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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 for human health [1, 2, 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, 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, 10, 11, 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 porins 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 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 inhibit bacterial growth in defined laboratory conditions), the effects of subinhibitory concentrations on bacterial physiology and AMR was mostly disregarded. Importantly, studies in this field have shown that low antibiotic concentrations affect bacteria at least at four different levels: 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]; (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 signaling resistance is the same as the target that is inhibited by the antibiotic. In case the antibiotic itself but not a secondary metabolite is the signaling 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 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 details in Davin *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 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 dramatic decrease in OmpF levels, as a first line of defense, 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 remodeling 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 for 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 key figure). Additionally, with the recent highlights and advances in RNA-based techniques [41], the repertoire of small regulatory RNAs (sRNAs) has vastly increased so as to and their impact on the OM is continuously emerging [15, 17]. sRNAs alter gene expression, allowing fast adjustment to different growth conditions [42]. Noteworthy, ESRs are often interconnected, regulate and are regulated by sRNAs in order control target genes both at the transcriptional and post-transcriptional levels [15-17, 43, 44] (see below and key figure).

Osmolarity was one of the earliest stresses described to influence 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 represses *ompF* transcription and activates *ompC* transcription [47]. This differential production of OmpF and OmpC is consistent with that in high osmolarity environments, such as in the hosts where nutrients are abundant the least permeable pore 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 anesthetics, 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 altered 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 are 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 has been 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 subjected to multiple signals and regulatory pathways [75]. Positive regulation includes EnvZ/OmpR in 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 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 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] (key figure). Of note all these sRNAs are *trans*-acting, function 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 leads 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 [92]. 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 [93]. This is an interesting example of a regulatory mutation that effects porin expression, and clinically favors AMR under antibiotic therapy.

A wide array of chemicals including disinfectants and antibiotics has been shown to modulate the OM permeability including expression of porins [94]. In addition, several studies have

described the effect of imipenem on porin loss or loss of function mutations in clinical isolates of *Enterobacteriaceae* [58, 95-100].

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, 101, 102]. 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 [101, 102]. Although the two porins share high sequence similarity, the pore interior is more negative in OmpC than in OmpF [102]. This can also account for the low permeability of OmpC for anionic β-lactams [103, 104]. 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 [105]. 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 [106, 97]. 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 [107]. 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 a cross resistance to carbapenems [106, 97]. 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 [108, 109]. Low *et al.* demonstrated that subtle changes in OmpC in clinical isolates of *E. coli* altered antibiotic permeability and thus cell viability [108]. 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 only been described 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 encounters the hosts. This gives an essential outlook on the host induced modifications that possibly occurs in these pathogens during infection. Heeding to 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 is completely skipped 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 minutes. 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 minutes 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

306 to inhibit Hfq-mediated repression of gene, by binding with proximal binding site of Hfq
307 [110]. Another approach will be to inhibit sRNA interfering with porin expression that is
308 involved in drug translocation. Recently, a small molecule was used to target human
309 microRNA (miR)-525 precursors as an anti-cancer strategy [111]. This promising discovery
310 can be repeated in bacteria for manipulating sRNA levels, which may save the failing
311 antibiotic therapies.
312 Predictability of an efficient drug based on the SICAR (Structure Intracellular Concentration
313 Activity Relationship) concept, is a step up to efficient drug designing. Briefly, SICAR
314 connects the physicochemical drug properties to the efficacy of translocation through the
315 bacterial membrane and the resulting intracellular accumulation. To achieve this goal, an
316 extensive knowledge of the OM permeability control, including the contribution of sRNAs, is
317 required.

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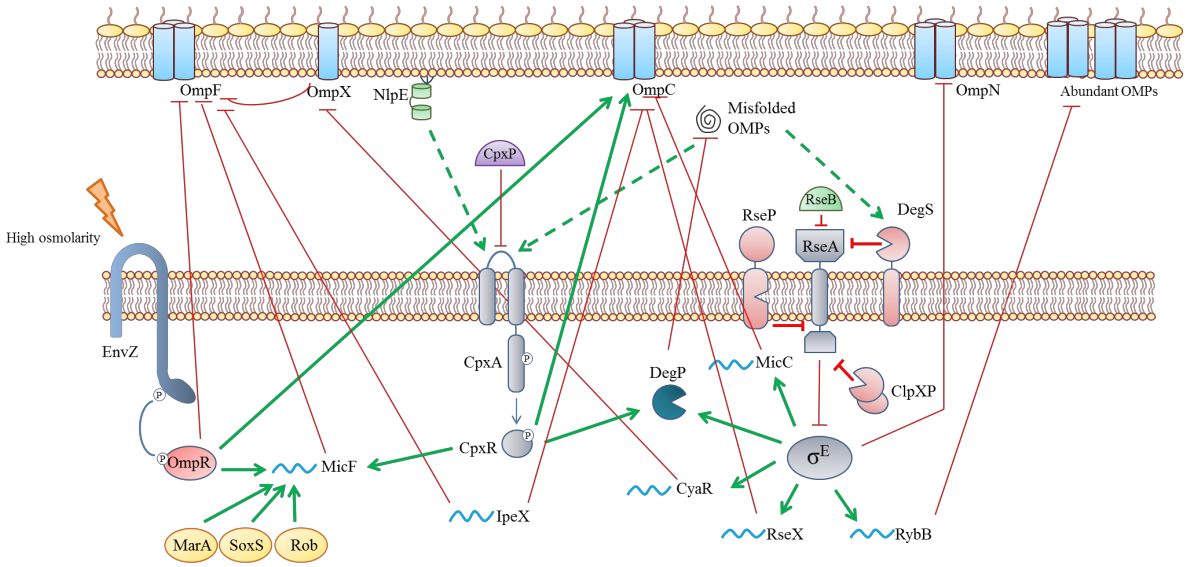
Key figure: Major regulatory pathways of porin regulation in *E. coli*: EnvZ/OmpR [46], CpxAR and sigma E (σ^E) [35] stress response systems are shown, along with known inducing cues and targets relevant to porin regulation. The upregulation is shown with thick green arrows, while the downregulation is shown with red lines. In the EnvZ/OmpR TCS, activation of the response regulator OmpR results in phosphorylated 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 σ^E responses are activated by a variety of envelope stresses. For clarity, only periplasmic misfolded OMPs are represented here. On one hand, CpxR~P alters 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 σ^E, leading to its release. A number of σ^E-regulated sRNAs are indicated: MicC [78] downregulates OmpC and is coupled with *ompN* upregulation [80]; sRNA regulation of porins via CyaR [90], IpeX [91], RseX [86] and RybB [84, 88] are shown accompanied by their activators and porin targets; CyaR negatively regulates the expression of single channeled 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.


OmpF
OmpX
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Abundant OMPs
Misfolded OMPs
CpxP
RseB
RseP
DegS
RseA
High osmolarity
EnvZ
CpxA
DegP
MicC
ClpXP
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RybB
MarA
SoxS
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