA program

We further compared our list of MicC putative targets from DESeq analysis to the pre computed list of MicC targets available on IntaRNA website. IntaRNA predicts RNA-RNA interactions by an energy-based approach that is based on two assumptions: (i) the

accessibility of the interaction sites is important for the interaction formation, and (ii) a seed region is required to initiate the interaction (e.g. the 5' seed region for miRNAs).

Gene target iadA was one of the candidates found to be common in both the lists, which encodes for a proteolytic enzyme Isoaspartyl dipeptidase (29, 30). Figure 2 shows a comparision between MicC binding to ompC mRNA and iadA mRNA. As seen in our work, MicC induction takes place in response to envelope stress; so modulation of peptidase activity by this sRNA is not surprising. The interaction energy between MicC- OmpC RNA-RNA binding was predicted to be -17.28810 kcal/mol and for MicC- IadA it was -11.96510 kcal/mol.

Conclusion

In summary, by using genome wide comparative transcriptome analysis, a global gene expression change profiles was detected when MicC sRNA was overexpressed in E. coli cells. Among 176 differentially expressed genes, 60 were downregulated and are putative MicC targets. GO enrichment analysis showed that the differentially downregulated GO terms are involved in different pathways including stress response, trans membrane transport and chemical stimulus, indicating the OMP modulating sRNA has a much diverse and global role in the bacterial physiology. Further validation of these targets using quantitative real time PCR (qRT-PCR) and reporter fusions will solidify its role in mechanisms of target regulation beyond porins.

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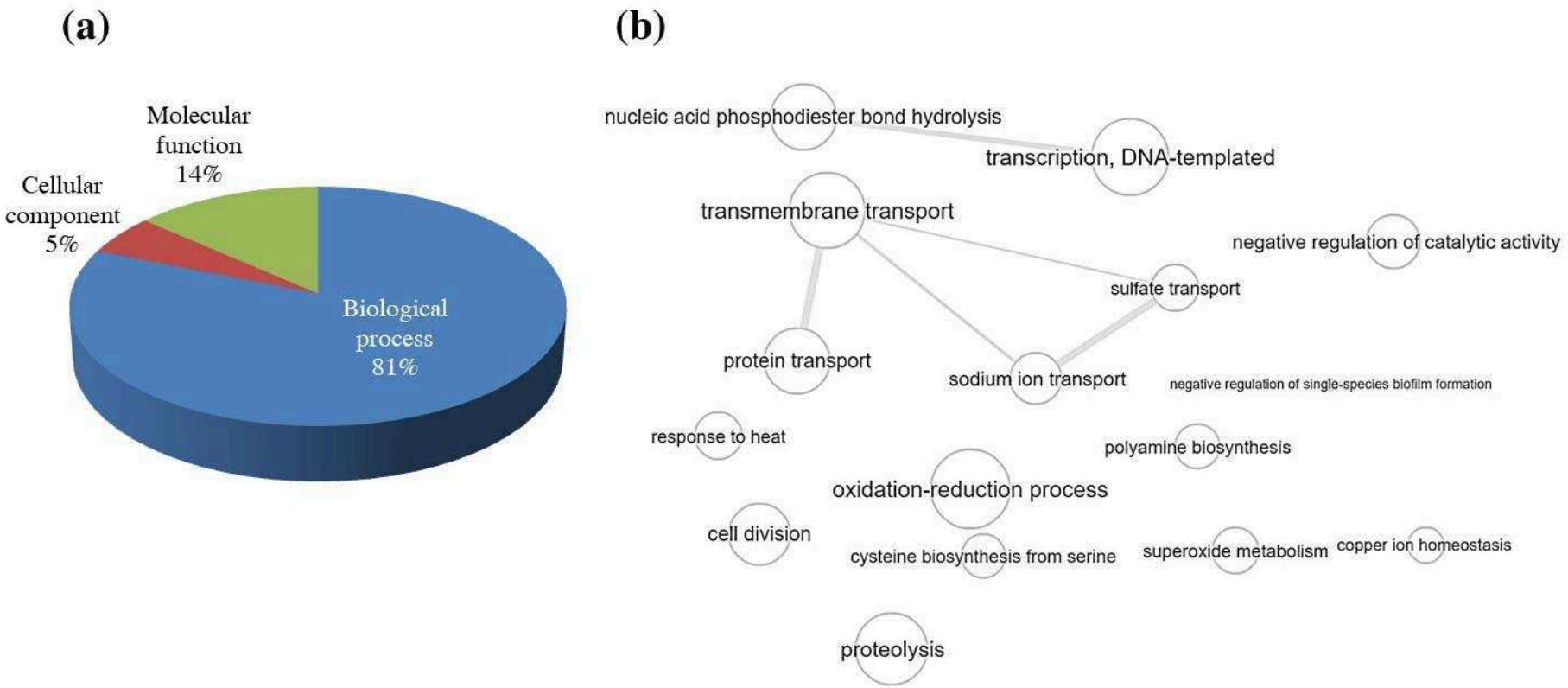
Gene name	FDR	Log2 expression change	Gene product
htpG	0,043	-1,219	protein refolding molecular co-chaperone Hsp90, Hsp70-dependent; heat-shock protein; ATPase [E. coli str. K-12 substr. MG1655]
ybiM	0,033	-1,223	hypothetical protein [E. coli IAI39]
glgS	0,049	-1,241	motility and biofilm regulator [E. coli str. K-12 substr. MG1655]
yhcO	0,017	-1,252	putative barnase inhibitor [E. coli str. K-12 substr. MG1655]
marC	0,027	-1,292	UPF0056 family inner membrane protein [E. coli str. K-12 substr. MG1655]
appB	0,041	-1,298	cytochrome bd-II oxidase subunit II [E. coli IAI39]
yifK	0,041	-1,304	putative APC family amino acid transporter [E. coli str. K-12 substr. MG1655]
ydcJ	0,038	-1,312	putative metalloenzyme [E. coli str. K-12 substr. MG1655]
yebV	0,017	-1,319	uncharacterized protein [E. coli str. K-12 substr. MG1655]
ycaP	0,017	-1,336	UPF0702 family putative inner membrane protein [E. coli str. K-12 substr. MG1655]
nhaA	0,047	-1,368	sodium-proton antiporter [E. coli str. K-12 substr. MG1655]
hspQ	0,008	-1,375	heat shock protein involved in degradation of mutant DnaA; hemimethylated oriC DNA-binding protein [E. coli str. K-12 substr. MG1655]
cutC	0,019	-1,376	cutC copper transporter homolog (E. coli) [Danio rerio(zebrafish)]
fic	0,033	-1,401	stationary phase-induced protein, putative toxin [E. coli str. K-12 substr. MG1655]
dnaK	0,022	-1,425	chaperone Hsp70, with co-chaperone DnaJ [E. coli str. K-12 substr. MG1655]
dppB	0,012	-1,436	dipeptide/heme ABC transporter permease [E. coli str. K-12 substr. MG1655]
iadA	0,008	-1,463	isoaspartyl dipeptidase [E. coli str. K-12 substr. MG1655]
zapA	0,023	-1,469	FtsZ stabilizer [E. coli str. K-12 substr. MG1655]
ompC	0,021	-1,469	Outer membrane protein (porin) [Cell wall/membrane/envelope biogenesis][E. coli str. K-12 substr. MG1655]

