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Extracytoplasmic function sigma factors in *Pseudomonas aeruginosa*

Sylvie Chevalier, Emeline Bouffartigues, Alexis Bazire, Ali Tahrioui, Rachel Duchesne, Damien Tortuel, Olivier Maillot, Thomas Clamens, Nicole Orange, Marc G.J. Feuilloley, et al.

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1 Transcriptional regulation of development in heterocyst-forming
2 cyanobacteria

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4

5 Enrique Flores*, Silvia Picossi, Ana Valladares and Antonia Herrero

6

7 Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC and Universidad de Sevilla, Seville,
8 Spain

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10 *E-mail address: eflores@ibvf.csic.es

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17 **ABSTRACT**

18

19

20 Filamentous, heterocyst-forming cyanobacteria are among the simplest multicellular systems
21 in Nature. In the absence of combined nitrogen, the filaments consist of vegetative cells that
22 fix CO₂ through oxygenic photosynthesis and micro-oxic heterocysts specialized for the fixation
23 of N₂ in a proportion of about 10 to 1. The development of a heterocyst-containing filament
24 involves differentiation of vegetative cells into heterocysts in a process that requires a distinct
25 gene expression program. Two transcription factors are strictly required, the CRP-family NtcA
26 and HetR. NtcA directly activates the expression of multiple genes during heterocyst
27 differentiation –in some cases assisted by coactivators including HetR– and in mature
28 heterocysts, whereas HetR is needed to build high NtcA levels in differentiating heterocysts
29 and directly activates some particular genes. A few other regulators of gene expression
30 participate at specific differentiation steps, and a specific transcription factor, CnfR, activates
31 *nif* gene expression under the micro-oxic conditions of the heterocyst.

32

33 1. Introduction

34

35 Multicellularity is a form of organization widely found in living organisms including evidently
36 eukaryotes but also prokaryotes. Two properties characterize multicellularity: cell-cell binding
37 and cell-cell communication. Additionally, organisms take advantage of multicellularity to
38 specialize cells in different functions, so that cellular differentiation is another hallmark of
39 multicellularity. Proterozoic microfossils frequently show a multicellular form, and filamentous
40 cyanobacteria showing cellular differentiation have been traced back to about 2.45 – 2.1
41 billion years ago [1]. Cyanobacteria are a phylogenetically coherent group of organisms
42 characterized by performing oxygenic photosynthesis, which allows a mainly photoautotrophic
43 mode of growth [2]. Additionally, many cyanobacteria can fix atmospheric N₂, making these
44 organisms capable to grow with very simple nutritional sources –air and water with a few
45 mineral salts. However, because the N₂-fixation machinery is very sensitive to oxygen [3],
46 cyanobacteria need to separate N₂ fixation from oxygenic photosynthesis. Whereas some
47 unicellular and filamentous cyanobacteria separate these two incompatible processes
48 temporally, restricting N₂ fixation to the dark period of diel cycles [3], some filamentous
49 cyanobacteria form cells specialized for N₂ fixation. Thus, in filaments of cells that fix CO₂
50 through oxygenic photosynthesis, some of these cells (usually less than 10 %) differentiate into
51 N₂-fixing heterocysts when no combined nitrogen is available [4]. Other developmental
52 options found in some heterocyst-forming cyanobacteria include the differentiation of
53 akinetes, a type of spores that resist drought and cold, and the production of hormogonia,
54 short motile filaments frequently made of small-sized cells that serve a dispersal function [5,
55 6]. Knowledge of the transcriptional regulation for the differentiation of akinetes and
56 hormogonia is however scarce [6]. This review will focus on the transcriptional regulatory
57 mechanisms that operate in heterocyst differentiation. We shall first briefly introduce the
58 structure of the cyanobacterial filament and the morphology and biochemistry of the
59 heterocyst, then describe the mechanisms of regulation of gene expression that operate
60 during the process of differentiation and in the mature N₂-fixing heterocyst, and finally discuss
61 the early regulation that leads to a defined pattern of heterocyst distribution in the filament.

62

63 2. Cyanobacterial filaments and heterocysts

64

65 Cyanobacteria of the order Nostocales –taxonomic section IV in the classification of Rippka *et*
66 *al.* [2]– are characterized by their ability to produce heterocysts when they lack a source of
67 combined nitrogen [2]. These cyanobacteria grow as filaments that can be hundreds of cells

68 long, and heterocysts can be found intercalary or at the end in the filaments, although some
69 strains make only terminal heterocysts [2]. Heterocysts are terminally differentiated cells, and
70 commitment to complete differentiation is established after the spatial distribution pattern of
71 heterocysts has already been settled [7, 8].

72

73 2.1. Structure of the cyanobacterial filament

74 The cyanobacteria are diderm bacteria, i.e., they bear a Gram-negative type of cell envelope.
75 The cells in a cyanobacterial filament are surrounded by their cytoplasmic membrane and
76 peptidoglycan layer(s), but the outer membrane does not enter into the septa between
77 consecutive cells and, therefore, is continuous along the filament [9]. This implies that the
78 periplasm –the space that lies between the cytoplasmic and outer membranes and contains
79 the peptidoglycan– is also continuous and may be a communication conduit between the cells
80 in the filament [10]. Consecutive cells in the filament are also joined by proteinaceous
81 structures known as septal junctions [9, 11], which appear to traverse the septal peptidoglycan
82 through perforations known as nanopores [12, 13]. These perforations are drilled in the
83 peptidoglycan by AmiC-type amidases [12, 14]. In the model heterocyst-forming strain
84 *Anabaena* sp. PCC 7120 (hereafter *Anabaena*), the SepJ (a.k.a. FraG), FraC and FraD proteins
85 are localized at the cell poles in the intercellular septa of the filament [15, 16]. They are
86 membrane proteins that, in the case of SepJ and FraD, have long extra-membrane sections
87 that appear to be located outside of the cells in the intercellular septa, and at least SepJ is
88 known to form multimers [17, 18]. Hence, they are candidate components of the septal
89 junctions (see below).

90

91 2.2. Heterocyst morphology

92 Under the light microscope, the heterocysts are seen larger than the vegetative cells and show
93 two refractile bodies at their poles (just one, proximal to the vegetative cells, in the case of
94 terminal heterocysts) (Fig. 1A). Under the electron microscope, differences become evident in
95 the global form of the heterocyst, in its envelope, and in its cytoplasm (Fig. 1B). The heterocyst
96 poles adjacent to vegetative cells take a narrow form known as “neck”, which makes the area
97 of contact between heterocysts and vegetative cells smaller than that between vegetative
98 cells. The cell envelope contains extra material that is deposited outside the outer membrane
99 and consists of an inner glycolipid (heterocyst-specific glycolipid, Hgl) layer and an outer
100 polysaccharide (heterocyst envelope polysaccharide, Hep) layer. Three characteristics
101 distinguish the heterocyst cytoplasm from the vegetative cell cytoplasm [6]: (i) intracellular
102 membranes are reorganized forming a structure known as “honeycomb” around the

103 heterocyst poles; (ii) glycogen granules and carboxysomes (the intracellular micro-
104 compartments that contain ribulose *bis*phosphate carboxylase/oxygenase [RuBisCO, the CO₂-
105 fixing enzyme]) disappear; and (iii) a large granule made of cyanophycin (multi-L-arginyl-poly
106 [L-aspartic acid]) is deposited at the heterocyst poles adjacent to vegetative cells –these
107 granules correspond to the refractile bodies observed by light microscopy.

108

109 2.3. Heterocyst biochemistry

110 The heterocyst has evolved to become a N₂-fixing factory. Hence, an essential goal of its
111 biochemistry is to keep a micro-oxic environment appropriate for the expression and function
112 of the N₂-fixing enzyme, nitrogenase, and accessory proteins. First, importantly, the oxygen-
113 releasing photosystem II (PSII) is non-functional in the heterocyst (reviewed in [19]). Second,
114 the extra envelope described above restricts gas diffusion contributing to reduce the amount
115 of oxygen that enters in the heterocyst as air (78 % N₂, 21 % O₂) [20]; indeed, O₂ has been
116 suggested to enter the heterocyst mainly through the vegetative cell-heterocyst connections
117 [20]. Third, dedicated terminal respiratory oxidases located in the honeycomb membranes and
118 cytoplasmic flavodiiron proteins consume O₂ contributing to create a micro-oxic environment
119 [21, 22]. The nitrogenase reaction consumes high amounts of ATP and reductant, and the
120 heterocyst keeps an electron transport chain and PSI activity that support
121 photophosphorylation and the reduction of ferredoxin that can provide nitrogenase with
122 electrons [19].

