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1 **Uncovering small membrane proteins in pathogenic bacteria: regulatory**
2 **functions and therapeutic potential**

3

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5

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12

13

14 **Abstract**

15

16 Bacterial small proteins (below 50 amino acids) encoded by small open reading frames
17 (sORFs) are recognized as an emerging class of functional molecules that have been largely
18 overlooked in the past. While some were uncovered serendipitously, global approaches have
19 recently been developed to detect these sORFs. A large portion of small proteins appears to be
20 hydrophobic and located in the bacterial membrane. In the present review, we describe
21 functional small hydrophobic proteins discovered in pathogenic bacteria and report recent
22 advances in the discovery of additional ones. Small membrane proteins contribute to bacterial
23 adaptation to changing environments and often appear to be implicated in negative feedback
24 regulation loops by modulating the function or stability of larger membrane proteins. A subset
25 of these proteins belongs to toxin-antitoxin modules. We highlight features of characterized
26 hydrophobic small proteins that may pave the way for identification of the functional small
27 proteins among novel sORFs discovered. Besides providing new insights into bacterial
28 pathogenesis, identification of naturally-occurring small hydrophobic proteins of pathogenic
29 bacteria can lead to new therapeutic interventions, as recently shown with the development of
30 synthetic peptides derived from natural small proteins that display antibacterial or antivirulence
31 properties.

32

33 **Keywords:** Small protein, pathogenic bacteria, membrane protein, regulation, antibacterial
34 molecule

35

36 **Introduction**

37 In bacteria, small non-coding RNAs (sRNAs) have been recognized for decades for their
38 regulatory role in a wide range of stress responses, including the response to host cells in the
39 context of pathogenic bacteria. More recently, increasing evidence has shown that some sRNAs
40 annotated as non-coding RNAs could in fact encode small proteins that were previously not
41 considered in both eukaryotes (Chen, Brunner et al. 2020) and prokaryotes (Hemm, Weaver et
42 al. 2020; Orr, Mao et al. 2020). Identification of small proteins that directly result from the
43 translation of small open reading frames (sORFs) rather than cleavage of larger protein
44 precursors adds to the pool of functional molecules. We use the term small protein for proteins
45 that are below 50-amino acids in size, while they are also named in other reports as “very small
46 proteins”, “microproteins”, “miniproteins” or “peptides”.

47 Small ORFs were historically missed in genome annotations (because of the minimal
48 ORF length cutoff commonly used) and proteomic studies. Several translated sORFs from
49 bacteria were discovered by serendipity. In addition, multiple global approaches, including
50 bioinformatics prediction, mass spectrometry and ribosome profiling, have been used to
51 identify bacterial small proteins (Sousa and Farkas 2018; Orr, Mao et al. 2020). These global
52 studies have been performed in the model organism *Escherichia coli* (Hemm, Paul et al. 2010),
53 in few pathogenic bacteria (see below) and more recently in bacteria of the microbiota (Sberro,
54 Fremin et al. 2019). A few bacterial small proteins associated with diverse functions have been
55 characterized and include toxins, chaperones, stabilizing factors of larger proteins and
56 regulators (Storz, Wolf et al. 2014; Duval and Cossart 2017; Orr, Mao et al. 2020).

57 A substantial fraction of bacterial small proteins are hydrophobic with a single alpha-
58 helix transmembrane domain and are predicted to localize in bacterial membrane, specifically
59 the inner membrane in the case of Gram negative bacteria (Alix and Blanc-Potard 2009;
60 Fontaine, Fuchs et al. 2011). In the present review, we focus on small membrane-associated

61 proteins identified in pathogenic bacteria (listed in Table 1) and their potential use in developing
62 therapeutic strategies. These proteins appear to belong to two main functional classes: i)
63 regulators, which modulate cellular functions like transport or antimicrobial resistance through
64 direct interaction with other larger proteins, and ii) toxins, which are part of toxin-antitoxin
65 modules and have antibacterial properties. Increasing evidence indicates that small membrane
66 proteins can interfere with bacterial virulence and be used for antivirulence strategies.
67 Moreover, common features of these small proteins will be highlighted here, which may
68 facilitate the identification of new functional proteins from global studies carried out on
69 pathogenic bacteria.

70

71 **Small hydrophobic proteins regulating membrane proteins in response to cation levels**

72 We describe here membrane-associated small proteins found in pathogenic bacteria that
73 regulate larger membrane proteins, including virulence factors, involved in the adaptation of
74 bacteria to changing concentration of cations in the environment (Table 1 and Table 2). These
75 small proteins, which were mostly identified by serendipity, are regulated by two-component
76 systems (TCS) (Table 2). As described below, implication of small proteins in negative
77 regulation of virulence proteins can provide a basis for novel therapeutic perspectives.

78

79 MgtR and MgtS: regulators of protein stability under Mg²⁺ limitation

80 MgtR and MgtS, both present in *Salmonella enterica* serovar Typhimurium (*S.*
81 Typhimurium), are two small hydrophobic proteins regulated by the PhoQP TCS, which is
82 induced by Mg²⁺ limitation (Groisman 2001).