lysA	0,023	-1,480	diaminopimelate decarboxylase, PLP-binding [E. coli str. K-12 substr. MG1655]
adhP	0,032	-1,482	ethanol-active dehydrogenase/acetaldehyde-active reductase [E. coli str. K-12 substr. MG1655]
yffR	0,008	-1,502	uncharacterized protein [E. coli str. K-12 substr. MG1655]
ygaU	0,023	-1,517	uncharacterized protein [E. coli str. K-12 substr. MG1655]
yibT	0,026	-1,541	uncharacterized protein [E. coli str. K-12 substr. MG1655]
dppA	0,040	-1,556	dipeptide/heme ABC transporter periplasmic binding protein; dipeptide chemotaxis receptor [E. coli str. K-12 substr. MG1655]
ygiW	0,016	-1,574	hydrogen peroxide and cadmium resistance periplasmic protein; stress-induced OB-fold protein [E. coli str. K-12 substr. MG1655]
yohC	0,011	-1,596	Yip1 family inner membrane protein [E. coli str. K-12 substr. MG1655]
yjbJ	0,006	-1,655	stress-induced protein, UPF0337 family [E. coli str. K-12 substr. MG1655]
rybA	0,009	-1,723	miscRNA [E. coli UMN026]
ybiI	0,009	-1,735	DksA-type zinc finger protein [E. coli str. K-12 substr. MG1655]
sodA	0,034	-1,757	superoxide dismutase, Mn [E. coli str. K-12 substr. MG1655]
ymdF	0,004	-1,780	KGG family protein [E. coli str. K-12 substr. MG1655]
yjfO	0,008	-1,813	putative biofilm stress and motility protein A [E. coli IAI39]
yjiG	0,002	-1,820	SpmB family inner membrane protein [E. coli str. K-12 substr. MG1655]
yncL	0,002	-1,858	stress-induced small inner membrane enterobacterial protein [E. coli str. K-12 substr. MG1655]
exbD	0,003	-1,879	membrane spanning protein in TonB-ExbB-ExbD complex [E. coli str. K-12 substr. MG1655]
exbB	0,000	-1,919	membrane spanning protein in TonB-ExbB-ExbD complex [E. coli str. K-12 substr. MG1655]
bfd	0,011	-1,944	bacterioferritin-associated ferredoxin [E. coli str. K-12 substr. MG1655]
yodC	0,001	-1,970	uncharacterized protein [E. coli str. K-12 substr. MG1655], NAD(P)H nitroreductase [Bacillus subtilis subsp. subtilis str. 168]

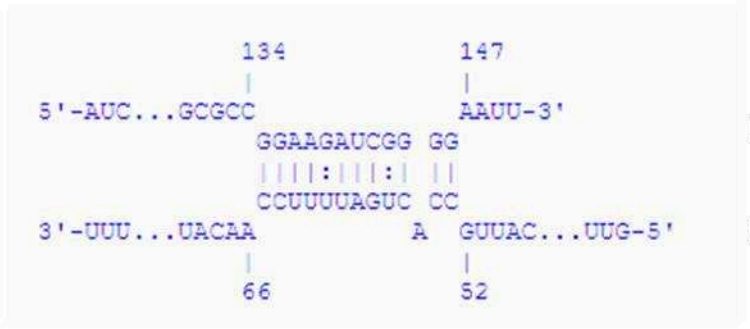
yiaG	0,000	-1,989	HTH_CROC1 family putative transcriptional regulator [E. coli str. K-12 substr. MG1655]
cbl	0,048	-2,031	ssuEADCB/tauABCD operon transcriptional activator [E. coli str. K-12 substr. MG1655]
ivy	0,000	-2,053	inhibitor of c-type lysozyme, periplasmic [E. coli str. K-12 substr. MG1655]
cysM	0,043	-2,055	cysteine synthase B (O-acetylserine sulfhydrolase B) [E. coli str. K-12 substr. MG1655]
fhuA	0,011	-2,133	ferrichrome outer membrane transporter [E. coli str. K-12 substr. MG1655]
cysP	0,023	-2,354	thiosulfate-binding protein [E. coli str. K-12 substr. MG1655]
yjiH	0,004	-2,409	nucleoside recognition pore and gate family putative inner membrane transporter [E. coli str. K-12 substr. MG1655]
cysW	0,029	-2,424	sulfate/thiosulfate ABC transporter permease [E. coli str. K-12 substr. MG1655]
cysK	0,011	-2,425	cysteine synthase A, O-acetylserine sulfhydrolase A subunit [E. coli str. K-12 substr. MG1655]
cysU	0,011	-2,445	sulfate/thiosulfate ABC transporter permease [E. coli str. K-12 substr. MG1655]
ryjA	0,000	-2,574	ncRNA [E. coli str. K-12 substr. MG1655]
cysA	0,023	-2,609	sulfate/thiosulfate transporter subunit [E. coli str. K-12 substr. MG1655]
cysJ	0,004	-2,749	sulfite reductase, alpha subunit, flavoprotein [E. coli str. K-12 substr. MG1655]
cysH	0,006	-2,958	phosphoadenosine phosphosulfate reductase; PAPS reductase, thioredoxin dependent [E. coli str. K-12 substr. MG1655]
cysN	0,007	-2,990	sulfate adenylyltransferase, subunit 1 [E. coli str. K-12 substr. MG1655]
sbp	0,003	-2,998	sulfate transporter subunit [E. coli str. K-12 substr. MG1655]
cysI	0,006	-3,029	HTH-type transcriptional regulator CysL [Bacillus subtilis subsp. subtilis str. 168]
cysD	0,007	-3,096	sulfate adenylyltransferase, subunit 2 [E. coli str. K-12 substr. MG1655]
cysC	0,008	-3,124	adenosine 5'-phosphosulfate kinase [E. coli str. K-12 substr. MG1655]
fhuF	0,000	-3,252	ferric iron reductase involved in ferric hydroximate transport [E. coli str. K-12 substr. MG1655]
yciW	0,001	-3,262	putative oxidoreductase [E. coli str. K-12 substr. MG1655]