123 In the heterocyst, dinitrogen is reduced by the nitrogenase complex producing
124 ammonium (and hydrogen as a byproduct). The ammonium resulting from the N₂-fixing
125 reaction is incorporated by glutamine synthetase into glutamate producing glutamine [23].
126 Glutamine is, at least in part, transferred to vegetative cells in exchange for glutamate, which
127 is not synthesized at high levels in the heterocyst [24, 25]. Further anabolic nitrogen
128 metabolism in the heterocyst includes the biosynthesis of aspartate and arginine, which are
129 incorporated by cyanophycin synthetase into cyanophycin that serves as a dynamic nitrogen
130 reservoir [26, 27]. In addition to glutamine, β-aspartyl arginine, the product of cyanophycin
131 degradation by cyanophycinase, is transferred to vegetative cells, in which β-aspartyl arginine
132 is hydrolyzed by isoaspartyl dipeptidase releasing aspartate and arginine [28]. Hence, both
133 glutamine and β-aspartyl arginine serve as nitrogenous nutrients for the vegetative cells. In
134 turn, because the heterocyst does not accomplish the photosynthetic fixation of CO₂, the
135 vegetative cells provide the heterocysts with reduced carbon in the form of a sugar, sucrose,
136 that in *Anabaena* is split by an invertase –InvB– producing glucose and fructose, which

137 support heterocyst metabolism [29, 30]. This exchange of reduced carbon by fixed nitrogen is
138 at the basis of the multicellular behavior of heterocyst-forming cyanobacteria.

139

140 2.4. Intercellular molecular exchange

141 During heterocyst differentiation and in the diazotrophic filament, regulators and metabolites
142 are transferred between cells. Intercellular molecular exchange in filamentous cyanobacteria
143 has been probed with fluorescent markers including calcein, 5-carboxyfluorescein and the
144 sucrose analog esculin [13, 31, 32]. The transfer has properties of simple diffusion, indicating
145 the existence of direct connections between adjacent cells [31, 32]. Mutants of *Anabaena*
146 lacking the septal proteins SepJ or FraC and FraD are impaired in the intercellular transfer of
147 those fluorescent markers and make less nanopores than the wild type, suggesting that these
148 proteins contribute to the formation of the septal junctions [13, 31]. Differences in the
149 impairment of transfer of the various fluorescent markers in different mutants have suggested
150 the existence of functionally-discernible SepJ- and FraCD-related septal junctions [13, 17].
151 Important for our discussion here is that septal junction complexes in the vegetative
152 cell/heterocyst septa may have specific characteristics as compared to those in vegetative
153 cell/vegetative cell septa. Thus, these structures appear to be longer in the former than in the
154 latter [33], and the SepJ protein visualized as a SepJ-GFP fusion is seen as a single fluorescent
155 spot between vegetative cells and as a double spot in the vegetative cell/heterocyst septa,
156 implying a more spread localization in the latter than in the former [11].

157

158 3. Key transcription factors and transcriptional regulation mechanisms

159

160 Two genes whose inactivation completely abolish heterocyst differentiation are *ntcA* [34, 35]
161 and *hetR* [36]. Whereas NtcA is a global regulatory transcription factor of cyanobacteria
162 mediating responses to variations in the C-to-N balance of the cells, HetR is a transcription
163 factor specifically found in filamentous cyanobacteria whose best-known role is in cell
164 differentiation.

165

166 3.1. NtcA

167 NtcA is a cyanobacterial transcription factor that orchestrates the genetic responses to the C-
168 to-N balance by directly regulating the expression of multiple genes involved in pathways for
169 nitrogen assimilation, but also of a number of other metabolic pathways [37, 38]. Indeed, by
170 means of chromatin-immunoprecipitation assays performed in *Anabaena*, an extraordinarily

171 large number of NtcA-binding sites on DNA were identified that could be ascribed to more
172 than 2,000 genes [38]. Besides genes involved in nitrogen scavenging and N₂ fixation, the NtcA
173 targets included genes involved in DNA metabolism, transcription and translation, central
174 metabolism and, remarkably, many predicted regulatory genes, which should extend the range
175 of NtcA-influenced cellular processes. NtcA belongs to the CRP (cAMP receptor protein) family
176 of transcriptional regulators, presenting a domain architecture similar to that of the well-
177 characterized *E. coli* CRP protein, including an N-terminal β -roll fold, which in CRP
178 accommodates the effector cAMP, a central long dimerization helix and a C-terminal helix-
179 turn-helix (HTH) motif for interaction with DNA [39] (Fig. 2A). These regulators are dimeric
180 proteins, so that each subunit interacts with half of a palindromic recognition sequence in
181 DNA.

182 NtcA can act as a transcriptional activator or repressor. For both activities, the NtcA
183 dimer binds to palindromic DNA sites including the consensus sequence GTAN₈TAC [37, 38]. In
184 many NtcA-activated promoters an NtcA-binding site is found separated by ca. 22 bp from a -
185 10 promoter determinant of consensus sequence TAN₃T, thus conforming to the so-called
186 Class II bacterial activated promoters, in which the regulator binds in place of a missing or
187 degenerated -35 determinant. This promoter structure is frequently found in genes of
188 unicellular cyanobacteria and, in heterocyst-forming strains, in vegetative cell-expressed genes
189 as well as in genes expressed in mature heterocysts [40]. Besides this, NtcA can activate gene
190 expression from DNA sites centered further upstream the -35 position, conforming to Class I
191 activated promoters, or from promoters with very degenerated NtcA-binding sites, which are
192 generally activated during heterocyst differentiation [41]. Finally, repression by NtcA has been
193 shown involving binding sites downstream from promoter determinants, overlapping the -10
194 box or even within the regulated ORF [41].

195 Regarding metabolic signals, NtcA activity is influenced by 2-oxoglutarate (2-OG) [42].
196 Because cyanobacteria lack the enzyme 2-oxoglutarate dehydrogenase, the 2-OG cellular
197 levels depend on its synthesis during CO₂ fixation and its consumption mainly to form
198 glutamate by the glutamine synthetase/glutamate synthase pathway, thus representing an
199 indicator of the cellular C-to-N balance [40]. Indeed, 2-OG has been shown to increase the
200 binding of NtcA to DNA in many regulated promoters *in vitro*, although in contrast to CRP,
201 NtcA can interact with DNA in the absence of the effector (see e.g. [37, 42, 43]). Structurally,
202 the positive effect of 2-OG in binding is explained by its effect on dimer stabilization by
203 decreasing the distance between the dimerization helices of each subunit, which in turn
204 repositions the two second helices in the HTH DNA-binding domains, making the global
205 conformation more suitable for interaction with DNA [39]. In the absence of the effector, the

206 NtcA apo-protein already presents in its DNA-binding domain some exposed positively-charged
207 residues that represent a DNA-binding patch. In addition, the conformation of the last helices
208 in this domain allows some interaction with DNA, whereas these helices are buried in the apo-
209 CRP [39].

210 Whereas a positive effect of 2-OG on NtcA binding to DNA *in vitro* has been found in all
211 types of NtcA-activated promoters, although at variable extents, an additional and stringent
212 requirement for 2-OG has been found for the step of promoter melting to form the tripartite
213 NtcA-RNA polymerase-DNA open promoter complex, and thus for transcript production, at
214 Class II NtcA-activated promoters [43]. However, no specific structural information is available
215 for this 2-OG effect, although it is reminiscent of that of cAMP during Class II activation by CRP.
216 Finally, the intrinsic capacity of NtcA to interact with DNA may be important for its action as a
217 repressor under physiological conditions that determine low 2-OG levels. This appears to occur
218 in the *cmpR* gene (encoding a LysR-type regulator of a bicarbonate transporter operon), which
219 under conditions that determine low 2-OG levels is expressed at higher levels in an *ntcA*
220 mutant than in the wild type [44].

221

222 3.2. HetR

223 For many years considered as a heterocyst-specific regulator, evidence is recently
224 accumulating in support of HetR activity also in undifferentiated cells, consistent with the
225 presence of HetR in non-heterocystous cyanobacteria as well as in heterocyst formers [45].
226 HetR is required for the expression of multiple genes involved in heterocyst differentiation,
227 and binding of HetR to DNA from the promoter region of a few of them has been detected *in*
228 *vitro*, including sequences from the promoters of *hetR*, *patS* and *hepA* ([46], see below) as well
229 as of *hetP*, which has been implicated in the commitment to irreversible differentiation [47,
230 48]. A global study of HetR targets by chromatin immunoprecipitation identified 26 direct
231 targets, of which only 10 % are related to known heterocyst-differentiation genes [49]. Finally,
232 a role of HetR in repression of several gene promoters in vegetative cells has been reported
233 [29, 50, 51].

234 Structurally, HetR represents a rather distinct type of transcription factor. The crystal
235 structure of a dimer of HetR from the heterocyst-forming cyanobacterium *Fischerella* sp.
236 shows a central DNA-binding region constituted by the N-terminal HTH motifs of both
237 subunits, and new folds conforming two globular "flaps" and a "hood" over the central core,
238 the latter formed by the two C-terminal parts [52] (Fig. 2B). However, HetR tetramers have
239 been detected *in vivo*, at higher levels during heterocyst differentiation, leading to the
240 proposal that the tetramer represents an active form of HetR [53]. This is consistent with (i)

241 the fact that in crystals assembled by HetR in complex with long synthetic DNA targets, HetR
242 tetramers formed by interaction of the flap domains were the predominant form [54], and (ii)
243 the occurrence of tandem repeats of the HetR DNA-binding consensus sequence in several of
244 the identified HetR targets [49].