83 MgtR is a 30-amino acid long hydrophobic protein found to regulate MgtC in *S.*
84 Typhimurium (Alix and Blanc-Potard 2008). MgtR is an inner membrane protein, with its N-

85 terminus in the cytoplasm and C-terminus in the periplasm ($N_{(in)}-C_{(out)}$ orientation), encoded in
86 the *mgtCBR* operon, downstream to the gene coding for the magnesium transporter MgtB
87 (Soncini, Garcia Vescovi et al. 1996; Groisman 2001). MgtR interacts with the inner membrane
88 protein MgtC and promotes its degradation by FtsH protease, thus providing a negative
89 feedback regulation (Alix and Blanc-Potard 2008). An Ala coil motif in the transmembrane
90 domain of MgtR is essential for the interaction between MgtR and MgtC (Alix and Blanc-
91 Potard 2008). The level of MgtB protein is not affected by MgtR, as shown by using *mgtR*
92 deletion mutant or *mgtR* overexpressing strains (Alix and Blanc-Potard 2008; Olvera, Garai et
93 al. 2019), but deletion of the *mgtR* gene slightly enhanced expression of MgtA, another Mg^{2+}
94 transporter of *Salmonella* regulated by PhoP, indicative of a negative regulation by MgtR (Choi,
95 Lee et al. 2012).

96 MgtC is a virulence factor that plays an important role in the adaptation to Mg^{2+}
97 deprivation and in the intramacrophage survival of *S. Typhimurium* (Blanc-Potard and
98 Groisman 1997). Although a *Salmonella mgtR* mutant is not significantly attenuated in
99 macrophages (Alix and Blanc-Potard 2008), the overexpression of MgtR decreased the survival
100 of *Salmonella* inside macrophages, likely due to a decreased level of MgtC protein (Olvera,
101 Garai et al. 2019). Hence, MgtR appeared as an antivirulence molecule. While MgtC has been
102 described in other bacterial pathogens (Alix and Blanc-Potard 2007; Belon, Soscia et al. 2015),
103 MgtR is naturally found only in *Salmonella* (Alix and Blanc-Potard 2008). However,
104 *Salmonella* MgtR interacts with MgtC from other species and heterologous expression of *mgtR*
105 in mycobacteria and *Pseudomonas aeruginosa* reduced intramacrophage survival, suggesting
106 that the antivirulence strategy may apply to these bacteria as well (Jean-Francois, Dai et al.
107 2014; Belon, Soscia et al. 2015; Belon, Rosas Olvera et al. 2016).

108 A synthetic MgtR peptide was used to test the antivirulence property of MgtR when
109 added exogenously to pathogenic bacteria. The synthetic MgtR peptide was successful in

110 reducing the pathogenicity of *S. Typhimurium* (Olvera, Garai et al. 2019). Interestingly, a
111 derivative lacking the Ala-coil interacting motif was also efficient in reducing *Salmonella*
112 intramacrophage survival and lowering not only the level of MgtC protein but also of other
113 inner membrane proteins. This suggested that the effect of synthetic MgtR is less specific than
114 that of endogenously produced MgtR. The growth of *S. Typhimurium* was not affected by
115 synthetic MgtR, indicating a mode of action that differs from classical antimicrobial peptides
116 (Olvera, Garai et al. 2019). Synthetic MgtR peptide was also found to reduce the level of MgtC
117 protein and the survival of *P. aeruginosa* inside macrophages without bactericidal activity
118 (Moussouni, Nogaret et al. 2019), supporting the therapeutic implication towards various
119 pathogens.

120 The 31-amino acid long inner membrane protein MgtS (formerly denoted YneM) was
121 first discovered in *E. coli*, where it acts to increase intracellular Mg^{2+} levels and maintain cell
122 integrity upon Mg^{2+} depletion (Wang, Yin et al. 2017). MgtS interacts with MgtA to increase
123 the levels of this P-type ATPase Mg^{2+} transporter under Mg^{2+} -limiting conditions.
124 Overexpression of MgtS also leads to induction of the PhoRB regulon because MgtS can form
125 a complex with a second protein, PitA, a cation-phosphate symporter (Yin, Wu Orr et al. 2019).
126 Interestingly, MgtS is also present in *S. Typhimurium* (Table 1), but its role has not been
127 studied. An effect of *Salmonella* MgtS on MgtA stabilization would antagonize the role of
128 MgtR. Moreover, it would be of interest to test whether *Salmonella* MgtS can also stabilize
129 MgtC or MgtB, which are not present in *E. coli*.

130

131 MgrB: modulator of Mg^{2+} -responsive signaling pathway

132 MgrB is a 47-amino acid long inner membrane protein, with an $N_{(in)}-C_{(out)}$ orientation,
133 first identified in *E. coli* and also found in enterobacterial pathogens including *Salmonella*
134 *enterica* (designated as *yobG*), *Shigella flexneri*, *Yersinia pestis* and *Klebsiella pneumoniae*

135 (Lippa and Goulian 2009). Its transcription is activated by phosphorylated PhoP under
136 magnesium limitation (Lippa and Goulian 2009).