Table 1: Differently downregulated genes (FDR cut-off = 0.05) identified in E. coli cells overexpressing MicC compared to E. coli WT MC4100.



(a) Fraction distribution of downregulated DEGs related GO terms based on molecular function, biological process, and cellular component. (**b**) Interactive graph of biological process of downregulated DEGs as a result of MicC overexpression in E. coli MC4100. The sizes of the circles are proportional to the number of genes associated with the specific term. The interactive network were summarized and plotted following published REVIGO protocol (http://revigo.irb.hr).

(a)



(b)

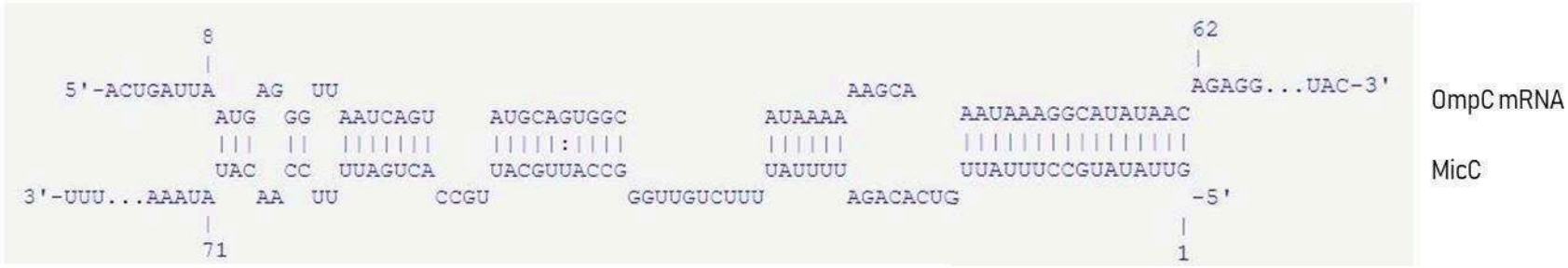


Figure 2: Comparision showing MicC binding to (a) IadA mRNA (b) OmpC mRNA. IntaRNA tool was used to predict the RNA interactions.

Chapter 3: General conclusion

General discussion

The recent discovery of sRNAs as a class of powerful regulators has revolutionized our understanding of gene regulation. They impact almost every aspect of the bacterial physiology. Advantage of RNA-based regulation over protein-based regulation is multifold. Reduced metabolic cost, additional levels of regulation, faster regulation and unique regulatory properties are some, to name a few (reviewed in 578). Furthermore the ability of regulation at two levels with both a transcription regulator and base-pairing sRNA, offers reduced leakiness of target gene expression. The expanding knowledge about these sRNA's particularly after the advent of advanced sequencing techniques will clarify if these molecules have multiple targets or roles in regulatory pathways.

Owing to the aforementioned advantages, sRNAs allows efficient adaptation of bacteria to their ever changing environment. Therefore, the involvement of sRNAs in antibiotic resistance is vital. There is accumulating evidence that *trans*-encoded sRNAs play an important role in regulatory circuits controlling antibiotic resistance. Adserschs they generate an immediate response, which is beneficial when antibiotic concentration increases rapidly. As illustrated in the review by Dersch *et.al,* 2017 (552), these circuits govern various processes, including functions required for antibiotic uptake, modifications of the cell envelope shielding against antimicrobials, drug efflux pumps expelling antibiotics, metabolic enzymes conferring resistance, production of biofilms protecting from antibiotics and DNA mutagenesis mechanisms facilitating evolution of novel resistances. The different *trans*-encoding sRNAs may regulate expression of resistance genes either directly by base-pairing or indirectly as members of regulatory cascades coordinating the response to antibiotics.