245

246 3.3. Mechanisms of transcriptional activation by NtcA and HetR

247 The expression of both *ntcA* and *hetR* increase early during heterocyst differentiation, upon
248 combined N step-down, in a mutually-dependent manner enhanced by positive
249 autoregulation, with *hetR* induction preceding that of *ntcA* [55]. Remarkably, induction takes
250 place in specific cells or cell clusters along the filament, marking the places of vegetative cell
251 differentiation into heterocysts ([56-58], see below). The response regulator-like factor NrrA,
252 which is directly induced by NtcA early upon N step-down from a canonical Class II promoter
253 [59], has been reported to bind the *hetR* promoter region *in vitro* and, hence, to mediate the
254 induction of *hetR* by NtcA [60].

255 Multiple genes whose products effect the morphological and biochemical
256 differentiation of heterocysts (see below), including secondary regulators, are also activated
257 upon combined N step-down at specific steps during differentiation [41, 61], and for some of
258 them induction could be ascribed to specific cells along the filament (see e.g. [29, 62]). In
259 contrast to *nrrA*, which is independent of HetR, *in vivo* activation of many other heterocyst-
260 differentiation genes depends on both NtcA and HetR, as they are not induced in strains
261 bearing inactivated *ntcA* or *hetR* genes. Mechanistically, a remarkable feature of heterocyst-
262 differentiation genes is that they present complex promoter sequences including several
263 successive promoters, which may include σ^{70} consensus-type promoters, likely directing a
264 basal level of gene expression in all the cells of the filament before N step-down, Class II and
265 Class I NtcA-activated promoters, and HetR-dependent promoters (see e.g. [41, 50, 63]). The
266 binding affinity of NtcA for the different NtcA-activated promoters and the response to HetR
267 are important in determining the temporal sequence of transcription activation during the
268 differentiation process that, as a result of the cell-confined increase of *hetR* and *ntcA*
269 expression, takes place in specific cells along the filament.

270 HetR appears to exert a positive effect on transcription activation by several
271 mechanisms. As mentioned above, direct binding of HetR *in vitro* has been reported for only a
272 few sequences upstream of heterocyst-differentiation genes [49], suggesting that the *in vivo*
273 requirement could be indirect in many promoters, e.g., by the requirement of high levels of
274 NtcA, attained by HetR-dependent activation of the *ntcA* gene, or of later secondary

275 regulators. In the case of the proximal promoter of the *devBCA* operon involved in deposition
276 of the Hgl layer (see below), HetR could not bind DNA by itself but was found to increase the
277 binding of NtcA at degenerated NtcA-binding sequences, thus behaving as an NtcA-coactivator
278 [50] (Fig. 2C). On the other hand, the *pipX* gene, which is activated directly by NtcA at late
279 stages of heterocyst differentiation, encodes a small protein required for full diazotrophic
280 growth and full activation of further late genes [62]. The structure of a complex of the NtcA
281 and PipX proteins from the unicellular cyanobacterium *Synechococcus elongatus* has been
282 determined, leading to the suggestion that binding of PipX stabilizes the active conformation
283 of NtcA [64]. With the *Anabaena* proteins, PipX has been shown to increase NtcA binding to
284 DNA and transcription from NtcA-dependent promoters *in vitro*, thus representing a late NtcA-
285 coactivator [65].

286 Regarding RNA polymerase sigma factors, NtcA-dependent transcription has been
287 achieved *in vitro* with the *Anabaena* RNA polymerase holoenzyme including the principal
288 group 1 σ^{70} -family factor SigA [43]. In *Anabaena*, the *sigA* gene is transcribed from one major,
289 proximal promoter that was first described to display constant activity [66] and later to be
290 repressed by NtcA after N step-down [67]. Additionally, several promoters induced upon
291 removal of combined nitrogen contribute to expression of *sigA*. This array of promoters
292 produces increased transcription of *sigA* in the differentiating cells, which is consistent with a
293 bulk of NtcA-dependent transcription during differentiation. Further, *Anabaena* has multiple
294 σ^{70} -family factors of groups 2 and 3 [68]. Although inactivation of none of them abolishes
295 heterocyst differentiation or diazotrophic growth, some of the single and double mutants that
296 have been isolated are affected in these processes [69]. Also, the *sigC* (group 2), *sigE* (group 2)
297 and *sigG* (group 3) genes are responsive to N step-down, with successive increases of
298 expression in the cells that are differentiating into heterocysts [70]. SigE is upregulated late
299 during differentiation, and both NrrA [71] and NtcA [72] directly bind to sequences upstream
300 of *sigE*. SigE is required for normal cellular differentiation and for expression of several
301 heterocyst-specific genes including *nifH* (encoding a nitrogenase subunit), *fdxH* (encoding a
302 putative electron donor to nitrogenase [73]), *hglE₂* (encoding a heterocyst-specific glycolipid
303 biosynthesis protein [72]), and the *xfp* operon involved in glycolysis [71]. Thus, in spite of
304 considerable redundancy, a succession of specific σ^{70} -family factors appears to cooperate with
305 SigA during heterocyst differentiation.

306 Finally, HetR is a target of phosphorylation *in vitro* and phosphorylation interferes with
307 the formation of HetR tetramers and HetR binding to DNA [53]. Consistently, overexpression in
308 *Anabaena* of a heterocyst-specific kinase, PknE, blocks heterocyst differentiation in a process

309 that appears to involve inactivation of HetR [74]. The *pknE* gene is induced late during
310 differentiation [61] with a direct participation of HetR [74] and NtcA [38], the latter using a
311 putative Class II promoter. These independent, *in vitro* and *in vivo* observations strongly
312 suggest that phosphorylation of HetR is a physiological process, leading to the proposal that
313 phosphorylation represents a mechanism of HetR inactivation at late stages of heterocyst
314 differentiation, when a direct action of HetR would be no longer required [53, 74].

315

316

317 4. Regulation of gene expression in the course of heterocyst 318 differentiation

319

320 The expression of genes involved in heterocyst differentiation and function is generally
321 dependent on NtcA and HetR. Although regulation by these transcription factors may in some
322 cases be indirect, evidence for direct regulation, especially by NtcA, is also available for many
323 genes. Additionally, a number of other regulators, some of them transcription factors, have
324 been shown to be involved in the expression of genes at specific stages of heterocyst
325 differentiation.

326

327 4.1. Biosynthesis and deposition of the heterocyst envelope

328 Key steps in the morphological and metabolic differentiation of heterocysts are the deposition
329 of the Hep and Hgl layers. During differentiation, the Hep layer is deposited before the Hgl
330 layer, allowing the latter to lie beneath the former.

331

332 4.1.1. Hep layer

333 In the chromosome of *Anabaena*, numerous genes involved in Hep layer formation are
334 clustered in a “gene expression island”, which covers ORFs *alr2825* to *alr2841* [75], and are
335 induced under nitrogen deprivation [76]. Because they are tightly packed together, the genes
336 *alr2825* to *alr2831* might constitute an operon, as might be the case for the genes *alr2835*
337 (*hepA*) to *alr2841* as well [61]. In spite of the presence of numerous NtcA binding sites in this
338 region [38], direct NtcA-activated expression has not been reported. On the other hand,
339 induction in response to nitrogen deprivation of the *hepA* gene within this cluster requires
340 HetR [56], which binds to a DNA fragment from the *hepA* promoter [46]. Direct regulation of
341 *hepA* by HetR establishes a link between formation of the Hep layer and the developmental
342 cascade of gene regulation that operates heterocyst differentiation (Fig. 3).

343 Expression of *hepA* is additionally dependent on HepK, a protein-histidine kinase of a
344 two-component regulatory system that shows homology to bacterial proteins involved in
345 acclimation to changing O₂ conditions [77]. Interestingly, a normal oxic atmosphere is required
346 for differentiation of mature heterocysts [78], but it remains to be determined whether HepK
347 actually mediates a response to O₂. A cognate response regulator of HepK is DevR [79], which
348 can be phosphorylated but lacks a C-terminal effector domain (RR* in Fig. 3). This suggests that
349 HepK/DevR participate in a phosphorelay transduction pathway that includes further protein
350 components, in particular a possible DNA binding protein [80].

351 Three additional putative regulatory genes that are required for production of the Hep
352 layer are *hepN* (*alr0117*) that encodes a histidine kinase that lacks a sensor domain (HK* in Fig.
353 3) [81, 82], *hepS* (*all2760*) that encodes a putatively membrane-anchored Ser-Thr kinase [82],
354 and *henR* (*alr1086*) that encodes a response regulator containing a CheY-like receiver domain
355 and a PPM-type (magnesium or manganese-dependent protein phosphatase that
356 dephosphorylates Ser and Thr residues) phosphatase domain [82]. The effect of inactivation of
357 some of these genes on global gene expression in *Anabaena* –analyzed with oligonucleotide
358 microarrays– has suggested that HepS and HenR belong to the same regulatory system, and
359 that HepN is part of the HepK/DevR regulatory pathway as well as of another undetermined
360 pathway [83]. To which environmental cues the HepS kinase and the HenR phosphatase
361 respond is unknown, although expression of *hepS* is activated under nitrogen deprivation [61].