137 MgrB negatively regulates the PhoQ sensor kinase activity by interacting with its
138 periplasmic domain, thus preventing any further phosphorylation of PhoP and therefore *mgrB*
139 expression (Lippa and Goulian 2009). This MgrB-mediated negative feedback serves as a mode
140 of a partial adaptation to achieve a steady state level of expression of genes induced by PhoP
141 (Salazar, Podgornaia et al. 2016). Interestingly, this modulation of function of PhoP, mediated
142 by MgrB, was observed even under high levels of magnesium, a condition that reduces PhoP
143 phosphorylation (Lippa and Goulian 2009). Intriguingly, another small membrane protein of
144 65-amino acids, SafA (formerly known as B1500), also binds to PhoQ to promote its kinase
145 activity and therefore opposes the action of MgrB (Eguchi, Itou et al. 2007; Eguchi, Ishii et al.
146 2012). MgrB has been proposed to mediate a redox sensitivity in the periplasm, which plays a
147 critical role in the regulation of the PhoQP circuit (Lippa and Goulian 2012). Disulfide bonding
148 by one or more MgrB cysteine residues seems important for the repressive effect of MgrB on
149 PhoQ and a model has been proposed where DsbA affects PhoQP signaling by oxidizing C28
150 and C39 of MgrB (Lippa and Goulian 2012). In *E. coli*, MgrB is also required for survival under
151 acid stress (Hobbs, Astarita et al. 2010), possibly due to a direct interaction between MgrB and
152 IraM, leading to accumulation of RpoS and induction of acid resistance mediated by GadABC
153 and HdeAB systems (Xu, Li et al. 2019).

154 In *S. Typhimurium*, where PhoQP plays a major role in virulence, MgrB acts as a
155 negative regulator of the expression of the effector *steA* by inhibiting PhoP-dependent
156 activation of *steA* (Cardenal-Munoz and Ramos-Morales 2013). In *K. pneumoniae* clinical
157 isolates, inactivation of the *mgrB* gene is responsible for acquired colistin resistance by
158 upregulating PhoQP activity, which, in turn, activates the PmrBA system responsible of

159 modification of the lipopolysaccharide polymyxin target (Cannatelli, D'Andrea et al. 2013;
160 Cannatelli, Giani et al. 2014).

161

162 PmrR: regulator of LPS in response to Fe³⁺ levels

163 PmrR is a 30-amino acid long hydrophobic protein located in the inner membrane of *S.*
164 *Typhimurium* (Kato, Chen et al. 2012). PmrR was identified as a PmrA-activated sORF located
165 immediately downstream to the *pmrAB* operon encoding the PmrBA TCS (Kato, Chen et al.
166 2012). It was also independently identified by a bioinformatics approach (Alix and Blanc-
167 Potard 2009). PmrBA, which responds to the presence of Fe³⁺ ions in the environment, is also
168 responsible for modifying LPS in *Salmonella* during the shift from Fe³⁺ poor to Fe³⁺ rich
169 environment in order to prevent toxicity by Fe³⁺ ions (Wosten, Kox et al. 2000; Kato, Chen et
170 al. 2012). PmrR interacts with and inhibits the activity of LpxT, an inner membrane enzyme
171 responsible for increasing the LPS negative charge. This prevents the generation of 1-PP lipid
172 A and decreases the negative charge of LPS, which further lowers Fe³⁺ binding to the bacterial
173 cell (Kato, Chen et al. 2012). Because Fe³⁺ is an activating ligand for the sensor PmrB,
174 transcription of PmrA-dependent LPS-modifying genes is reduced by PmrR. This mechanism
175 acts as a negative feedback regulation for the expression of PmrA and enables bacteria to
176 regulate their cell surface modifications based on the availability of an inducing signal.

177 The importance of LPS in virulence of *Salmonella*, including intracellular replication of
178 the pathogen, is well established (Nagy, Danino et al. 2006). Hence PmrR-dependent
179 modification of LPS could be exploited for the development of antivirulence therapeutic agents
180 against *Salmonella* or other related pathogens, like *K. pneumoniae*, which are known to contain
181 PmrR (Kato, Chen et al. 2012).

182

183 KdpF: stabilizer of a K⁺ transporter

184 KdpF is a 30-amino acid long membrane protein with an N_(in)-C_(out) orientation, which
185 is part of the P-type K⁺ transporter complex KdpFABC (Huang, Pedersen et al. 2017).
186 Expression of *kdpFABC* is induced by the KdpDE TCS under K⁺ limitation (Greie and
187 Altendorf 2007; Gannoun-Zaki, Alibaud et al. 2013). KdpF, mainly studied in *E. coli*, but
188 present and annotated in other bacterial genomes, is proposed to be a subunit involved in the
189 stabilization of the KdpABC complex *in vitro*, possibly acting as a lipid-like peptide for the
190 holoenzyme (Greie and Altendorf 2007). However, the role of KdpF *in vivo* remains elusive
191 because it is not essential for the K⁺-stimulated ATPase activity of the Kdp complex.