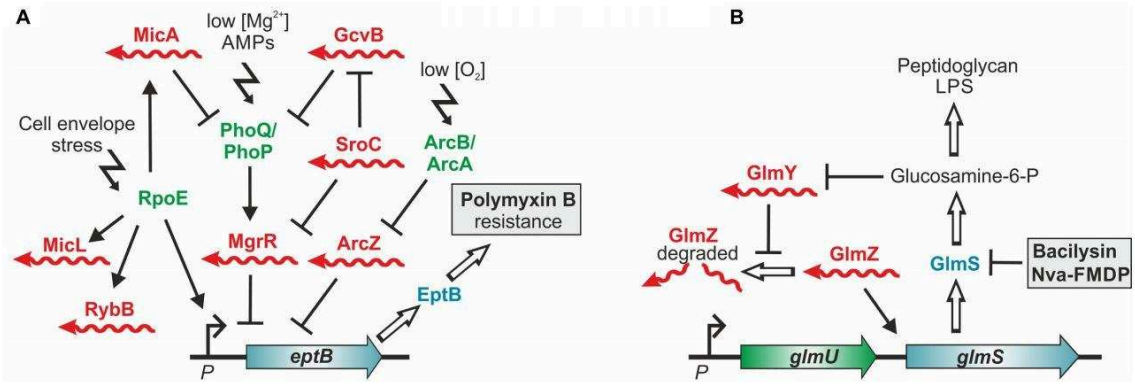


Figure 31 : Control of antibiotic resistance by trans-encoded sRNAs. (A) A well known example of control of polymyxin resistance in *E. coli*. Enzyme EptB provides resistance to polymyxin B by modification of LPS with phosphoethanolamine. Translation of *eptB* mRNA is inhibited by sRNA MgrR, which is itself repressed by base pairing with the sponge sRNA SroC. Consequently, loss of MgrR increases and loss of SroC decreases resistance to polymyxin B. In addition, *eptB* is repressed by sRNA ArcZ (548), whose levels are controlled by the aerobic/anaerobic-sensing ArcA–ArcB two-component system (579). Counterintuitively, deletion of Hfq, which is required for activity of these sRNAs increases susceptibility to polymyxin B. This might be explained by a defective cell envelope stress response executed by RpoE. RpoE not only activates transcription of *eptB* but also of further Hfq-dependent sRNAs, which control LPS biogenesis and modification. Complexity is further increased by the fact that mgrR transcription is activated by the two-component system PhoPQ, which is repressed by sRNAs MicA and GcvB. (B) sRNA-mediated resistance to antibiotics targeting the cell wall biosynthesis enzyme GlmS. In Enterobacteriaceae small RNAs GlmY and GlmZ feedback-regulate GlmS synthesis to achieve homeostasis of the essential metabolite GlcN6P. Inhibition of GlmS by bacilysin and other antibiotics depletes GlcN6P, which is sensed by sRNA GlmY triggering its accumulation. By a mimicry mechanism GlmY counteracts degradation of the homologous sRNA GlmZ, which in turn selectively activates translation of glmS encoded within the glmUS operon. As a result, higher GlmS levels are produced compensating for its inhibition (figure acquired from Dersch *et.al,* 2017)**.**

To date, systematic genome-wide searches have led to the identification of approximately 80 sRNAs in *E. coli*, the majority of which are conserved in *Salmonella* and other closely related species. Alteration of OM composition, particularly OMPs, represents one of the major mechanisms for antibiotic resistance. Interestingly about one-third of the reported sRNAs repress synthesis of OM proteins. So sRNAs influencing bacterial membrane synthesis or permeability could lead to antibiotic resistance. sRNAs such as MicF or MicC, which target the major porins OmpF and OmpC respectively, are probable to mediate antibiotic resistance. A few other known sRNA regulators of OMPs are InvR, MicA, OmrA/B, RseX, and RybB. Many parallels have been drawn between the

MicC and MicF sRNAs. Both repress the expression of porins by base pairing near the ribosome binding site, thereby blocking the translation, and both are encoded opposite to another porin gene (i.e. the *micF-ompC* and *micC-ompN* regions). Both the sRNAs are also conserved, together with their omp target sequences in *Salmonella, K. pneumoniae,* and *Enterobacter spp*. However, major questions such as (i) environmental conditions and/or intracellular regulatory pathways that promote maximal expression of MicC; (ii) the co-regulation of MicC and OmpN; (iii) the impact of such regulation on antibiotic susceptibility; and (iv) the prevalence of MicC/OmpN in MDR clinical isolates remain unanswered.

In this work, I had first set up a reporter gene assay by constructing *micC-lacZ* and *ompN-lacZ* translational fusions and evaluated their promoter activities under numerous stress conditions and in multiple genetic backgrounds. We optimized the assay by using 96-well microtiter plates, and screened the entire collection of compounds provided by the Biolog Phenotype MicroArrays™ for bacterial chemical susceptibility, in order to extend the range of putative inducing cues. The response of *micC–lacZ* to antimicrobials was measured during growth over a 6 h time period. As seen in our work, we identified carbapenems and cephalosporins along with *rpoE* depleted mutant background to induce the *micC-lacZ* induction maximally. This data is significant as activation of *micC* promoter by carbapenems points towards a potential porin based antibiotic resistance response that is possibly prevalent in strains exposed to this drug. Carbapenems, among the β-lactams, is a broad spectrum antimicrobial prescribed for treatment of both Gram-positive and Gram-negative bacteria. Carbapenems are considered to be the most reliable last-resort treatment for bacterial infections, presenting fewer adverse effects, and are safer to use than other last-line drugs such as the polymyxins. However, *Enterobacteriaceae* have become increasingly resistant to carbapenems owing to extensive and irrational use in clinical practice. Well known mechanisms of carbapenem resistance includes the enzyme-mediated resistance by production of β-lactamases such as carbapenemases (101), or by the production of ESBL or AmpC. Another bacterial strategy is to limit the drug entry by porin modification. OprD for example, is an OMP of *P. aeruginosa* through which carbapenems enter its periplasmic space where PBPs are located (580). Liposome swelling assays have also exhibited carbapenems such as meropenem and imipenem to be of high relative permeability through porins (306). So the induction of MicC promoter in presence of these β-lactams for suppressing the major porin OmpC to limit drug entry is possibly a viable mechanism for limiting drug entry and hence evading antibiotic mediated killing. Porin loss alone has also been implicated in causing carbapenem resistance in few studies (344, 581). The effect of MicC overexpression on *ompC* expression was examined by monitoring OmpC protein levels directly by western blot analysis. *E. coli* MC4100 cells were transformed with MicC overexpression plasmid and OM extracts were prepared from cultures collected at exponential phase. The levels of OmpC were analyzed by Western blot with specific anti-peptide antibodies.