362 In summary, genes involved in formation of the Hep layer are subjected to regulation
363 within the cascade of gene expression that operates during heterocyst differentiation
364 promoted at least by HetR, but also to pathway-specific regulation involving two-component
365 regulatory system(s) and protein kinases/phosphatases. A main goal of future research should
366 be to find out whether any of these pathway-specific regulators responds to oxygen, and a
367 point of general interest is whether any of them are subjected to direct NtcA/HetR-dependent
368 regulation.

369

370 4.1.2. Hgl layer

371 For a detailed discussion of the heterocyst envelope Hgl layer, the excellent review of Awai et
372 al [84] is recommended. As is the case for the Hep layer, numerous genes involved in the
373 formation of the Hgl layer are clustered in the chromosome of *Anabaena* (ORFs *all5341* to
374 *all5359*), including genes involved in the biosynthesis of the glycolipids and genes involved in
375 their deposition outside of the outer membrane [85, 86]. Further genes necessary for the
376 formation of the Hgl layer are located outside this cluster, and these include the *devBCA*
377 operon encoding and ABC exporter that, together with a TolC-like protein (HgdD), constitutes a

378 Type I secretion system involved in the export of glycolipids outside of the outer membrane in
379 the differentiating heterocyst [87]. Along the Hgl cluster, NtcA binds to several sites, including
380 sites upstream of genes *all5341* (*hglT*) and *asr5350* as well as in the region between *all5347*
381 and *all5348*, which could constitute activator sites [38]. On the other hand, as described
382 above, the promoter of the *devBCA* operon is a well-characterized, complex NtcA-dependent
383 promoter [50, 88, 89]. It contains one canonical Class II NtcA-activated promoter and one non-
384 canonical promoter that appears to be activated by NtcA assisted by HetR [50].

385 Inactivation of the *hepS* and *henR* genes involved in formation of the Hep layer also
386 down-regulates expression of genes involved in the formation of the Hgl layer such as *hglE_A*
387 (*alr5351*) [83]. On the other hand, DevH is a CRP-family transcriptional regulator whose
388 expression is increased under nitrogen deprivation in an NtcA- and HetR-dependent mode
389 [90]. Inactivation of *devH* affects specifically the expression of Hgl-biosynthesis genes such as
390 *hglE_A* and the production of Hgl [91]. Whether HepS/HenR and DevH act in concert in the
391 regulation of expression of *hgl* genes is unknown (discussed in [83]). *Anabaena* has two
392 heterocyst-specific glycolipids. Production of the minor one appears to be dependent also on
393 two Ser/Thr kinases, Pkn44 (All1625) and Pkn30 (All3691) [92], and production of the major
394 one appears to be dependent on a specific protein phosphatase, PrpJ1 (All1731), that also
395 affects expression of *hglE_A* [93]. The possibility that Pkn44/Pkn30 and PrpJ1 belong to two
396 different regulatory branches for production of the minor and major Hgls, respectively, acting
397 downstream of DevH has been discussed [92].

398 In summary, expression of genes for the formation of the Hgl layer involves
399 transcription factors including the well-known NtcA and HetR proteins discussed above and
400 DevH. In the case of DevH, the mechanism of dependence on NtcA and HetR and the direct
401 DNA targets remain to be elucidated. Additionally, protein kinases and phosphatases affect the
402 formation of the Hgl layer as a whole and specifically the major and minor glycolipids of that
403 layer. The environmental cues to which such kinases and phosphatases respond and how they
404 affect glycolipid production remain to be determined (e.g., they could affect transcription
405 factors or directly the activity of biosynthetic enzymes).

406

407 4.2. Oxygen-consuming enzymes and protection against ROS

408 Oxygen that enters the heterocyst is reduced by at least two types of enzymatic processes:
409 respiration [4] and reduction by flavodiiron proteins [94]. Additionally, a ruberythrin, RbrA,
410 protects against oxidative stress in the heterocyst [95]. In *Anabaena*, two terminal respiratory
411 oxidases, Cox2 and Cox3, substitute in the heterocyst for the terminal respiratory oxidase,

412 Cox1, expressed in vegetative cells [21]. Cox2 is an aa_3 -type cytochrome *c* oxidase encoded by
413 the *coxB2-coxA2-coxC2* operon and Cox3 appears to be an heme-copper quinol oxidase that is
414 encoded by the *alr2729-alr2730-coxB3-coxA3-coxC3* operon. The contribution of Cox3 to
415 protection of nitrogenase appears to be more important than that of Cox2 [21, 96]. The
416 expression of both operons is strictly dependent on NtcA and HetR. The *cox3* promoter region
417 has been characterized in detail and found to contain both Class I and Class II NtcA-dependent
418 promoters in which NtcA appears to work assisted by PipX [65].

419 Flavodiiron proteins directly reduce O₂ to water without the production of deleterious
420 reactive oxygen species (ROS) [97]. In *Anabaena*, two flavodiiron proteins, Flv1B and Flv3B, are
421 specifically expressed in the heterocysts [94], being Flv3B especially relevant for diazotrophic
422 performance [22]. These flavodiiron proteins are the products of two contiguous genes,
423 *all0178* (Flv3B) and *all0177* (Flv1B), which may constitute an operon expressed from an NtcA-
424 dependent promoter [38].

425 Interestingly, scavenging of O₂ by terminal respiratory oxidases and flavodiiron
426 proteins appears not to be redundant [38]. Whereas the terminal respiratory oxidases likely
427 remove O₂ in the heterocyst poles, since respiratory activity is located at the honeycomb
428 membranes [98], the heterocyst flavodiiron proteins are cytoplasmic [94]. Photosystem I-
429 generated reductants can also reduce O₂, but in this case producing ROS. In *Anabaena*, the
430 rubrerythrin RbrA is a heterocyst-specific peroxidase that transfers electrons from NADPH to
431 H₂O₂ with the participation of ferredoxin-NADP⁺ oxidoreductase [95]. The *rbrA* gene is
432 expressed from two promoters one of which directs expression in the heterocysts, is
433 dependent on HetR and binds HetR *in vitro* [95].

434

435 4.3. Particular heterocyst metabolism genes

436 The metabolism of the heterocyst is focused on the fixation of N₂ and the assimilation of the N
437 fixed in that reaction. We shall address now the transcriptional regulation of *nif* genes and of
438 nitrogen assimilation genes such as those encoding glutamine synthetase and cyanophycin
439 metabolism enzymes, as well as other relevant genes.

440

441 4.3.1. *nif* gene expression

442 In diazotrophic bacteria, the nitrogenase structural genes, *nifH-nifD-nifK*, are generally
443 clustered together with other nitrogen fixation genes –including, e.g., genes encoding
444 nitrogenase electron donors such as specific ferredoxins– and nitrogenase maturation genes –
445 such as FeMo-cofactor biosynthesis genes (99). The *Anabaena* genome bears a large *nif* gene

446 cluster including *nifB-fdxN-nifS-nifU-nifH-nifD-nifK-nifE-nifN-nifX* and a few other genes
447 downstream. Notably, *fdxN* and *nifD* are interrupted by long DNA elements that are excised
448 during heterocyst differentiation [100]. After excision, the *nifHDK* operon is mainly expressed
449 from a promoter located upstream from *nifB* [101], although the large mRNA produced is
450 subjected to processing so that *nifHDK*, *nifHD* and *nifH* transcripts are produced (see e.g. [96]).
451 In *Anabaena* and other diazotrophic cyanobacteria, including non-heterocystous strains, the
452 *nifB* promoter is activated by CnfR [102], previously known as PatB [103]. The CnfR protein has
453 N-terminal sequences for binding two 4Fe-4S clusters, resembling bacterial-type ferredoxins,
454 and a C-terminal HTH motif for binding to DNA [103, 104]. It is thought that CnfR can activate
455 *nif* gene expression in response to a low-oxygen tension [104, 105], and putative CnfR-binding
456 sites have been characterized in the promoter region of *nifB* [105, 106].

457 In *Anabaena*, expression of *cnfR* (*patB*) is induced in response to nitrogen deprivation
458 [61, 103], exclusively in heterocysts [107]. Based on the presence of consensus NtcA-binding
459 sites upstream of *cnfR*, it can be suggested that this gene is subjected to NtcA-dependent
460 expression in both heterocyst-forming and N₂-fixing, non-heterocystous cyanobacteria [38,
461 105]. *Anabaena variabilis* harbors two *nif* gene clusters, *nif1* that is expressed in the
462 heterocysts and *nif2* that is expressed in the vegetative cells under micro-oxic conditions (see
463 e.g. [106]), and expression of the *nif2* cluster regulatory gene *cnfR2* has been shown to require
464 NtcA [104]. Therefore, expression of *cnfR2* is activated when combined nitrogen is scarce, but
465 the Fe-S clusters may be formed only when the oxygen tension is low resulting in a protein
466 that can promote expression of the *nifB* operon only under micro-oxic conditions [104, 105].

467

468 4.3.2. Expression of *petH*

469 The *petH* gene encodes ferredoxin-NADP⁺ oxidoreductase that is expressed at much higher
470 levels in heterocysts than in vegetative cells [108], consistent with a very relevant role of this
471 enzyme in the distribution of electrons in the heterocyst [19]. In heterocysts, *petH* is expressed
472 from an NtcA-dependent promoter to which NtcA directly binds [109]. No good NtcA-binding
473 site is however found in this promoter, suggesting the involvement of an NtcA coactivator such
474 as PipX.