192 Interestingly, overexpression of KdpF in *Mycobacterium bovis* BCG reduced
193 intramacrophage growth and altered cording morphology (Gannoun-Zaki, Alibaud et al. 2013).
194 This lower replication rate within macrophages is linked to an increased bacterial susceptibility
195 to nitrosative stress (Rosas Olvera, Vives et al. 2017). Accordingly, *Mycobacterium* KdpF can
196 interact with membrane proteins involved in nitrosative stress detoxification or induced by
197 nitrosative stress. Exogenous addition of KdpF synthetic peptide correlated with reduced level
198 of these interacting target proteins (Rosas Olvera, Vives et al. 2017). Moreover, exogenous
199 KdpF peptide presented similar biological effects as the endogenously expressed KdpF
200 including nitrosative stress susceptibility and reduced intramacrophage replication rate for *M.*
201 *bovis* BCG, highlighting KdpF as a potent molecule with antivirulence properties. However,
202 this is not common to all intracellular pathogens because, in contrast to mycobacteria, *kdpF*
203 overexpression in *S. Typhimurium* increases intramacrophage multiplication, which may be
204 related to opposite roles reported for the KdpDE TCS in the virulence of these two pathogens
205 (Gannoun-Zaki, Belon et al. 2014).

206

207 **Small membrane proteins implicated in adaptation to environmental stresses and**
208 **antibiotic resistance**

209 This section deals with additional small regulatory proteins (Table 1) that interact with
210 larger proteins and participate in the coping strategies of pathogens during environmental
211 stresses like oxidative stress or are involved in the development of resistance to antibiotics.
212 These proteins appear to be beneficial for bacterial pathogens and although they have not been
213 implicated in therapeutic strategies so far, one can propose that preventing interaction with their
214 protein targets using mimicking peptides (Stone and Deber 2017) may reduce bacterial
215 virulence.

216

217 CydX: cytochrome oxidase component involved in oxidative stress adaptation

218 CydX was first identified in *E. coli* during the biochemical study of the cytochrome *bd*-
219 I oxidase (Miller and Gennis 1983) and its gene was identified as a sORF in the *cydAB* operon,
220 previously named *ybgT* (VanOrsdel, Bhatt et al. 2013). CydX is a 37-amino acid long protein
221 shown to be part of the oxidase complex by cryo-EM (Safarian, Hahn et al. 2019), required for
222 the cytochrome *bd* oxidase activity and proposed to be involved in the folding and stabilization
223 of the complex. CydX homologues are found in various bacteria, with a high degree of sequence
224 variation in the C-terminal region (Allen, Brenner et al. 2014). The CydAB oxidase is known
225 to play a role in pathogen survival within hosts and functional studies to specifically address
226 the contribution of CydX have been carried out in two bacterial pathogens, *Brucella abortus*
227 and *S. Typhimurium*. A *B. abortus cydX* mutant showed increased sensitivity to H₂O₂ and
228 decreased acid tolerance (Sun, de Jong et al. 2012). *S. typhimurium* CydX plays an essential
229 role in cytochrome *bd* function under stress conditions, including exposure to nitric oxide, and
230 is required for bacterial replication within macrophages (Duc, Kang et al. 2020).

231

232 Prli42: activator of stressosome for adaptation to oxidative stress

233 Prli42 is a 31-amino acid long tail-anchored membrane protein identified in *Listeria*
234 *monocytogenes* through a global approach of N-terminal proteogenomics (Impens, Rolhion et
235 al. 2017) (see below). Prli42 relays oxidative stress signals to the stressosome to activate the
236 general sigma factor B (σ^B) regulon mediated stress-sensing pathway. In contrast to the other
237 small regulatory proteins described here, Prli42 directly interacts with a cytosolic component
238 of the stressosome, RsbR, and not a membrane protein. This interaction involves the basic
239 amino acids K4, K5 and K8 of the N-terminus of Prli42 and the acidic residues E109, E110 and
240 E113 of N-terminus of RsbR (Impens, Rolhion et al. 2017). Cryo-electron microscopy revealed
241 that this interaction enables RsbR to sense oxidative stress in the external environment leading
242 to the activation of the stressosome. This is followed by a phosphorylation signaling cascade to
243 activate σ^B and express stress response genes required for the survival of *L. monocytogenes*
244 under oxidative stress (Williams, Redzej et al. 2019). The contribution of Prli42 in adaptation
245 to oxidative stress explains its role in the survival of *L. monocytogenes* inside macrophages
246 (Impens, Rolhion et al. 2017).

247

248 AcrZ: Regulator of drug efflux pump

249 AcrZ, a 49-amino acid long protein (previously known as YbhT) found in the inner
250 membrane of *E. coli*, modulates the efflux activity of AcrAB-TolC drug efflux pump, which is
251 required for resistance against several antibiotics (Hobbs, Yin et al. 2012). Accordingly, a
252 mutation of AcrZ leads to sensitivity towards many antibiotics including chloramphenicol and
253 tetracyclin (Hobbs, Yin et al. 2012). Transcription of *acrZ* is coregulated with *acrAB* (Hobbs,
254 Yin et al. 2012). Cryoelectron microscopy showed that AcrZ interacts with the inner membrane

255 protein AcrB (Wang, Fan et al. 2017) and AcrZ cooperates with lipids to allosterically modulate
256 AcrB activity by inducing conformational changes in the drug-binding pocket (Du, Neuberger
257 et al. 2020). AcrZ is highly conserved across enteric bacteria including enteropathogens like
258 *Salmonella* and *Klebsiella* and therefore holds potential in the context of studying multi-drug
259 resistance development by these pathogens.