Downregulation of *ompC* expression was confirmed by MicC sRNA. Although sub inhibitory concentrations of antimicrobial compounds identified for *micC* induction by reporter assays were not able to decrease *ompC* expression by a large margin, 10-30% reduction were observed in presence of imipenem and ertapenem. A recent work by Hao *et. al.* has confirmed the involvement of MicC in carbapenem resistance among clinical *E. aerogenes* isolates (581). They studied CRE isolates and evaluated the contribution of the major OMPs to carbapenem resistance. A two fold increase in MIC against carbapenems in clinically carbapenem susceptible isolate was observed when *micC*was overexpressed. The minor impact on the MIC results demonstrates the play of other unknown regulatory mechanisms.

We also employed Digoxigenin (DIG) labelled RNA probes for evaluating the MicC expression in the conditions inducing *micC* fusion. The total RNA extracted from the cells was transferred using Northern blot and the RNA expression was detected using an anti-DIG alkaline-phosphatase conjugated antibody. MC4100 cells overexpressing *micC* under IPTG inducible promoter harvested in the exponential phase was taken as a positive control. The analysis could not detect MicC in the total RNA extracted from cells grown under different stress condition, except in the positive control. We believe the DIG labeling system is not amply sensitive for identifying sRNAs in total RNA extracts and a more appropriate tool would be to use radiolabeled probes. Enriching the mRNA in total RNA sample as used for transcriptomics experiments might also be helpful in increasing the sensitivity.

Due to similar genetic organization of *ompN–micC* and *ompC–micF*, and the co-induction of *ompC* and *micF* under specific conditions (i.e., high osmolarity via EnvZ–OmpR), it has been suggested that *ompN* and *micC* could also be subjected to dual regulation. In our study the 18 compounds that were found to induce *micC–lacZ* fusions, also maximally induced the *ompN–lacZ* fusions. This hinted the possible existence of a dual co-regulation of *micC* and *ompN* in specific conditions. OmpN is a quiescent porin in *E. coli*, and has the same trimeric structure as OmpC and OmpF (320). It has a ~70% sequence identity to OmpC which is one of the preferred route for the uptake of β-lactam antibiotics across the OM of *E. coli* (368). Furthermore, the orthologous OmpK37 of *K. pneumoniae* has been shown to be expressed at low levels under standard laboratory growth conditions, but highly expressed in a β-lactam-resistant clinical isolate (389).

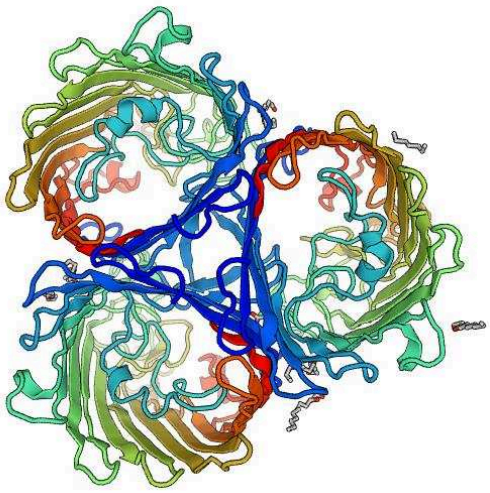
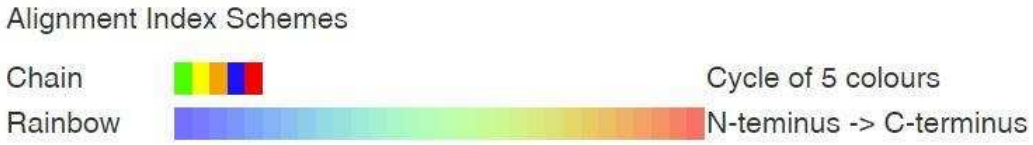


Figure 32 : Structure of the OmpN porin of *E. coli* acquired from the protein structure homology-modelling server SWISS MODEL. View of the trimer from the top. The range of color from blue to red represents the length of the peptide from blue being the N-terminus and red being the C-terminus of the peptide. The regions depicted in red color are also the ones which are most hydrophobic. The color scheme is as follows:



In the course of this study we have generated peptide specific antibodies for detection of OmpN with the help from Thermo Scientific Pierce custom antibody service, France. These specific antibodies were directed against the GGADNPAGVDDKDLVKYAD amino acid sequence found in the loop 7, and did not have cross reaction with the other major OMPs. Hence this can be a very useful tool for screening porin less MDR clinical isolates for examining expression of OmpN or its orthologs. This will shed light on existing but unknown porin based MDR mechanisms. Co-induction of *ompN-lacZ* as *micC-lacZ* fusions with the selected compounds led us to question the actual protein level of OmpN in the cells. However, OmpN production was undetectable in OM extracts prepared from cells grown in the presence of the sub- inhibitory concentrations of *micC* inducing compounds. It is worthy to note that Western blot analysis only provides steady-state levels of OmpC and OmpN. Additional reverse transcription PCR and pulse chase experiments are needed to conclude the effects of *micC* and *ompN* inducing conditions on the expression of OmpC and OmpN at the transcriptional and post-transcriptional levels, respectively.

To further discover the genetic factors and corresponding regulatory pathways that promotes the expression of MicC and their impact on OmpC/OmpN expression, we used chromosomal null mutants or overexpressing plasmids of various regulatory

factors in *E. coli* MC4100 background. Global regulators such as EnvZ-OmpR or MarA did not appear to play any role in *micC/ompN-lacZ* fusion activation. Meanwhile, envelope stress response regulator σ^E had an effect on them. When cells were depleted of σ^E upon the overexpression of its cognate anti-sigma RseA, the activity of both the *micC*– and *ompN–lacZ* fusions resulted in a 3–4-fold increase. Additionally, OmpN was also detected in OM extracts of cells grown under the same conditions. We suggest this regulation is most likely indirect, as the *micC–ompN* intergenic region does not contain a σ^E core promoter motif (582). As we have discussed above, σ^E is widespread among pathogenic and non-pathogenic bacteria, and becomes activated when bacterial envelope homeostasis is perturbed due to the misfolding of OMPs in the periplasm or severe OM damage by external stresses. *degP*, which encodes a periplasmic protease required for viability at high temperatures was also found to be activated in these conditions. Our data has shown a connection between antimicrobials impacting the OM, the envelope stress σ^E pathway, and MicC/OmpN expression. Our working hypothesis for explaining this phenomenon is that in the event of antibiotic exposure (carbapenems) and in the absence of the σ^E envelope stress response pathway, the cell uses sRNA based mechanism to maintain membrane integrity by possibly reducing the translation of misfolded porins. Translation of the major porin is overall reduced by Deep post-transcriptional regulation through MicC which also reduces the chances of antibiotic uptake, as this channel has been shown to do so. Meanwhile the cell simultaneously activates the *ompN* expression which has been found in our study to have no antibiotic translocation properties, but keeping the influx of nutrient intact for survival of the bacteria.

