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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

9

10 \*E-mail address: [eflores@ibvf.csic.es](mailto: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 CO<sub>2</sub> through oxygenic photosynthesis and micro-oxic heterocysts specialized for the fixation  
23 of N<sub>2</sub> 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 N<sub>2</sub>, making these  
44 organisms capable to grow with very simple nutritional sources –air and water with a few  
45 mineral salts. However, because the N<sub>2</sub>-fixation machinery is very sensitive to oxygen [3],  
46 cyanobacteria need to separate N<sub>2</sub> fixation from oxygenic photosynthesis. Whereas some  
47 unicellular and filamentous cyanobacteria separate these two incompatible processes  
48 temporally, restricting N<sub>2</sub> fixation to the dark period of diel cycles [3], some filamentous  
49 cyanobacteria form cells specialized for N<sub>2</sub> fixation. Thus, in filaments of cells that fix CO<sub>2</sub>  
50 through oxygenic photosynthesis, some of these cells (usually less than 10 %) differentiate into  
51 N<sub>2</sub>-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 N<sub>2</sub>-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 CO<sub>2</sub>-  
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 N<sub>2</sub>-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 N<sub>2</sub>-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 % N<sub>2</sub>, 21 % O<sub>2</sub>) [20]; indeed, O<sub>2</sub> 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 O<sub>2</sub> 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 N<sub>2</sub>-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 CO<sub>2</sub>, 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 N<sub>2</sub> 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  $\beta$ -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 GTAN<sub>8</sub>TAC [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 TAN<sub>3</sub>T, 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 CO<sub>2</sub> 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  $\sigma^{70}$  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  $\sigma^{70}$ -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  $\sigma^{70}$ -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]), *hglE<sub>2</sub>* (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  $\sigma^{70}$ -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 O<sub>2</sub> 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 O<sub>2</sub>. 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 an 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<sub>A</sub>*  
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<sub>A</sub>* 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<sub>A</sub>* [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 O<sub>2</sub> 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 O<sub>2</sub> by terminal respiratory oxidases and flavodiiron  
426 proteins appears not to be redundant [38]. Whereas the terminal respiratory oxidases likely  
427 remove O<sub>2</sub> 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 O<sub>2</sub>, but in this case producing ROS. In *Anabaena*, the  
430 rubrerythrin RbrA is a heterocyst-specific peroxidase that transfers electrons from NADPH to  
431 H<sub>2</sub>O<sub>2</sub> with the participation of ferredoxin-NADP<sup>+</sup> 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 N<sub>2</sub> 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 N<sub>2</sub>-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<sup>+</sup> 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 N<sub>2</sub>-  
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  $\beta$ -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 CO<sub>2</sub>, 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 N<sub>2</sub> 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 N<sub>2</sub> 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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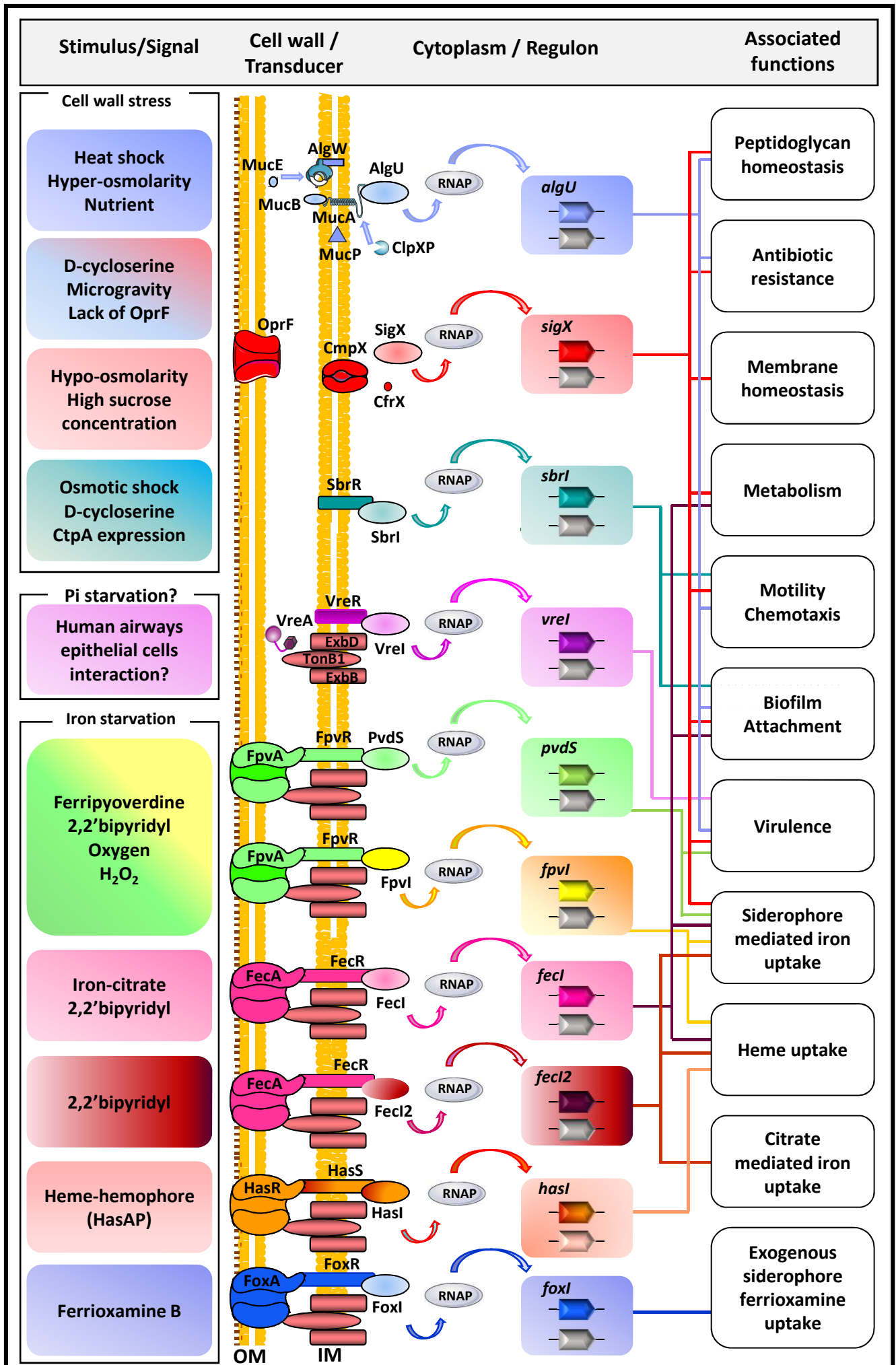