260

261 **Small hydrophobic proteins as part of type I toxin-antitoxin modules**

262 Chromosomally encoded toxin-antitoxin (TA) systems are abundantly present in
263 bacteria. TA loci encode a stable toxin whose ectopic overexpression either kills cells or confers
264 growth stasis, and an unstable antitoxin. In type I TA systems, the antitoxin is a small antisense
265 RNA that base-pairs with the toxin-encoding mRNA (Brantl 2012; Brielle, Pinel-Marie et al.
266 2016). We describe here two examples of transmembrane type I toxic peptides present in *S.*
267 *Typhimurium* and *Staphylococcus aureus* (Table 1). Many additional toxic hydrophobic small
268 proteins are known in *E. coli* and shared by enteropathogens (Fozo, Hemm et al. 2008; Hemm,
269 Weaver et al. 2020). In terms of biological activity, these toxins are related to bacteriocins,
270 which are the subset of antimicrobial peptides produced by bacteria, consisting of small
271 amphipathic peptides that interact with bacterial membranes leading to cell death (Yang, Lin et
272 al. 2014). However, bacteriocins are secreted, whereas toxins of type I TA systems usually
273 remain membrane associated.

274

275 TisB: role in persistence and antibiotic tolerance

276 TisB, an SOS-induced membrane associated small protein identified in *E. coli*, is part
277 of TisB/IstR1 TA system, where IstR is a non-coding RNA that inhibits the translation of the
278 TisB toxin mRNA (Vogel, Argaman et al. 2004; Wagner and Unoson 2012). In addition to

279 slowing bacterial growth down, TisB also plays a role in the formation of persister cells (Dorr,
280 Vulic et al. 2010; Wagner and Unoson 2012), a subset of a bacterial population that enters a
281 dormant state. Persister cells become refractory to the action of antibiotics and bacteria
282 producing TisB toxin are tolerant to multiple antibiotics (Dorr, Vulic et al. 2010). TisB was
283 shown to decrease proton motive force and ATP levels (Unoson and Wagner 2008), consistent
284 with its role in formation of dormant cells. TisB forms an anion channel in artificial membranes
285 (Gurnev, Ortenberg et al. 2012), thus functions as a typical antimicrobial peptide. The pattern
286 of charges on the amphiphilic TisB helix suggested the presence of antiparallel dimers
287 assembled via a ladder of salt bridges in the form of electrostatic charge-zipper (Steinbrecher,
288 Prock et al. 2012). A computational-experimental approach found TisB to be inserted upright
289 in the membrane as a tetramer (antiparallel dimer-of-dimers) (Schneider, Wadhvani et al.
290 2019).

291 Interestingly, TisB is also found in *Salmonella* (Table 1) but its role has not been
292 investigated. A *Salmonella* mutant lacking the regulatory sRNA *istR* showed small, but
293 reproducible, reduction in fitness during competitive infection in mice (Santiviago, Reynolds
294 et al. 2009). Considering the role of TisB in *E. coli*, a deletion of *tisB* in *S. Typhimurium* may
295 reduce persistence frequency in macrophages and play a role in antibiotic tolerance. Moreover,
296 synthetic peptides derived from TisB may display antimicrobial potential against *Salmonella*.

297

298 PepA1, PepG1 and PepG2: role in toxicity towards bacteria and host cells

299 Several functional type I toxin-antitoxin (TA) modules with membrane peptide toxins
300 are expressed by the human pathogen *S. aureus*. PepA1 (part of the SprA1/SprA1AS module)
301 localizes in the membrane and triggers *S. aureus* death (Sayed, Nonin-Lecomte et al. 2012). Its
302 solution structure was solved as a long bent, interrupted helix. When inserted into the *S. aureus*
303 membrane, the PepA1 conformation switches to a continuous helix, presumably forming pores

304 to alter membrane integrity. PepA1 expression is favored in an acidic environment and during
305 oxidative stress, which may play a role in the survival of *S. aureus* after internalization into host
306 immune cells (Sayed, Nonin-Lecomte et al. 2012). *S. aureus* also expresses a related toxin
307 PepA2 (part of the SprA2/SprA2AS module), not redundant with PepA1, which internally
308 triggers bacterial death (Germain-Amiot, Augagneur et al. 2019). Although PepA2 does not
309 affect bacteria when it is present in the extracellular medium, it is highly toxic to other host
310 cells such as polymorphonuclear neutrophils and erythrocytes.

