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 targetting 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 phylog of γ-proteobacteria [13]. Additionally, tripartite RND (Resistance-Nodulation-cell Division) efflux pumps, such as AcrAB-ToIC 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
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 sub-inhibitory 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 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 σB, and the phase shock response [34–37] in *E. coli* and closely related *Enterobacteriaceae*. 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 σB-dependent stress response in *E. coli*, as the rpoE gene, which encodes σB, 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 σB (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

**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.
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 σE. σE 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 σE but not Cpx and vice versa. Recent studies rather suggest that Cpx responds to IM perturbations, while σE 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 σE 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, σE is held at the cytoplasmic side of the

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**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.
IM by the anti-sigma factor RseA. At the periplasmic side of the IM, RseB binds to RseA, thus enhancing the inhibition of σE. Upon activation, σE 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 σE-dependent repression of porin synthesis only occurs at the post-transcriptional level, wherein base-pairing 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 σE-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), OmpA 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 σE-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 affects 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, cephr- ome, 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 carbapenem [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 R120 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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