Our results have also showed that H-NS has a role in repressing the expression of MicC and OmpN. The expression of approximately 5% of the genes and/or the accumulation of their protein in *E. coli* is directly or indirectly controlled by H-NS. H-NS is a major component of the bacterial nucleoid and is known to have pleiotropic effects on gene expression, genome stability, and DNA recombination. A genomic study by Hommais *et. al.* on H-NS deleted mutants found that one third of the genes controlled by H-NS were predicted to encode cell envelope components or proteins that are usually involved in bacterial adaptation to changes in environmental conditions (583). So it is not surprising that it is connected with *micC/ompN* induction.

An interesting observation was made during our *lacZ* fusion experiments. The concentration of the compounds exhibiting highest Miller units for *micC/ompN* promoter activity was way beyond the MIC values (2X-8X) for the respective compound against the *lacZ* fusion strains. As a consequence the data was representing a fraction of the sub population of bacteria that was surviving the bacterial killing at such a high concentration. This led us to ponder about the possible involvement of persister cells (see 1.2.4.4). Persisters are suggested to arise either stochastically and continuously during population growth (so-called type II or stochastic), or are formed in response to an external (i.e. environmental) trigger (type I or determinative). So it is a possible

scenario that the envelope stress perpetuated by our screened compounds promotes persister formation. ESBLs and carbapepnemase producing strains have been implicated in biofilm formation and cause chronic infections which are known indicators of persisters. A recent work with *P. aeruginosa* clinical strains has demonstrated a change from a sensitive strain to a persister while applying carbapenems (Imipenem and meropenem) treatment (584). Interestingly at the same time porin OprD2 expression was lost which has been reported to be a prominent phenotype observed to evolve in 34 clinically isolated carbapenems resistant *P. aeruginosa* strains in a study done by the same group (584). This goes with our line of hypothesis of carbapenem treatment leading to suppression of major porin OmpC via MicC expression. Further studies looking for connection between carbapenem exposure and persister formation in *E. coli* will enable more understanding of these interconnected regulatory pathways in MDR.

Deep sequencing transcriptomics have been a godsend to understand the role of sRNAs as crucial elements for the bacterial response to antibiotics. Antibiotics are found to elicit much more extensive significant changes in the bacterial sRNA repertoire than previously thought. Many antisense RNAs are upregulated in methicillin-resistant *S. aureus* as well as in *M. tuberculosis* upon exposure to antibiotics frequently used to treat corresponding infections (585, 586). Similarly, in a MDR *Pseudomonas putida* strain 140 candidate sRNAs were detected, which responded to at least one of multiple tested antibiotics (587). Of note, each antibiotic generated a unique sRNA expression profile. All these observations are also in favor of a bacterial program in which sRNAs orchestrate responses to antibiotics. Further work is required to discriminate direct from indirect effects and to determine whether provoked sRNA profile changes contribute to drug tolerance. Our work with sRNA micC and carbapenems inspired us to look for the *E. coli* transcriptome under the effect of sub lethal concentrations of five clinically relevant carbapenems namely doripenem, ertapenem, meropenem, imipenem and biapenem. The objective was to have an understanding of any common sRNA regulatory pathways that is being trigerred by this class of β- lactams. An unforeseen clumping of cells was observed repeatedly when the cells were being stored in RNAprotect® Cell Reagent solution. This possibly led to an error prone collection of total RNA and the subsequent transcriptomic analysis was not conclusive. A refined protocol of a balanced antibiotic concentration enough for producing a stress response at the sRNA level and non-reactiveness of RNA stabilizing solution will possibly allow a more successful transcriptomic analysis.

As *micC* exists in almost all the enterobacterial genomes, it suggests that conserved targets exist to serve a wide range of cellular functions. I used RNA sequencing to determine the impact of the MicC overexpression on *E. coli* transcriptome and phenotype regarding antibiotic resistance. We discovered about 60 mRNA targets negatively regulated by MicC apart from its original target *ompC.* These targets were involved in diverse cellular functions ranging from protein processing, amino acid

metabolism, biofilm formation and motility to stress response. Many membrane proteins were also found to be transcriptionally inhibited along with OmpC. A further validation of these transcriptional targets by reporter fusions will help us in connecting stress response pathways with unknown bacterial physiology via MicC.

Antibiotics at sub-MIC concentrations not only trigger adaptive responses that enables bacteria to survive successive exposures to higher antibiotic concentrations and other lethal stresses (588, 589), but even have effects beyond. They increase mutation rates promoting emergence of novel antibiotic resistances and also stimulate the spread of resistance genes by horizontal transfer (590, 591). In *E. coli*, sub-MICs of antibiotics activate the master regulator of the general stress response, RpoS, which holds a key role in the latter processes. The *rpoS* mRNA represents a hub for post-transcriptional regulation as it is positively and negatively controlled by base pairing with multiple sRNAs including RprA (592, 593). One of these sRNAs apparently contributes to the induction of RpoS in response to ampicillin (**Figure 33**) (207). The cell wall damages caused by β-lactam antibiotics are sensed by the Rcs phosphorelay signal transduction system, which triggers activation of RpoS (**Figure 33**). However, upregulation of RpoS is indirect and occurs through an Hfq-dependent sRNA. The likely sRNA candidate is RprA because its expression is positively controlled by the Rcs system. Induction of RpoS not only activates genes counteracting stress, but also upregulates the error-prone DNA polymerase IV (PolIV), which incorporates spontaneous mutations (**Figure 33**) (564, 588). Moreover, RpoS activates expression of sRNA SdsR, which down-regulates the DNA mismatch repair protein MutS, thereby favoring fixation of the mutations introduced by PolIV (564). This mechanism increases genetic diversity, which could lead to mutations conferring antibiotic resistance (**Figure 33**). According to a study in *Salmonella*, RpoS and the sRNA RprA are also important for plasmid conjugation and could potentially have an impact on horizontal transfer of antibiotic resistance genes (594).