311 Due to its antibacterial activity, PepA1 has been a starting point for the development of
312 novel therapeutics. Exogenously provided PepA1 has activity against both Gram-positive and
313 Gram-negative bacteria (Solecki, Mosbah et al. 2015). However, PepA1 was significantly
314 hemolytic, thus limiting its use as an antibacterial agent. To overcome these limitations, PepA1
315 was converted into a cyclic heptapseudopeptide derivative with no toxicity to human cells, but
316 sharp antibacterial activity. Mechanistically, linear and helical PepA1 derivatives form pores at
317 the bacterial and erythrocyte surfaces, while the cyclic peptide induces bacterial envelope
318 reorganization with insignificant action on the erythrocytes (Solecki, Mosbah et al. 2015). Other
319 optimized cyclic heptapseudopeptides with unnatural amino acids that strengthen dynamic
320 association with bacterial lipid bilayers are effective against both MRSA and *P. aeruginosa* in
321 severe sepsis and skin infection models, respectively, thus confirming that they are promising
322 lead candidates for new antibacterial agents (Nicolas, Bordeau et al. 2019).

323 *S. aureus* also produces an unconventional type I TA system composed of the cis-
324 antisense RNA SprF1 and the stable SprG1 RNA, which encode two toxins (PepG1 and PepG2,
325 of 44 and 31 amino acids, respectively) that accumulate at the membrane and trigger *S. aureus*
326 death (Pinel-Marie et al., 2014). These two toxins can be secreted out by the pathogen to lyse
327 human host cells, a process performed more efficiently by PepG1. Both secreted toxins also
328 inactivate Gram-negative and -positive bacteria, with PepG2 being more effective (Pinel-Marie,

329 Brielle et al. 2014). Further studies are required to better characterize the role, kinetics of
330 expression and processing of these two toxins during infection, as well as to evaluate their
331 therapeutic potential using synthetic peptides.

332

333 **Global analyses leading to identification of sORFs in pathogenic bacteria**

334 The technologies to identify sORFs in bacteria have recently been improved (Miravet-
335 Verde, Ferrar et al. 2019; Weaver, Mohammad et al. 2019), which can allow analysis of the
336 small proteome of pathogenic bacteria and extend the identification of novel small proteins. As
337 detailed below, at present, global analysis leading to identification of sORFs in bacterial
338 pathogenic species has been carried out in *S. Typhimurium*, *L. monocytogenes*, *Mycoplasma*
339 *pneumoniae* and *S. aureus*.

340 For *S. Typhimurium*, a combination of *in silico* softwares allowed the identification of
341 14 putative sORFs below 50 amino acids with a single trans-membrane domain (Alix and
342 Blanc-Potard 2009). Nine of them were conserved in *E. coli*, whereas others were *Salmonella*-
343 specific. MgtR and PmrR, which were identified independently in other studies (Alix and
344 Blanc-Potard 2008; Kato, Chen et al. 2012), were also recovered. Later, an experimental global
345 study based on ribosome profiling identified translated sORFs in *S. Typhimurium* (Baek, Lee
346 et al. 2017). 113 small proteins below 50 amino acids were identified. Expression of 25 small
347 proteins was validated in several conditions, including infection-relevant conditions of low
348 Mg²⁺ or pH 5.8. Interestingly, few sORFs were specifically expressed in infection-relevant
349 conditions and hence are good candidates for playing a role during infection. This study
350 however did not specify the proportion of predicted hydrophobic proteins, nor investigated the
351 cellular location of small proteins. In addition, a mass spectrometry-based proteomics approach
352 used to explore the RpoS proteome (alternative sigma factor involved in stress response)
353 allowed the identification of several small proteins. This included small hydrophobic proteins

354 identified in other studies and present in *E. coli* (YncL and YohP), as well as 4 uncharacterized
355 ORFs smaller than 50 amino acids controlled by RpoS (Lago, Monteil et al. 2017).

356 A genome-wide N-terminomic approach allowed unambiguous detection of bacterial
357 translation initiation sites (TISs) in *Listeria* and led to the discovery of 19 internal translation
358 initiation sites and 6 small proteins, including Prli42 (see above) (Impens, Rolhion et al. 2017).
359 The method of N-terminal COFRADIC (COmbined FRActional DIagonal Chromatography)
360 isolates N-terminal peptides via two sequential chromatographic separations. In prokaryotes,
361 N-terminal peptides are formylated and this modification can be exploited to identify TISs upon
362 treatment with a peptide deformylase inhibitor.

363 A combination of novel bioinformatics tool (RanSEPs) with omics approaches allowed
364 the description of 109 small ORFomes in *M. pneumoniae* (Miravet-Verde, Ferrar et al. 2019).
365 RanSEPs is a random forest-based tool for the prediction of small open reading frame-encoded
366 proteins (SEPs). The functional study of SEPs highlighted an enrichment in the membrane,
367 translation, metabolism and nucleotide-binding categories (Miravet-Verde, Ferrar et al. 2019).

368 Ribosome profiling was also used in *S. aureus* and examining the read coverage across
369 the entire genome allowed the identification of 19 novel ORFs of less than 70 -amino acids in
370 length (Davis, Gohara et al. 2014). Most of them contained weak Shine-Dalgarno sequences
371 and although some initiated with an AUG start codon, a vast majority of the newly identified
372 ORFs initiated with alternative codons. Using an *in vitro* translation system with T7 promoter-
373 driven templates carrying the native SD and initiation codons, the protein products of 10 ORFs
374 was detected. Among them, PepA1 peptide, which was found independently (Sayed, Nonin-
375 Lecomte et al. 2012), was recovered as Orf9.

