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

3

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23 lipopolysaccharide (LPS), antimicrobial resistance (AMR), multidrug resistance (MDR),
24 envelope stress response (ESR), two-component system (TCS), small regulatory RNA
25 (sRNA).

26

27 **Abstract**

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

41 **Introduction**

42 Antimicrobial resistance (AMR) is broadly recognized as a growing threat for human health
43 [1, 2, 3]. As such, increasing antibiotic treatment failures due to multidrug resistant (MDR)
44 bacteria have stirred the urgent need to better understand the underlying molecular
45 mechanisms and promote innovation with the development of new antibiotics and alternative
46 therapies [4, 5]. The efficacy of antibacterial compounds depends on their capacity to reach
47 inhibitory concentrations at the vicinity of their target. This is particularly challenging for
48 drugs directed against Gram-negative bacteria, which exhibit a complex envelope comprising
49 two membranes and transmembrane efflux pumps [6, 7]. The Gram-negative envelope
50 comprises an inner membrane (IM), which is a symmetric phospholipid bilayer; a thin
51 peptidoglycan (PG) layer ensuring the cell shape; and an outer membrane (OM) that is an
52 asymmetric bilayer, composed of an inner leaflet of phospholipids and an outer leaflet of
53 lipopolysaccharide (LPS) [8]. The OM is a barrier to both hydrophobic and hydrophilic
54 compounds, including necessary nutrients, metabolic substrates and antimicrobials, but
55 access is provided by the water filled β -barrel channels called porins [6, 9, 10, 11, 12]. In
56 *Escherichia coli*, the channels of the general porins OmpF and OmpC are size restricted and
57 show a preference for passage of hydrophilic charged compounds, including antibiotics such
58 as β -lactams and fluoroquinolones. These porins are conserved throughout the phylum of γ -
59 proteobacteria [13]. Additionally, tripartite RND (Resistance-Nodulation-cell Division)
60 efflux pumps, such as AcrAB-TolC in *E. coli*, play a major role in removing antibiotics from
61 the periplasm [7, 12]. Not surprisingly, MDR clinical isolates of *Enterobacteriaceae*
62 generally exhibit porin loss and/or increased efflux, which act in synergy to reduce the
63 intracellular accumulation of antibiotics below the threshold that would be efficient for
64 activity [10].

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

73

74 **Antibiotic stresses**

75 Bacteria are present in a wide range of environments in which they are exposed to diverse
76 toxic compounds or growth-limiting conditions. These include antibiotics used in the medical
77 environment and agricultural settings. The last few decades have been marked by the constant
78 increase of (multi)drug resistant clinical isolates to which we responded by increasing
79 antibiotic dosing. Therefore, antibiotics are present almost everywhere at different
80 concentrations [18]. Although MDR still emerges from bacterial exposure to antibiotic
81 concentrations that are higher than the minimal inhibitory concentrations (MIC, defined as
82 the lowest concentration of a drug that inhibit bacterial growth in defined laboratory
83 conditions), the effects of subinhibitory concentrations on bacterial physiology and AMR was
84 mostly disregarded. Importantly, studies in this field have shown that low antibiotic
85 concentrations affect bacteria at least at four different levels: as selectors of resistance (by
86 enriching resistant bacteria within populations and selecting for *de novo* resistance mutations)
87 [19]; (ii) as contributors of genetic and phenotypic heterogeneity [20]; (iii) as intercellular
88 signals [21]; (iv) as inducers of persistence [22]. In this regard, Viveiros and colleagues have
89 demonstrated the induction of high-level resistance to tetracycline (TET) in susceptible *E.*
90 *coli* K12 obtained by gradual, step-wise increase exposure to subinhibitory concentrations of
91 the antibiotic [23]. Increased expression of the AcrAB efflux pump was found responsible for
92 resistance to TET, which could also be reversed by the use of the efflux pump inhibitor
93 phenylalanine-arginine- β -naphthylamide (PA β N). Interestingly, the TET-resistant strain also
94 exhibited MDR due to repression of OmpF and OmpC expression [24]. Important questions
95 arise from this and other related studies. First is whether the target for signaling resistance is
96 the same as the target that is inhibited by the antibiotic. In case the antibiotic itself but not a
97 secondary metabolite is the signaling molecule, this could be determined by examining
98 whether the response is alleviated by a target mutation that prevents drug binding. Second is
99 whether and how the antibiotic (or a secondary metabolite) interferes with the ESRs
100 described above. Here, comparative transcriptomics between susceptible and resistant strains
101 would be a valuable tool to answer this question.