475

476 4.3.3. Expression of *glnA*

477 Glutamine synthetase, which incorporates into glutamate the ammonium produced in the N₂-
478 fixation reaction to synthesize glutamine, is present at higher levels in the heterocysts than in
479 vegetative cells [110]. The *glnA* gene encoding glutamine synthetase is expressed at higher

480 levels in the absence than in the presence of combined nitrogen, being transcribed from a
481 complex promoter region [111]. The promoter most proximal to the gene is a Class II NtcA-
482 dependent promoter that is used during growth with nitrate and under deprivation of
483 combined N [34, 35]. This is the *glnA* promoter that is used in the heterocysts, and its
484 dependence on NtcA has been corroborated by mutation of its NtcA-binding site [112].

485

486 4.3.4. Cyanophycin metabolism genes

487 As mentioned earlier, the dynamic N reservoir cyanophycin is accumulated conspicuously at
488 the heterocyst poles. In *Anabaena*, the genes encoding the main cyanophycin synthetase
489 (*all3879*, *cphA1*) and cyanophycinase (*all3880*, *cphB1*) are clustered together. These genes are
490 expressed as an operon (*cphB1-cphA1*) from a complex promoter region localized upstream of
491 *cphB1*, and *cphA1* is also transcribed monocistronically from a complex promoter present in
492 the intergenic region. In these regions, NtcA-dependent promoters comprising high- and low-
493 affinity NtcA-binding sites are found which are active in heterocysts [113]. The gene *all3922*,
494 encoding isoaspartyl dipeptidase that hydrolyzes the dipeptide β -aspartyl arginine, is induced
495 under nitrogen deprivation [61] and is expressed preferentially in vegetative cells [28],
496 consistent with the transfer of the dipeptide from the heterocysts to the vegetative cells.

497

498 4.3.5. Expression of RuBisCO

499 As mentioned earlier, the RuBisCO enzyme is not active in the heterocysts thus avoiding the
500 investment of energy and reductant in the fixation of CO₂, a process confined to the vegetative
501 cells. Lack of RuBisCO in the heterocysts results from repression of gene expression [114], and
502 binding of NtcA to a DNA site overlapping the translation start of the *rbcLXS* operon encoding
503 RuBisCO appears to represent a mechanism of repression [38, 115]. This mechanism of cell-
504 specific repression of RuBisCO stresses the relevant role of NtcA in regulation of gene
505 expression, not only by induction but also by repression, in the mature heterocyst.

506

507 5. Heterocyst patterning

508

509 Heterocyst distribution is not random in the cyanobacterial filaments, but instead it follows a
510 relatively fixed pattern. In most strains of the order Nostocales, heterocysts are separated by
511 ca. 10 to 15 vegetative cells along the filament. Importantly, this pattern is established early
512 upon N step-down, implying that products of N₂ fixation are not required for generation of at
513 least the initial pattern.

514

515 5.1. Coupled fluctuations of HetR expression

516 Several theoretical models have been put forward to try a mathematical description of the
517 generation of the heterocyst distribution pattern, and most of those models are based on the
518 operation of an activator and an inhibitor of differentiation with different intercellular
519 diffusivities (revised in [116]). The HetR transcription factor has been considered as the
520 activator in these models. As mentioned above, basal expression of *hetR* takes place in the
521 filament even in the presence of ammonium, and expression fluctuations are coupled in 2-4
522 nearby cells influenced by positive autoregulation ([117]; J. Stavans, personal communication).
523 Moreover, this pattern persists during the initial stages of development upon N step-down
524 [117], which could explain the classical observation that differentiation is first observed in
525 small clusters of cells that are later resolved to single heterocysts [8]. Thus, coupled
526 fluctuations of *hetR* expression after N step-down would build-up upon the basic pattern
527 already latent under N-replete conditions. At intermediate stages of differentiation, an
528 increase in autocorrelation functions of *hetR* expression is observed [117], which would result
529 from increased *hetR* autoregulation in response to *ntcA* (and *nrrA*) induction.

530

531 5.2. The PatS morphogen and the ABC exporter HetC

532 The *patS* gene is induced early in specific cells, depending on HetR [46], and its overexpression
533 abolishes heterocyst differentiation, whereas its inactivation leads to the formation of
534 heterocysts in groups (the so called MCH, *multiple contiguous heterocysts* phenotype). Hence,
535 it was suggested that a *patS* gene product effects a lateral inhibition of differentiation [118].
536 The primary product of the *patS* gene is a 17-amino acid peptide that is processed to render a
537 smaller active peptide including the C-terminal part of the primary gene product [118, 119],
538 likely consisting of the C-terminal seven residues [119, 120]. Nonetheless, a peptide consisting
539 of only the five C-terminal amino acids of PatS can also suppress heterocyst differentiation
540 when added to *Anabaena* cultures [118]. The active PatS peptide (which we will denote
541 hereafter PatS*) is transferred to the neighboring cells to establish a concentration gradient
542 with highest levels in the cells adjacent to the producing cell, in which the concentration
543 remains negligible, and decreasing thereon, thus behaving as a morphogen [119]. The N-
544 terminal half of the *patS* primary product is important for processing of the peptide and for
545 conferring immunity against the inhibitory activity in the producing cell, which would involve
546 peptide excretion [119].

547 The *hetC* gene encodes an ABC-type exporter that includes a putative peptidase
548 domain required for heterocyst differentiation [121, 122]. *hetC* is expressed from a complex
549 promoter region that includes a canonical NtcA-dependent promoter and a HetR- and NtcA-
550 dependent promoter, similar to the promoter region of the *devBAC* operon [43, 89]. HetC
551 localizes mostly to the heterocyst poles [122], and the possibility of its involvement in export
552 of PatS* from the differentiating heterocysts has been considered [122, 123], as has also been
553 considered the participation of the septal junction protein SepJ in the intercellular transfer of
554 PatS* between vegetative cells [124]. Regarding the inhibitory activity of the morphogen,
555 PatS* has been shown to interact with HetR [125, 126], to influence the turnover of HetR
556 [127], and to inhibit HetR tetramer accumulation [53] and HetR binding to DNA [46]. All these
557 observations are fully consistent with the reported isolation of a suppressor mutant of *patS*
558 overexpression, which bore a mutation in the *hetR* gene [128]. Thus, at least one component
559 of the inhibition of heterocyst differentiation by PatS* is the inhibition of HetR activity. Figure
560 4 presents a hypothetical model in which (i) HetC participates in processing of PatS and
561 transfer of PatS* from the (pro)heterocyst to its neighboring vegetative cell, and (ii) SepJ
562 participates in spreading of PatS* between vegetative cells along the filament. How PatS* is
563 prevented from moving back to the (pro)heterocyst through SepJ-related conduits is unknown,
564 but may be related to the distinct architecture of the septal junctions in vegetative cell-
565 heterocyst septa described above.

566 Besides being dependent on *hetR* auto-regulation, the spatial correlation of *hetR*
567 expression fluctuations along the filament, both in the presence of ammonium and early after
568 N step-down, is influenced by expression of *patS* and intercellular communication dependent
569 on SepJ [117]. This implies the existence of a basal expression of *patS* in the presence of
570 ammonium, consistent with the reported detection of *patS* transcription in the presence of
571 combined nitrogen [8]. Thus, fluctuations in the expression of *hetR* encoding a positive
572 regulator of differentiation, HetR positive autoregulation, lateral inhibition by PatS (whose
573 synthesis depends on HetR), and cell-to-cell communication are fundamental components of
574 the developmental process.

575

576 5.3. Other proteins influencing pattern formation

577 Finally, other protein factors –including HetL, HetN, PatA, PatL and PatN– have been described
578 to influence the spatial pattern of heterocyst distribution. The *hetN* gene influences the
579 pattern of heterocysts especially at advanced stages of differentiation [129, 130]. This effect
580 likely involves an internal hexapeptide sequence identical to the C-terminal sequence of PatS
581 [130, 131]. Indeed, at late, but not at early times after N step-down, expression of *hetN* has

582 been shown to influence the coupling of *hetR* expression fluctuations through the filament
583 [117]. At times when heterocyst differentiation is already completed, the FraC/FraD proteins
584 involved in intercellular molecular exchange impact *hetR* expression correlations in
585 neighboring cells in a manner similar to SepJ [117]. This effect of FraC/FraD could involve
586 transfer of a HetN-derived product or other regulators or N₂ fixation-derived metabolites.

587 Whereas inactivation of *patN* results in an increased heterocyst frequency [132, 133],
588 inactivation of *patA* [134] or *patL* [135] results in the preferential production of terminal
589 heterocysts in the filaments (implying a decreased heterocyst frequency). On the other hand,
590 overexpression of *hetL* increases heterocyst frequency [136]. PatL and HetL are pentapeptide
591 repeat proteins of unknown function, PatA and PatL have been shown to interact with each
592 other [135], and PatN has been shown to influence the expression of *patA* [132]. Significantly,
593 the *patA* gene is transcribed in differentiating heterocysts from two NtcA-activated promoters
594 [137]. PatA is a response regulator that lacks a DNA binding domain and counteracts PatS and
595 HetN inhibition of heterocyst differentiation [138].