376

377 **Uncovering novel functional hydrophobic small proteins in pathogenic bacteria**

378 With the increasing number of global analyses to uncover sORFs, the next challenge
379 will be to define which ORFs are expressed, the required conditions for their expression and
380 their function. Based on the amino acid sequence of known ORFs, one can distinguish some
381 features that may help developing tools to identify additional functional sORFs (Table 1).
382 Firstly, the size of these small proteins is often around 30-amino acids and few of these proteins
383 are around 45-50-amino acids. Secondly, their amino acid composition displays some common
384 features. Toxic peptides from TA systems have a C-terminal extremity enriched with positively
385 charged lysine residues, especially as last amino-acid (Table 1). Positively charged residues
386 (lysine and arginine) adjacent to the trans-membrane domain favor an N_(out)-C_(in) orientation,
387 according to the “positive inside” rule (von Heijne 1992). The positive inside rule applies for
388 single transmembrane domain proteins, as shown by the mutational analysis of conserved
389 positive residues close to the transmembrane domain (Fontaine, Fuchs et al. 2011).

390 In contrast to toxic peptides, regulatory small membrane proteins are usually found with
391 an N_(in)-C_(out) orientation (see above) and are not enriched with positively charged residues in
392 the C-terminal part (Table 1). In addition, with the exception of CydX, we noticed that these
393 proteins harbor aromatic residues at the C-terminal extremity and/or at the end of the trans-
394 membrane domain (Table 1). This may have an impact on the membrane localization of the
395 protein, as shown by the translocation of some outer membrane proteins (Soprova, Sauri et al.
396 2010). Tryptophan residues in the tail of small membrane protein may also contribute to the
397 stabilization of important structural features, as known for bacteriocins whose activity is
398 dependent on a tryptophan residue that helps in folding back of the C-terminal tail into the
399 central helical part (Fimland, Eijsink et al. 2002). Moreover, tryptophan residues located in the
400 transmembrane helices, which localize preferentially to the interfacial regions of phospholipid
401 bilayers, can influence interactions between transmembrane proteins (White and von Heijne
402 2005). Accordingly, PmrR’s interaction with its partner LpxT is dependent on a single

403 tryptophan residue at the position 25 (W25) (Kato et al., 2012). In addition, the W29 residue of
404 MgtS plays a role in the regulatory activity of MgtS towards MgtA (Wang, Yin et al. 2017).

405 Taking the size and amino acid sequence near the C-terminus into account may thus
406 help to infer sORFs of interest among those identified with predicted transmembrane domain
407 in global studies and to extend the number of small membrane proteins playing a role in
408 pathogenic bacteria.

409

410 **Concluding remarks**

411 With the development of new approaches to identify small ORFs, the number of putative
412 small proteins discovered in pathogenic bacteria is increasing and the current knowledge on
413 functional hydrophobic sORFs provides insights into prediction of additional ORFs of interest.
414 In functional studies, deletion of sORFs can have mild or unclear phenotypes, whereas
415 overexpression of sORFs can provide better insights into biological relevance (keeping in mind
416 that overproduction of such proteins can also drive non-specific effects on membrane lipids or
417 proteins). Functionally characterized hydrophobic small proteins contribute to the adaptation of
418 bacteria to changing environment and stress conditions, and are often implicated in negative
419 feedback regulation loops through direct interaction with diverse membrane proteins (Figure
420 1). Negative regulation of virulence factors, such as MgtC, supports that a tiny regulation is
421 required for optimal pathogenesis and opens the way for developing antivirulence strategies
422 (Figure 2).

423

424 The increasing characterization of small proteins involved in the regulation of bacterial
425 virulence, antibiotic resistance or bacterial toxicity provides new perspectives for therapeutic
426 strategies against bacterial pathogens (Figure 1), as shown recently with the use of synthetic

427 peptides derived from the natural small hydrophobic proteins PepA1, MgtR and KdpF.
428 Derivatives of toxic peptides, as PepA1, induce membrane permeability, leading to bacterial
429 death. On the other hand, synthetic peptides derived from regulatory small proteins MgtR and
430 KdpF act by lowering bacterial virulence (Figure 2). The therapeutic potential of synthetic
431 transmembrane domains is gaining interest as they can disrupt the folding of membrane proteins
432 or compete for protein-protein interactions in membranes (Stone and Deber 2017). Chemical
433 modifications, such as cyclization or use of non-natural amino acids, are required to improve
434 membrane insertion and stability of synthetic hydrophobic peptides (Stone and Deber 2017).
435 Tests of such chemically modified peptides in suitable animal models, as performed for cyclic
436 pseudopeptides derived from PepA1 (Nicolas, Bordeau et al. 2019), will then be performed to
437 validate their efficiency. Strategies to reduce bacterial growth or virulence may also be derived
438 from other known membrane proteins such as TisB or MgrB, which have been identified, but
439 not yet studied, in pathogenic bacteria. Discovery of additional small membrane proteins in
440 pathogenic bacteria will further increase the number of novel antibacterial or antivirulence
441 peptidic molecules available for therapeutic uses.