102

103 **Global regulators**

104 In *Enterobacteriaceae*, the development of MDR is under positive regulation by global
105 transcriptional activators that include members of Ara/XylS superfamily such as MarA,
106 RamA (absent in *E. coli*) and Rob as well as the oxidative stress regulon SoxSR [10, 25-29].
107 Mutations in the corresponding genes are well-documented and induce the overproduction of

108 efflux pumps with concomitant repression of porin expression both directly and indirectly
109 [10]. These mechanisms are reviewed in details in Davin *et al.* [10]. Negative regulation by
110 repressors of porins also plays a major role. OmpX is a small OM channel [30], of which
111 overexpression is associated with a decreased expression of Omp36 (the OmpC ortholog of
112 *Enterobacter aerogenes*) and a decreased susceptibility to β -lactams [31, 32]. Studies have
113 indicated that expression OmpX itself is controlled by a number of environmental factors
114 including salicylate via MarA and paraquat via SoxS [33] A very rapid MarA-dependent
115 response pathway for upregulation of *ompX* has been shown to occur within 60-120 min upon
116 cell exposure to salicylate [32]. This work by Dupont *et al.* identified dramatic decrease in
117 OmpF levels, as a first line of defense, with simultaneous development of resistance to β -
118 lactams and fluoroquinolones by altering OM permeability.

119

120 **Envelope stress responses**

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

141 Osmolarity was one of the earliest stresses described to influence OmpF and OmpC
142 expression via the EnvZ/OmpR two-component system (TCS) [45, 46]. EnvZ is a membrane-
143 bound sensor kinase, and OmpR is a cytosolic response regulator, which binds to the
144 promoter region of the porin genes. Upon activation, EnvZ autophosphorylates and the high
145 energy phosphoryl group from EnvZ is subsequently transferred to a conserved Asp residue
146 on OmpR. Phosphorylated OmpR (OmpR~P) serves as a transcription factor that
147 differentially modulates the expression of the *ompF* and *ompC* porin genes [45]. At low
148 osmolarity, high levels of OmpR~P activates *ompF* transcription, whereas at high osmolarity,
149 low levels of OmpR~P represses *ompF* transcription and activates *ompC* transcription [47].
150 This differential production of OmpF and OmpC is consistent with that in high osmolarity
151 environments, such as in the hosts where nutrients are abundant the least permeable pore
152 channel OmpC is predominant, thus limiting the uptake of toxic bile salts; whereas in low
153 osmolarity environments where nutrients are scarce, the most permeable pore channel OmpF
154 is expressed [6]. OmpF and OmpC transcriptional regulation by EnvZ/OmpR is also triggered
155 by local anesthetics, pH, and nutrient limitation [46].

156 Accumulation of misfolded OM proteins in the periplasm, presumably reflecting problems in
157 protein assembly or transport across the IM, can be detected by regulatory sensors that
158 activate either the Cpx TCS or the alternative sigma factor σ^E . σ^E and Cpx are the two major
159 regulation pathways that control the envelope integrity with overlapping regulon members
160 [48-51] but respond to different inducing cues [35]. It is possible that these poorly defined
161 signals (see below) act by causing accumulation of misfolded proteins. However, misfolded
162 proteins are not the inducing signal *per se*, as some induce σ^E but not Cpx and *vice versa*.
163 Recent studies rather suggest that Cpx responds to IM perturbations, while σ^E is activated by
164 signals at the OM. The Cpx system comprises the CpxA sensor kinase and response regulator
165 CpxR. Envelope stresses including alkaline pH, periplasmic protein misfolding, IM
166 abnormalities such as misfolded transporters or accumulation of the lipid II precursor, induce
167 the dissociation of the accessory protein CpxP from CpxA, trigger CpxA-mediated
168 phosphorylation of CpxR, and altered expression of protein foldases and proteases,
169 respiratory complexes, IM transporters, and cell wall biogenesis enzymes [37, 48, 49], all of
170 which affect resistance to a number of antibiotics, particularly aminoglycosides and β -
171 lactams [37, 49, 52-54]. The Cpx-mediated regulation of porins occurs at several levels. At
172 the transcriptional level, CpxR~P has been shown to bind directly the *ompF* and *ompC*
173 promoters [55]. More recently, it has been found that the small IM protein MzrA connects