Figure 2. Mechanism of activation of AlgU (RpoE)

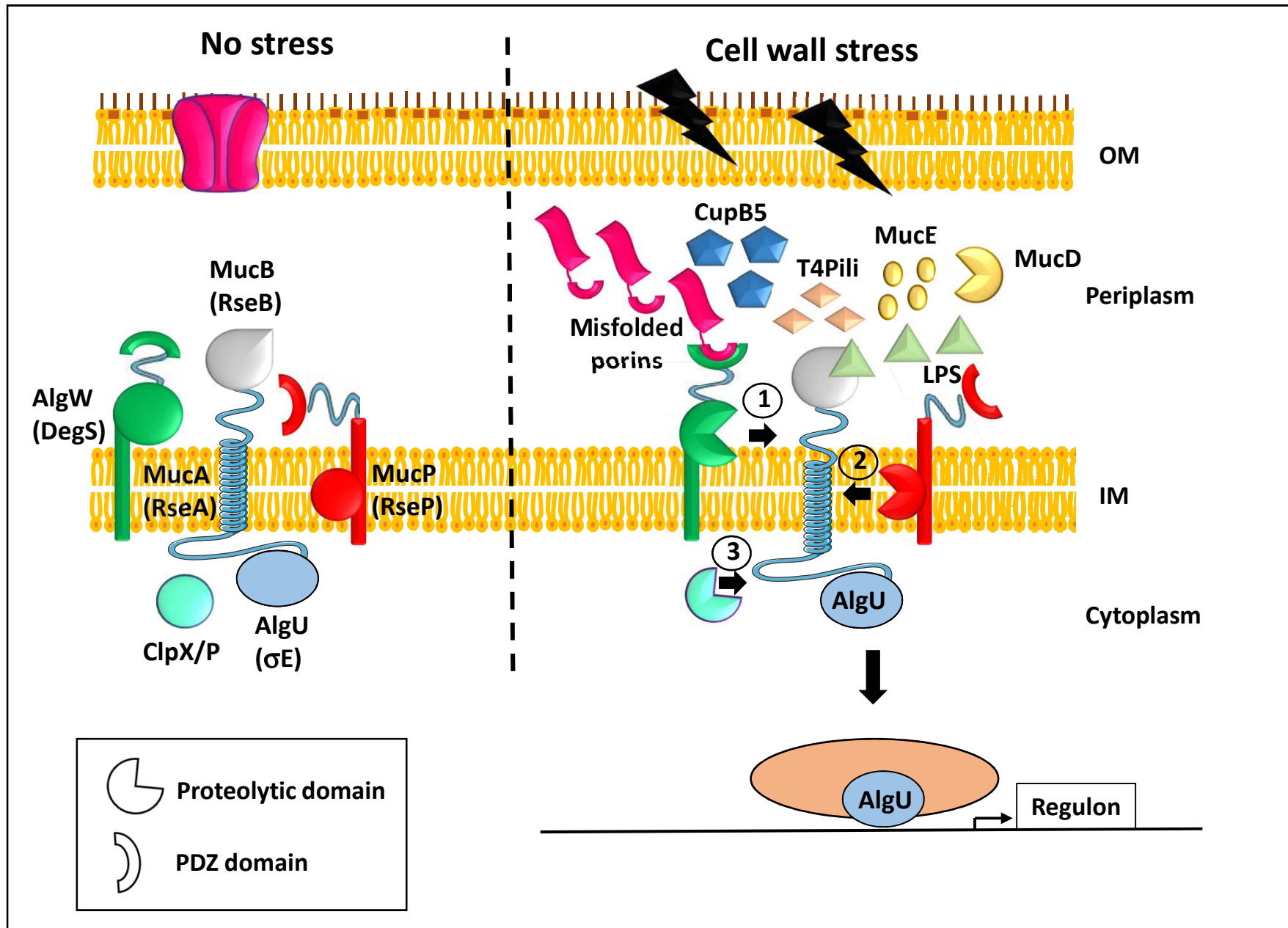


Figure 3: ECF sigma factors involved in