442

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448

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- 639
- 640
- 641

642 **Table 1. Amino acid sequences of functional hydrophobic small proteins from**
643 **pathogenic bacteria**

Regulatory small proteins	Amino acid sequence
MgtR (<i>S. Typhimurium</i>) (30 aa)	MNRSPDKIIALIFLLISLLVLCLAL <u>WQIVF</u>
MgtS (<i>S. Typhimurium</i>) (31 aa)	MLGSINL <u>FIVV</u> LGILFSGFLAA <u>WFSHKWDD</u>
PmrR (<i>S. Typhimurium</i>) (29 aa)	MKSYYKSLTTLCSVLIVSSFIYV <u>WVTTY</u>
MgrB (<i>S. Typhimurium</i>) (47 aa)	MKKFRWVVLGIVVVVCLLLWAQVFNIMCDQDVQFFSGICAINK <u>FIPW</u>
KdpF (<i>S. Typhimurium</i>) (29 aa)	MSAGVITGIVLVFLLLGYL ^V YALINAEAF
KdpF (<i>M. tuberculosis</i>) (30 aa)	MTTVDNIVGLVIAVALMAFLFAALL <u>FPEKF</u>
CydX (<i>S. Typhimurium</i>) (37 aa)	MWYFAWILGTLLACAFGIITALALEHVEAGKTGQEE <u>S</u>
Prli42 (<i>L. monocytogenes</i>) (31 aa)	MTNKKVVRVVVILMLIAIVLSSVLTGVLM <u>F</u> L
AcrZ (<i>S. Typhimurium</i>) (49 aa)	MLELLKSLVFAVIMVPVVMAILGLIYGLGEVFNIFSGIGQKDQSRQNR
Toxic hydrophobic small proteins	
TisB (<i>S. Typhimurium</i>) (29 aa)	MSVVDITILILKLIVAALQLLDAVL <u>KYLK</u>
PepA1 (<i>S. aureus</i>) (30 aa)	MLIFVHIIAPVISGCAIAFFSY <u>WLSRRNTK</u>
PepA2 (<i>S. aureus</i>) (35 aa)	MFNLLINIMTSAISGCLVAFFAH <u>WLRTRNNKKGDK</u>
PepG1 (<i>S. aureus</i>) (44 aa)	MVALLKSLERRRLMITISTMLQFGLFLIALIGLVI <u>KLIELSNKK</u>
PepG2 (<i>S. aureus</i>) (31 aa)	MITISTMLQFGLFLIALIGLVI <u>KLIELSNKK</u>

644 For each small protein, the number of amino-acids (aa) is indicated in parenthesis and the
645 predicted TM domain based on TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>) and
646 TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html) servers is underlined.

647 Aromatic residues in the C-terminus are in green. Positively charged residues in the C-terminus

648 are in red

649

650 **Table 2. Characterized small membrane proteins regulating larger membrane proteins in**
 651 **response to cation levels**

652

Small membrane protein	Regulation by TCS	Target	Function	Therapeutic potential upon exogenous addition
MgtR	PhoQP	MgtC, MgtA	Destabilization of MgtC	Antivirulence action (reduced intramacrophage survival)
MgtS	PhoQP	MgtA, PitA	Stabilization of MgtA Modulation of PitA activity	Unknown
PmrR	PmrBA	LpxT	Inhibition of LpxT activity	Antivirulence potential (LPS modification)
MgrB	PhoQP	PhoQ	Reduction of PhoQP activity	Antivirulence potential (lower PhoP phosphorylation)
KdpF	KdpDE	KdpABC	Stabilization of KdpABC	Antivirulence action (increased susceptibility to nitrosative stress)

653

654

655 **Figure legend**

656

657 **Figure 1. Small membrane proteins in pathogenic bacteria: from function as regulators**
658 **or toxins to therapeutic potential.** Regulatory small membrane proteins are generally
659 expressed in response to particular environmental cues in order to regulate specific functions
660 by interacting with their target protein within the membrane. Such interactions can either result
661 in stabilization or destabilization of the target protein to help the pathogen regulate its virulence
662 and adapt to certain environmental stress. Toxins are part of toxin-antitoxin modules, which
663 cause cell toxicity by protein accumulation and pore formation in the membrane, and also
664 contribute in the formation of persister cells. Therapeutic interventions against bacterial
665 pathogens are developed using synthetic peptides derived from natural small membrane
666 proteins known to destabilize virulence factors or display antibacterial action.

667

668 **Figure 2. Model of action of the regulatory small protein MgtR when endogenously**
669 **expressed or exogenously added in the context of therapeutic intervention.** MgtR is
670 regulated by the PhoQP TCS, which is activated under magnesium limitation (left panel). A
671 direct interaction between MgtR and the MgtC virulence factor negatively regulates MgtC (left
672 panel), which provides the basis for an antivirulence strategy. Addition to pathogenic bacteria
673 of synthetic MgtR peptide has an antivirulence action by reducing the amount of MgtC protein
674 (right panel) and possibly other membrane proteins (shown in blue).