174 Cpx and EnvZ/OmpR [56]. In this pathway and upon the activation of Cpx, MzrA interacts
175 directly with EnvZ, which in turn, stabilizes OmpR~P [57]. In sensing different signals, the
176 interconnection between Cpx and EnvZ/OmpR allows cells to adapt to diverse environmental
177 stresses. Finally, although Cpx contributes to AMR by regulating a number of genes [37, 49,
178 52-54], its precise role and that of other TCSs in the development of MDR in clinical isolates
179 are still poorly documented [58]. On the other hand, the stress responsive sigma factor σ^E is
180 induced by stresses that disturb the OM and its regulon members comprise genes that
181 facilitate the biogenesis of OM components, including proteins, lipoproteins and LPS [59-
182 67]. In the absence of inducing signals, σ^E is held at the cytoplasmic side of the IM by the
183 anti-sigma factor RseA. At the periplasmic side of the IM, RseB binds to RseA, thus
184 enhancing the inhibition of σ^E . Upon activation, σ^E is released from RseA by a proteolytic
185 cascade that starts with the sequential degradation of the periplasmic and transmembrane
186 domains of RseA by DegS and RseP, respectively, followed by the degradation of the
187 cytoplasmic domain of RseA by ClpXP [68]. Interestingly, proteolysis of RseA is triggered
188 by the binding of a conserved peptide found at the C-terminus of OM proteins, which is
189 normally buried in folded porin trimers, to DegS in conjunction with the release of RseB
190 from RseA upon binding of LPS intermediates [69, 70]. Of note, the σ^E -dependent repression
191 of porin synthesis only occurs at the post-transcriptional level, wherein base-pairing sRNAs
192 inhibits translation of *omp* mRNAs (see below) in order to maintain the envelope homeostasis
193 under stress conditions, as porins are major abundant proteins under normal growth
194 conditions [6].

195 The post-transcriptional repression of OmpF by the sRNA MicF has been discovered in 1984
196 [71-73]. This 93-nucleotide (nt) RNA is transcribed in the opposite direction to the *ompC*
197 gene and acts by direct base-pairing to a region that encompasses the ribosome binding site
198 (RBS) and the start codon of the *ompF* mRNA, thus preventing translation initiation [74].
199 The expression of the MicF sRNA itself is subjected to multiple signals and regulatory
200 pathways [75]. Positive regulation includes EnvZ/OmpR in high osmolarity conditions [76],
201 SoxS in response to oxidative stress [77] and MarA in response to antibiotic stress [25]. The
202 109-nt MicC sRNA has been identified more recently and shown to repress OmpC by direct
203 base-pairing to a 5' untranslated region of the *ompC* mRNA [78]. Interestingly, *micC* is
204 transcribed in the opposite direction to the *ompN* gene that encodes a quiescent porin
205 homologous to OmpF and OmpC [79]. We have recently shown that *ompN* and *micC* are
206 subjected to dual regulation upon exposure to certain antimicrobials such as β -lactams in a

207 σ^E -dependent manner [80]. This is consistent with that *ompN-micC* and *ompC-micF* share
208 similar genetic organization and that *ompC* and *micF* are co-induced under specific
209 conditions (i. e. high osmolarity via EnvZ/OmpR). The last decade has been marked by the
210 identification and characterization of several sRNAs. These are differentially expressed and
211 have been assigned to various important regulatory pathways in *E. coli* and *Salmonella*.
212 Interestingly, many pathways regulate and are regulated by sRNAs [43, 44]. A prime
213 example is EnvZ/OmpR, which activates the expression of MicF (that target *ompF*), OmrA
214 and OmrB (that target *ompT* and mRNA of OM channels for iron-siderophore complexes)
215 [81]; OmrA and OmrB, in turn, repress the translation of the *ompR* mRNA, creating a
216 negative feedback loop [82]. Others examples include the well-conserved σ^E -regulated
217 sRNAs RybB (that target *ompC* and *lamB* in *E. coli*; *ompN* and *ompW* in *Salmonella*), MicA
218 (*ompA*), RseX (*ompC* and *ompA*), CyaR (*ompX*) and MicL (that represses translation of the
219 major OM lipoprotein Lpp) [43, 66, 83-90] (key figure). Of note all these sRNAs are *trans*-
220 acting, function by imperfect base pairing with multiple mRNA targets and require the help
221 of the RNA chaperone Hfq [15-17].