596

597 6. Concluding remarks

598

599 Heterocyst differentiation from a vegetative cell involves changes in expression of several
600 hundred genes [139], including repression and induction (thoroughly reviewed by Xu *et al.*
601 [140]). Several transcription factors effecting this regulation have been identified mainly in the
602 model organism *Anabaena* (Table 1). NtcA and HetR are absolutely needed for heterocyst
603 differentiation, whereas other transcription factors participate in specific steps of the
604 differentiation process. NtcA and HetR are however not equivalent. Whereas NtcA is a global
605 transcription factor that activates numerous gene promoters at different steps of
606 differentiation, and represses some promoters as well, HetR has been shown to bind to few
607 DNA sequences possibly activating transcription. Additionally, the strict requirement for HetR
608 may rely on (i) its effect on localized induction of *ntcA* in the developing heterocyst and (ii) a
609 role as a coactivator of NtcA at specific promoters. An additional coactivator of NtcA is PipX,
610 which being expressed late in heterocyst differentiation supports NtcA-dependent activation
611 of late genes, including some that are expressed in mature heterocysts. In the mature
612 heterocyst, the *rbcL* operon promoter is directly repressed by NtcA, and a highly-expressed
613 gene such as *glnA* is transcribed from a canonical Class II NtcA-dependent promoter, raising
614 the possibility that expression in the mature heterocyst no longer requires differentiation-
615 specific transcription factors. This is consistent with an inactivation of HetR by phosphorylation

616 as discussed above. Finally, in the mature heterocyst, expression of the *nif* genes requires a
617 specific transcription factor (CnfR) that may link expression to the micro-oxic conditions
618 prevailing in the differentiated cell.

619

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621

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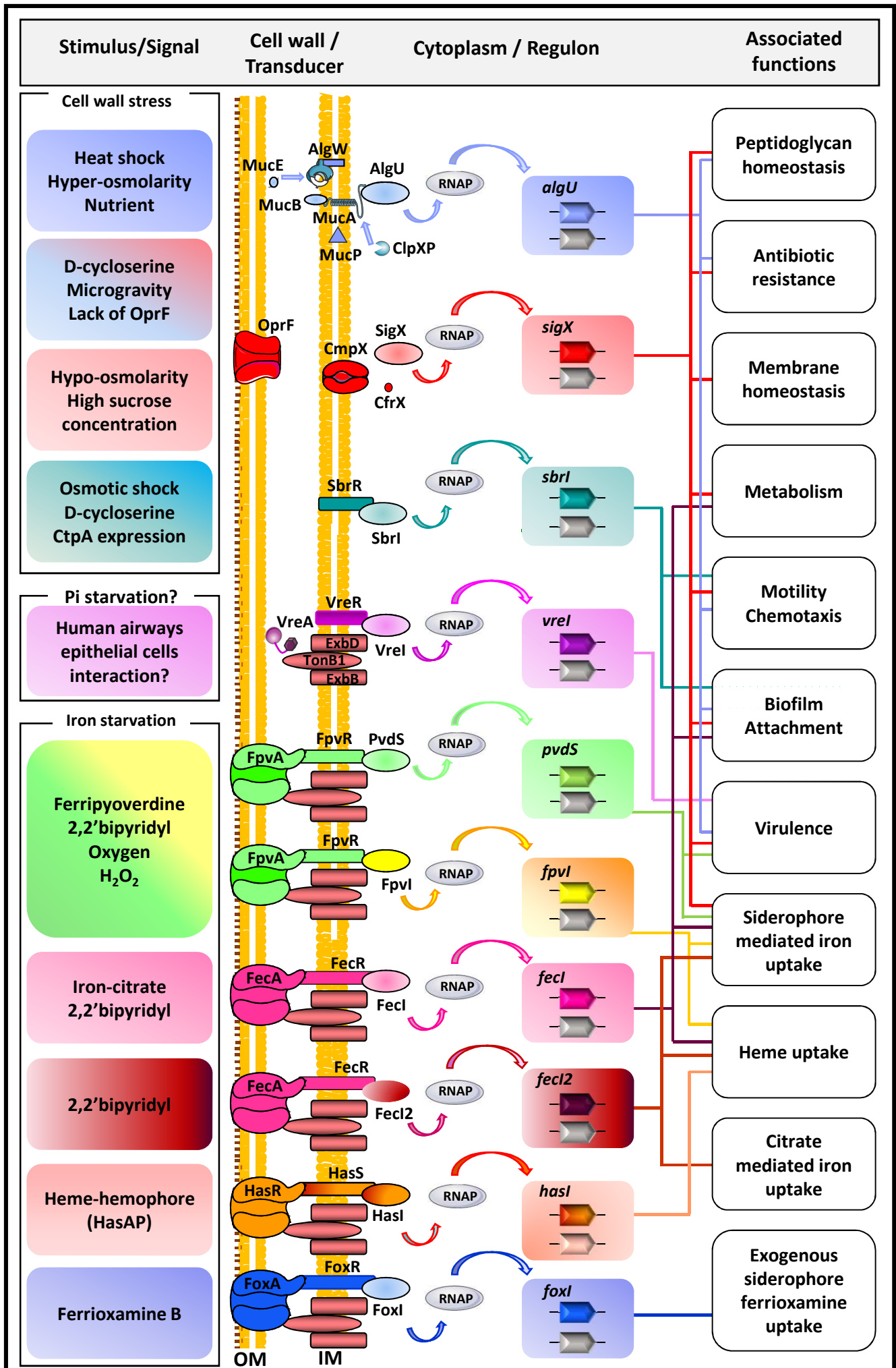


Figure 2. Mechanism of activation of AlgU (RpoE)