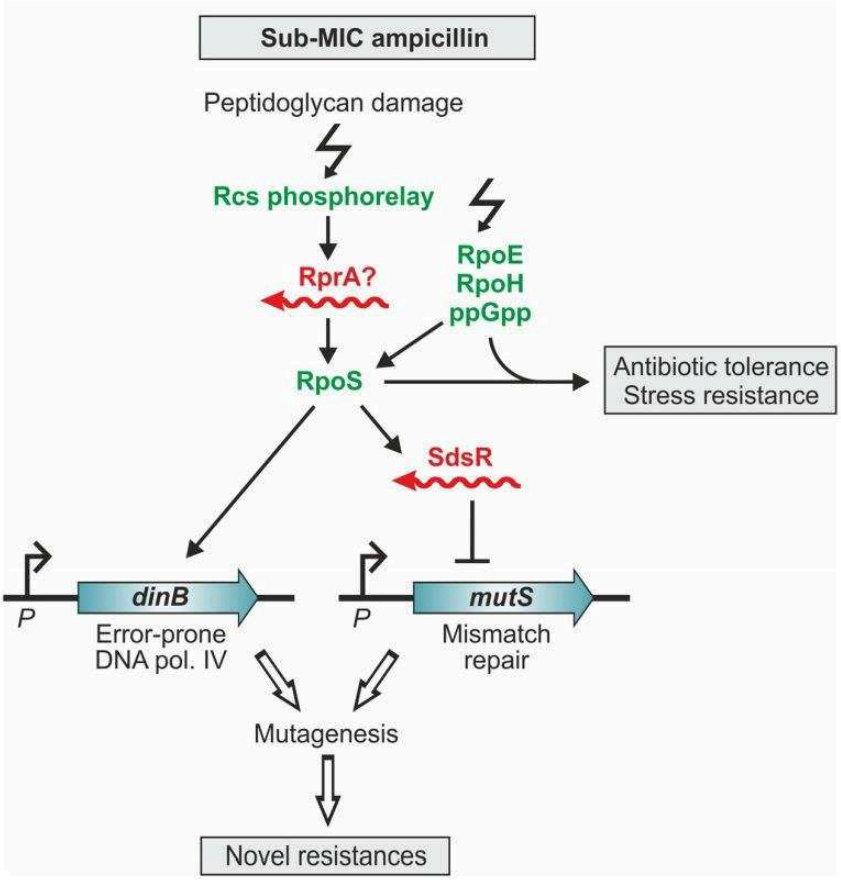


Figure 33 : The response of *E. coli* to sublethal concentrations of ampicillin involves small RNAs. Sub-MIC concentrations of ampicillin induce the stress regulons controlled by RpoS, RpoE, RpoH, and the alarmone ppGpp (588). The resulting response renders cells resistant to higher ampicillin concentrations and other stresses. Induction of the RpoS-regulated general stress response occurs via accumulation of ppGpp and the Rcs phospho-relay system. Rcs senses peptidoglycan damage caused by ampicillin and activates RpoS via an Hfq-dependent sRNA, presumably RprA (595). Induction of the RpoS regulon also increases the level of the error-prone polymerase IV, which generates base-substitutions in the DNA (564). The introduced mutations become fixed because the levels of the mismatch repair protein MutS decrease upon ampicillin treatment. RpoS represses *mutS* indirectly by activating expression of sRNA SdsR, which downregulates *mutS* by base pairing. This mechanism leads to increased mutagenesis, which can generate mutations conferring antibiotic resistance**.**

General conclusion

The relationship between sRNAs and antibiotic resistance is very straightforward. As discussed in length in section 1.2.2, antibiotics can use miscellaneous mechanisms of action to target vital cellular processes such as nucleic acid and protein synthesis, as well as envelope integrity. All the while, sRNAs are strongly involved in these functions. As a consequence, perturbation of sRNA activity may lead to alteration of cellular processes, and a potential outcome is modulation of bacterial antibiotic resistance. As Hfq and CsrA are essential for the activity of numerous sRNAs, their inhibition was shown to down-regulate sRNA networks controlling multiple virulence relevant processes, which eventually can render bacteria not only non-infective but also more susceptible to antibiotics.

Importantly, inactivation of Hfq not only attenuates virulence but also increases susceptibility to antibiotics (596), which could also reflect the roles of Hfq-dependent *trans*-encoded sRNAs in this process. However, the effect of Hfq inactivation on individual resistance genes is difficult to predict, because they are often controlled by extensive regulatory networks involving multiple sRNAs (**Figure 31**). For instance, *eptB*, which provides resistance to polymyxin B, is repressed by the Hfq-dependent sRNA MgrR (**Figure 31**). However, deletion of Hfq counterintuitively increases susceptibility of uropathogenic *E. coli* to polymyxin B (597). The reason for this opposing effect is unclear, but might be attributable to the influence of Hfqdependent sRNAs on the RpoE-dependent cell envelope stress response and thus envelope integrity, or the control of the MgrR sRNA by the two-component system PhoP/PhoQ, which is also regulated by Hfq-dependent sRNAs (**Figure 31**) (492, 512, 548). One of these sRNAs is GcvB (549), which is repressed by basepairing with the sponge sRNA SroC, similar to MgrR (598). However, whether downregulation of GcvB by SroC affects eptB expression remains to be clarified. This example illustrates that thorough knowledge of the complex regulatory network governing a resistance gene is a prerequisite to avoid unpredictable effects of this class of inhibitors.