222

223 **Porin alterations in clinical isolates**

224 Combined regulations contributed by different stressors leads to hampering of the drug
225 accumulation inside cells under the threshold for bacterial death. In one such study in *K.*
226 *pneumoniae*, preferential expression of OmpK37 was detected in porin-deficient strains [92].
227 Amino acid sequencing showed that OmpK37 is highly homologous to quiescent porins
228 OmpS2 from *Salmonella enterica* serovar Typhimurium and OmpN from *E. coli*. Liposome
229 swelling assay with purified porins determined that OmpK37 also has a narrower pore, which
230 was responsible for higher MICs of cefotaxime and ceftazidime antibiotics because of lower
231 drug diffusion. A very recent study identified mutation in the *pho* regulon of an extensively
232 drug resistant strain of *K. pneumoniae* demonstrating downregulation of *phoE* gene by
233 mutations in *phoR* and *phoB*. Here the PhoE porin, which is normally involved in phosphate
234 transport, promotes restoration of ceftazidime and carbapenem resistance [93]. This is an
235 interesting example of a regulatory mutation that effects porin expression, and clinically
236 favors AMR under antibiotic therapy.

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

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

241 Porins are trimers of 16-stranded β -barrels, each monomer formed of a central channel
242 constricted by loop 3 that folds inward, thereby restricting the size of the channel. The
243 presence of acidic residues in loop 3 facing a cluster of basic residues on the opposite side of
244 the pore creates a strong transversal electric field [6, 101, 102]. This so-called eyelet or
245 constriction region determines the channel size and ion selectivity, with OmpF being more
246 permeable than OmpC. This latter observation was first attributed to the OmpC pore being
247 slightly more constricted in this porin compared to OmpF [101, 102]. Although the two
248 porins share high sequence similarity, the pore interior is more negative in OmpC than in
249 OmpF [102]. This can also account for the low permeability of OmpC for anionic β -lactams
250 [103, 104]. Moreover, the replacement of all ten titratable residues that differ between OmpC
251 and OmpF in the pore-lining region leads to the exchange of antibiotic permeation properties
252 [105]. Together, these structural and functional data clearly demonstrate that the charge
253 distribution at pore linings, but not pore size, is a critical parameter that physiologically
254 distinguishes OmpC from OmpF.

255 Functional mutations in porin genes leading to reduced permeability are another strategy
256 found in MDR bacteria. In two documented cases, β -lactam-resistant clinical isolates of *E.*
257 *aerogenes* contained Omp36 (an OmpC homologue) that carried the mutation G112D in L3
258 [106, 97]. The homologous mutation G119D in OmpF of *E. coli* narrows the size of the
259 channel as the large side chain of Asp protrudes into the channel lumen and confers a drastic
260 reduction in β -lactam susceptibility [107]. Consistently, the Omp36 G112D mutant of *E.*
261 *aerogenes* was characterized by a 3-fold decrease in ion conductance and a significant
262 decrease in cephalosporin sensitivity (*e. g.* MICs of cefotaxime, cefpirome, cefepime and
263 ceftazidime were 7 to 9 fold higher in the clinical isolate as compared to that in a sensitive
264 reference strain) and a cross resistance to carbapenems [106, 97]. Recent studies also found a
265 series of OmpC mutants that were isolated from a patient with chronic *E. coli* infection and
266 additive mutations that conferred increased resistance to a variety of antibiotics, including
267 cefotaxime, ceftazidime, imipenem, meropenem and ciprofloxacin [108, 109]. Low *et al.*
268 demonstrated that subtle changes in OmpC in clinical isolates of *E. coli* altered antibiotic
269 permeability and thus cell viability [108]. Seven isolates collected over a two year clinical
270 treatment exhibited increased levels of antibiotic resistance. These isolates exhibited the same
271 two mutations (D18E and S274F) in the OmpC porin with increased levels of antibiotic

272 resistance, thus pointing towards the possible functional role of these mutations in antibiotic
273 influx.