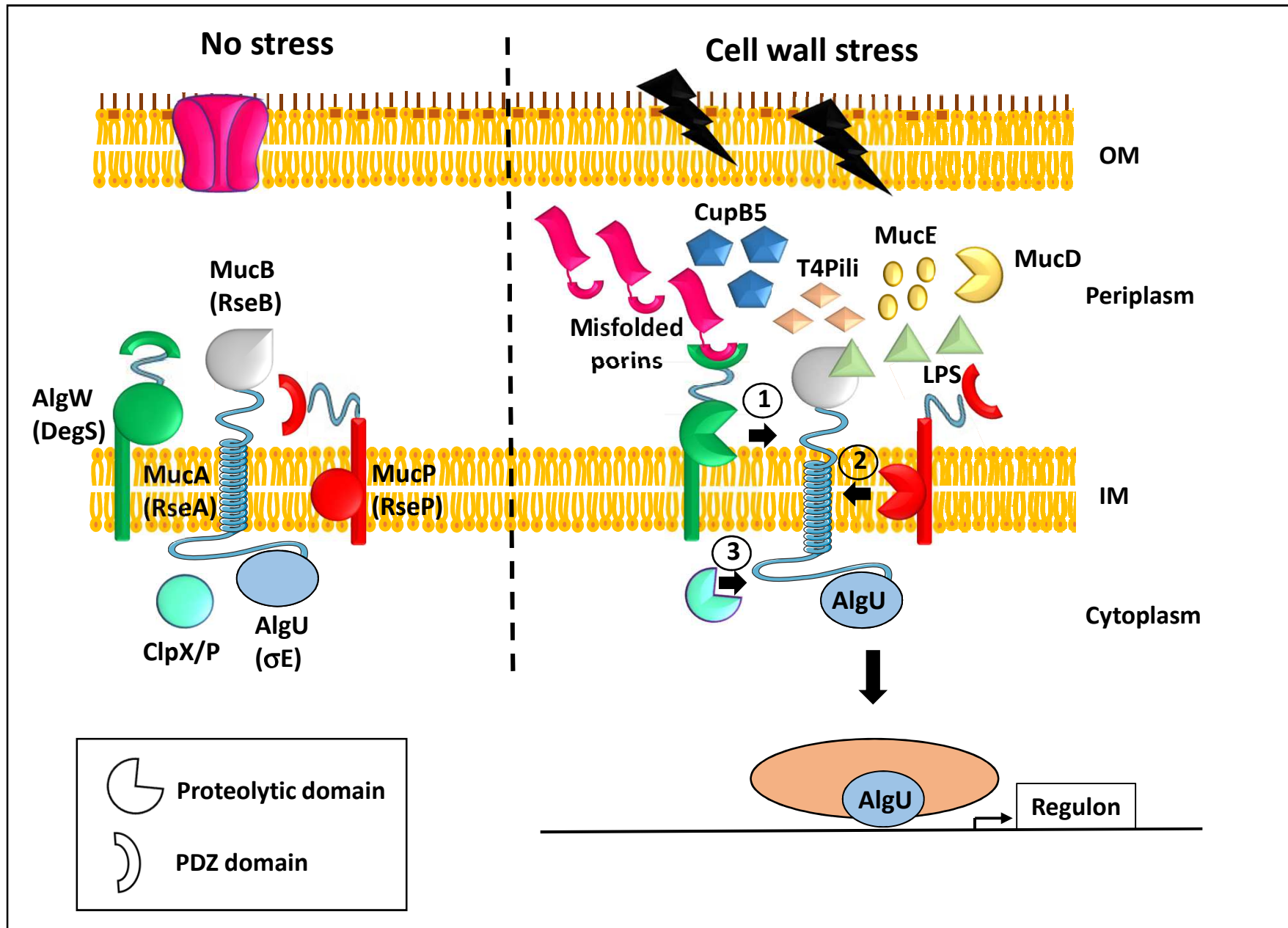


Figure 3: ECF sigma factors involved in regulators expression

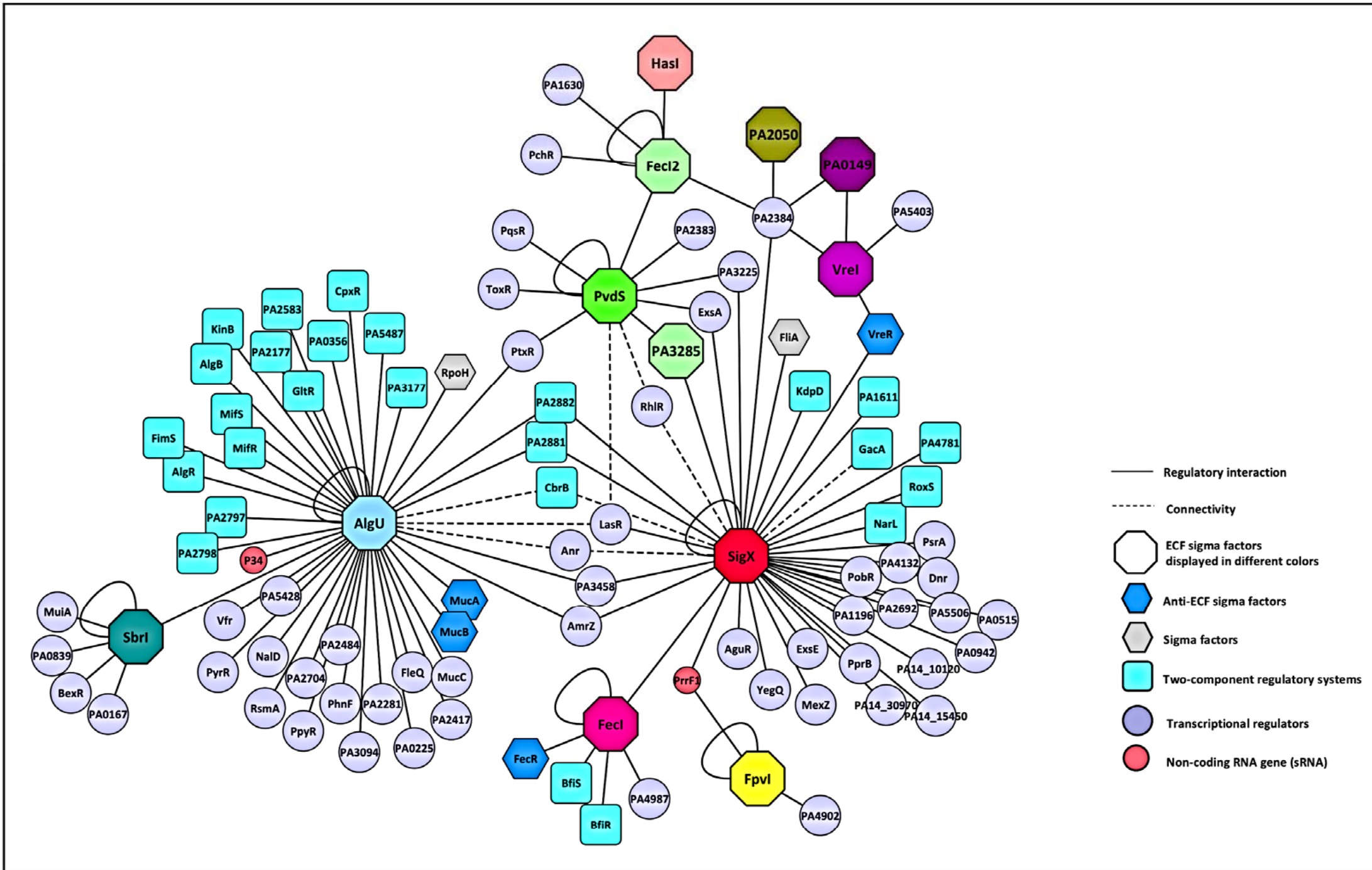


Figure 4: Regulators involved in ECF sigma factors expression

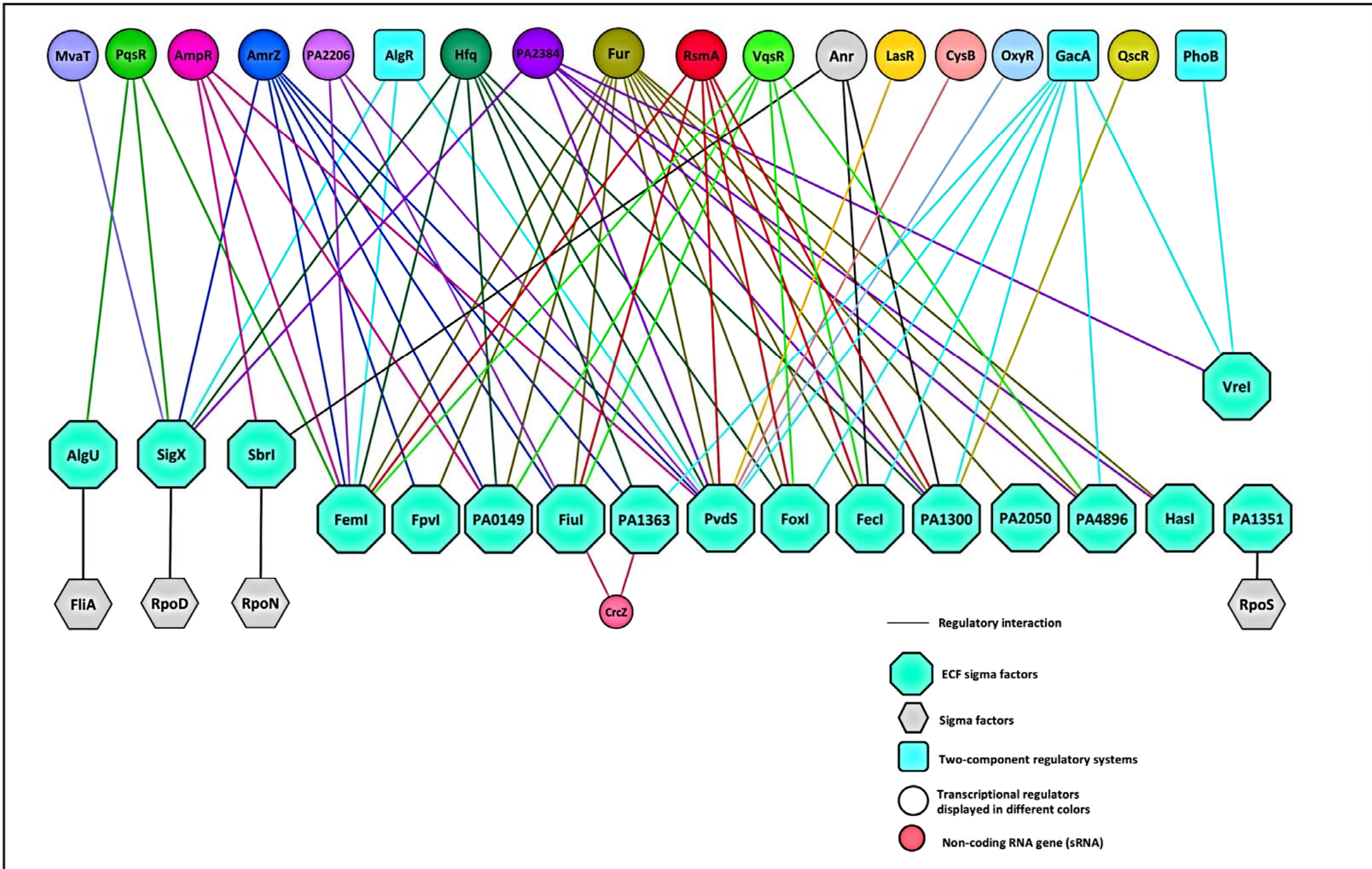


Figure 5. FpvA cell surface signaling

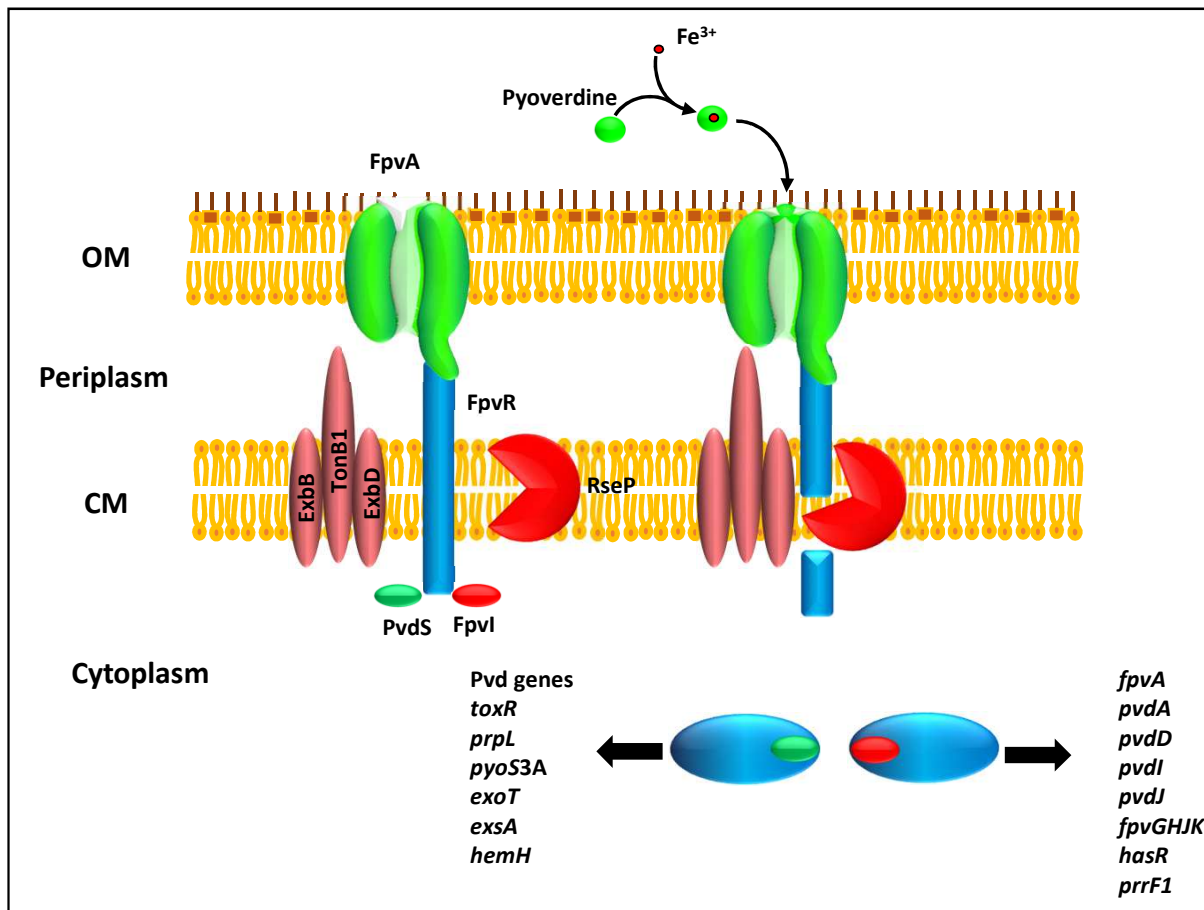