As of now, a cyclic peptide RI20 has been identified to inhibit Hfq-mediated repression of gene, by binding with proximal binding site of Hfq (599). Another approach will be to inhibit sRNA interfering with porin expression that is involved in drug translocation. Recently, a small molecule was used to target human microRNA (miR)-525 precursors as an anti-cancer strategy (600). This promising discovery can be repeated in bacteria for manipulating sRNA levels, which may save the failing antibiotic therapies.

Emerging data has confirmed the fast and efficient translocation of antibiotics of different classes through bacterial membranes via OM pores or transporters (porins or efflux pumps). The concept of Structure Intracellular Concentration Activity

Relationship (SICAR) for explaining the physiochemical drug properties to the efficacy of translocation through membrane and the resulting intracellular accumulation will be an essential tool for designing new drugs. In addition to this, the knowledge of underlying regulatory mechanism objecting this intracellular mechanism will be a big benefit to counteract MDR. Antibiotic efficacy could be improved by manipulating the levels of sRNAs involved in resistance. Targeting the regulatory RNAs provides the opportunity to increase efficacy of existing antibiotics by rapid silencing of corresponding resistance genes in combined therapy. Recent progress in targeting microRNAs as discussed above (600), which are the eukaryotic counterparts of sRNAs, is in favor of this idea.

Altogether, our work has given compelling evidence that exposure to β-lactams induce a complex stress response to reduce the translocation of these antibiotics across the OM in *Enterobacteriaceae*. Further work will analyze how external stresses, such as β-lactams, interact with the σ^E envelope stress response and H-NS in laboratory strains, as well as in MDR clinical isolates. A refined knowledge of this pathway now awaits further optimization and evaluation with appropriate infection models.

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Régulées de manière post-transcriptionnelle de l'expression de la porine chez *Escherichia coli* et son impact sur la résistance aux antibiotiques

La résistance aux antibiotiques est une menace sérieuse et grandissante pour la santé publique, causant approximativement 700 000 décès annuels. Chez les bactéries à Gram-négatif, l'imperméabilité de la membrane externe et ainsi l'incapacité des antibiotiques à pénétrer l'enveloppe bactérienne pour atteindre leur cible est un facteur majeur contribuant au développement de la résistance. Chez *Escherichia coli*, les porines OmpF et OmpC sont des protéines de la membrane externe qui forment des canaux pour la diffusion de petites molécules hydrophiles tels que les antibiotiques. Les modifications des porines telles que la diminution de leur expression ou des altérations structurales se retrouvent dans de nombreux isolats cliniques résistants, limitent la translocation des antibiotiques, diminuent leur concentration intracellulaire et leur activité. L'expression des porines est soumise à une régulation complexe à plusieurs niveaux. Notamment, la régulation transcriptionnelle de ompF et ompC est bien connue et fait intervenir le système à deux composants EnvZ-OmpR en réponse à l'osmolarité du milieu. Au niveau post-transcriptionnel, plusieurs études ont également montré le rôle des petits ARN non-codants (sRNAs, small RNAs). Parmi ceux-ci, MicF et MicC modulent l'expression respective de OmpF et OmpC. Ils fonctionnent par appariement de bases avec le site de liaison du ribosome du messager cible, bloquant ainsi l'initiation de la traduction. De manière intéressante, les gènes codant ces deux sRNAs sont adjacents à deux gènes codant des porines — micF-ompC et micC-ompN — suggérant une co-régulation.

Dans ce cadre, et en utilisant *E. coli* comme bacterie modele, les objectifs de mon travail de these etaient : (1) de caractériser la régulation du sRNA MicC et la co-régulation putative de la porine quiescente OmpN; (2) d'examiner l'effet global de MicC sur le transcriptome; (3) d'analyser l'impact de l'expression de MicC sur la sensibilite aux antibiotiques. Dans un premier temps, nous ainsi avons etudie le role de plusieurs facteurs environnementaux et des voies de regulation connues pouvant conduire a une augmentation de l'expression de MicC. Pour cela, nous avons mesuré l'activité β-galactosidase d'une fusion transcriptionnelle micC-lacZ dans de nombreuses conditions de croissance et dans de nombreux contextes génétiques. Nous avons également optimisé le test du gène rapporteur à un format microplaque afin de cribler plusieurs collections de molécules fournis par la compagnie Biolog. Les résultats obtenus montrent l'induction de MicC en présence d'antibiotiques de la famille des β-lactamines (spécifiquement les carbapénèmes et les céphalosporines) ainsi qu'en déplétant le facteur de transcription sigma spécifique au stress de l'enveloppe, σE. Ces mêmes conditions activent aussi l'activite d'une fusion ompN-lacZ, indiquant une regulation transcriptionnelle commune de micC et ompN. De plus, la production de OmpN a ete confirmee par une analyse en immunoblot avec des anticorps specifiques. Ainsi, MicC pourrait agir conjointement avec σE pour contrôler l'expression de OmpC et OmpN en réponse à la présence de β-lactamines, une famille d'antibiotiques qui cible justement la synthèse du peptidoglycane et l'intégrité de l'enveloppe. Etant donnée la conservation de MicC chez les entérobacteries, nous avons effectue une etude par RNASeq pour déterminer l'impact de la surexpression de MicC sur le transcriptome d'*E. coli* et identifie 60 ARNm regules par MicC en plus de sa cible initiale ompC. L'identification des spectres cibles globaux des sRNAs est importante pour comprendre leur importance dans la physiologie bacterienne, ici celui de MicC dans la resistance aux antibiotiques. Les travaux a venir viseront a etudier cet aspect en détail ainsi que le lien putatif entre la résistance aux β-lactamines, la perte d'OmpC et la surexpression de MicC dans des isolats cliniques d'entérobactéries.

Mots clés : Entérobactéries, enveloppes bactériennes, perméabilité membranaire, résistance aux antibiotiques, porines, petits ARNs régulateurs non-codants.