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

281

282 **Conclusion**

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

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

298 Targeting the early transcriptional step of antibiotic stress response regulatory mechanism is
299 much more logical, especially when we have reports of OMP expression being regulated
300 (both up and downregulation) within 60 minutes of stress appearance [32]. This will
301 especially promote bypassing of aforementioned mutations in porins in clinical strains that
302 are selected during antibiotic treatment. Targeting of sRNA or sRNA regulators such as
303 MicF or Hfq can rejuvenate failing antimicrobial therapies in regards with membrane
304 impermeability. They can be original targets for increasing the efficiency of existing drugs by
305 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
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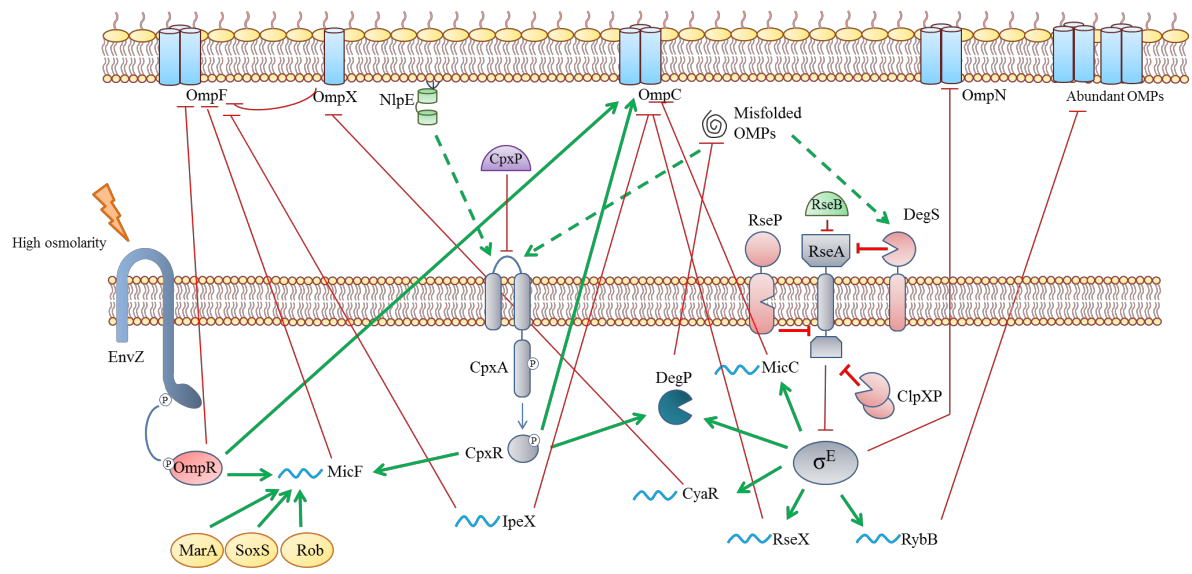
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608 **Key figure: Major regulatory pathways of porin regulation in *E. coli*:** EnvZ/OmpR [46],
609 CpxAR and sigma E (σ^E) [35] stress response systems are shown, along with known inducing
610 cues and targets relevant to porin regulation. The upregulation is shown with thick green
611 arrows, while the downregulation is shown with red lines. In the EnvZ/OmpR TCS,
612 activation of the response regulator OmpR results in phosphorylated and OmpR~P
613 downregulates the expression of OmpF both at the transcriptional and post-transcriptional
614 levels, the latter via the MicF sRNA. The *mar-sox-rob* regulons also downregulate OmpF
615 expression via MicF. Both the CpxAR and σ^E responses are activated by a variety of
616 envelope stresses. For clarity, only periplasmic misfolded OMPs are represented here. On one
617 hand, CpxR~P alters expression of multiple genes, including that of *micF*. On the other hand,
618 the anti-sigma factor RseA is degraded by the successive action of two proteases, DegS and
619 RseP at the periplasmic and the cytoplasmic site. Another protease, ClpXP specifically
620 degrades the cytoplasmic RseA portion bound to σ^E , leading to its release. A number of σ^E -
621 regulated sRNAs are indicated: MicC [78] downregulates OmpC and is coupled with *ompN*
622 upregulation [80]; sRNA regulation of porins via CyaR [90], IpeX [91], RseX [86] and RybB
623 [84, 88] are shown accompanied by their activators and porin targets; CyaR negatively
624 regulates the expression of single channeled porin OmpX [30], which in turn negatively
625 regulates the major porin OmpC. The details of all these interconnected pathways are
626 discussed thoroughly in the text.

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