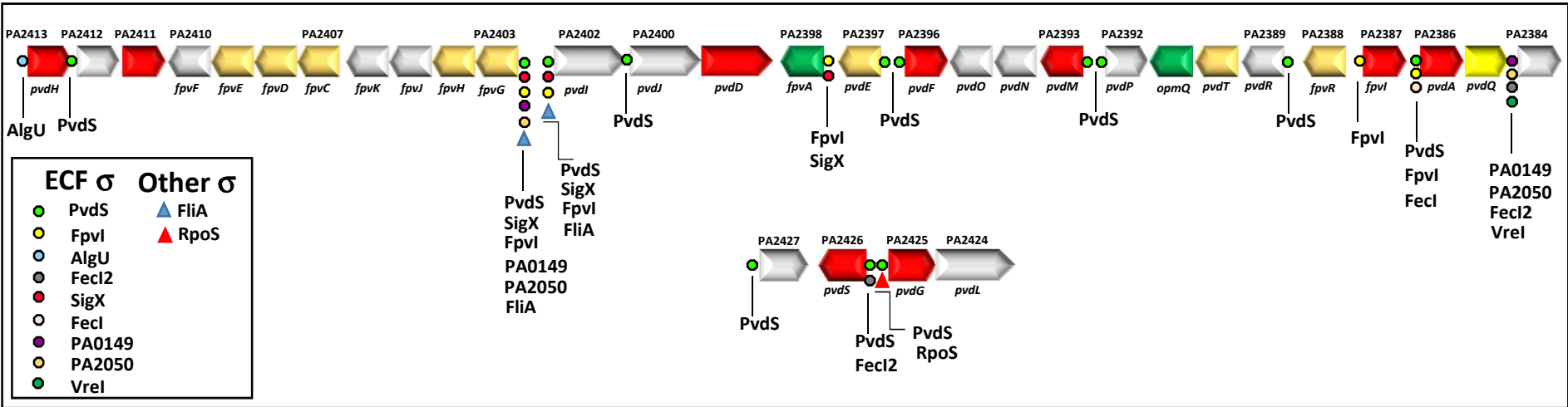
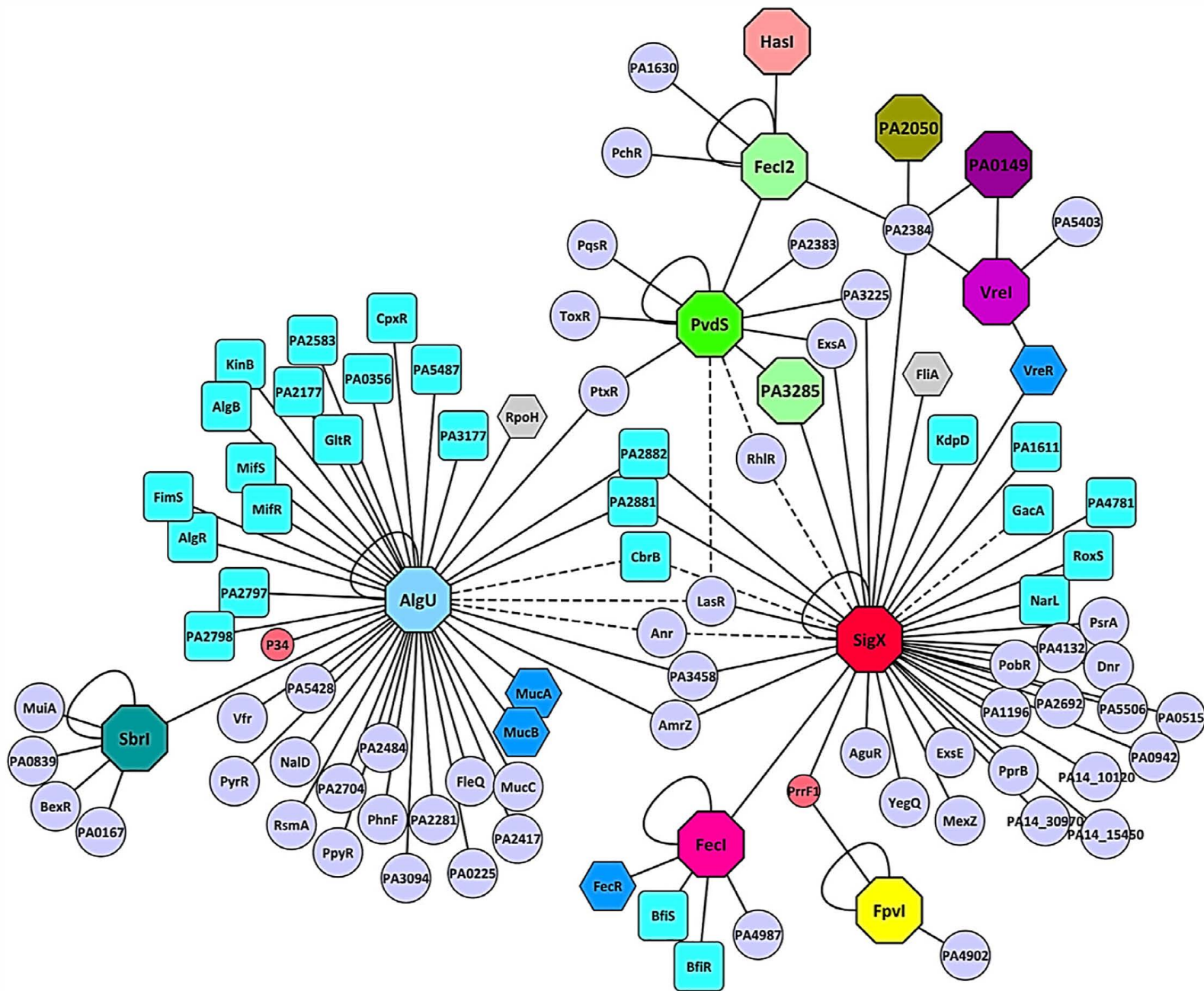


Figure 6: Pyoverdine cluster





- Regulatory interaction
- - - Connectivity
- ⬡ ECF sigma factors displayed in different colors
- ⬡ Anti-ECF sigma factors
- ⬡ Sigma factors
- Two-component regulatory systems
- Transcriptional regulators
- Non-coding RNA gene (sRNA)