regulators expression

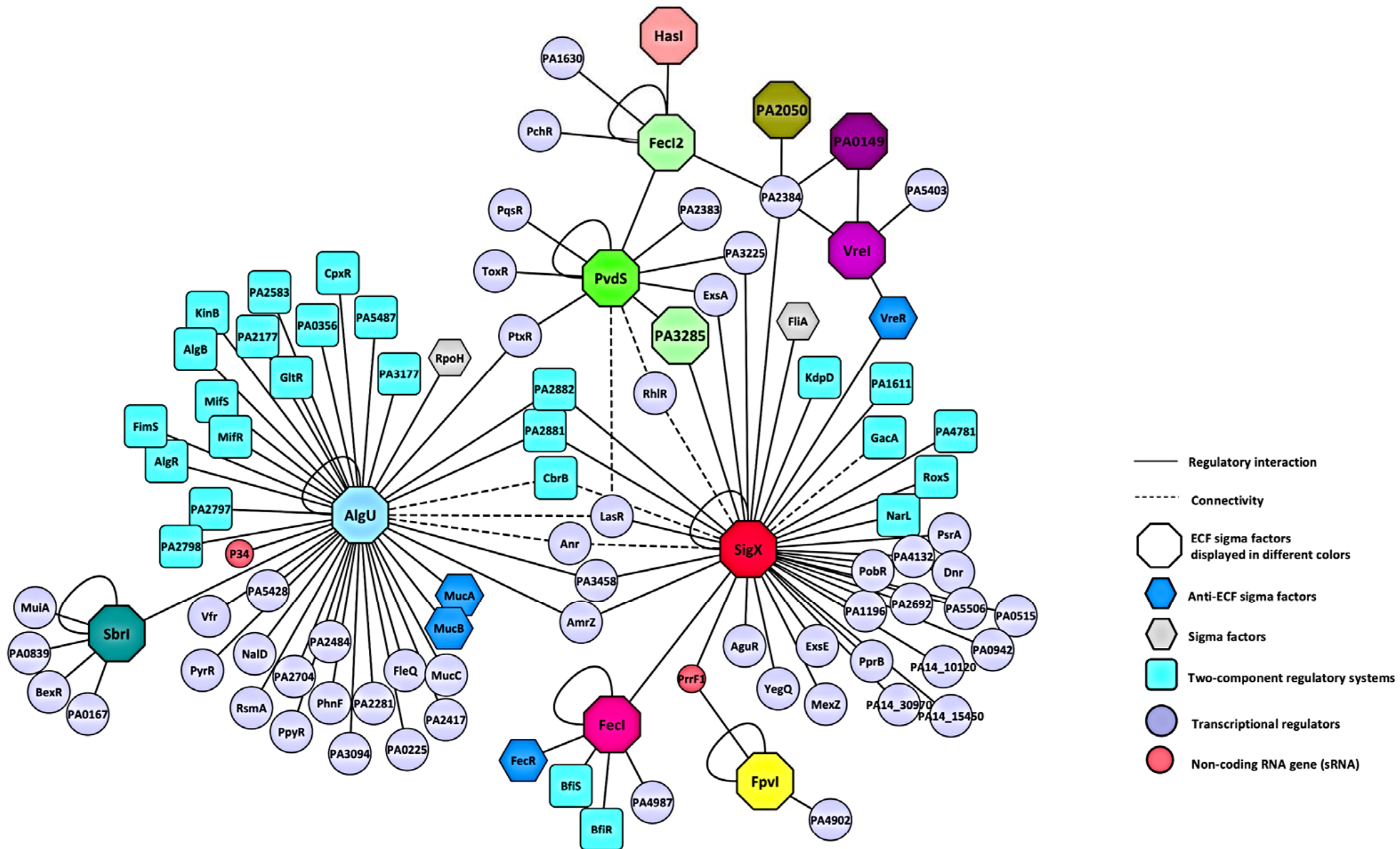




Figure 4: Regulators involved in ECF sigma factors expression

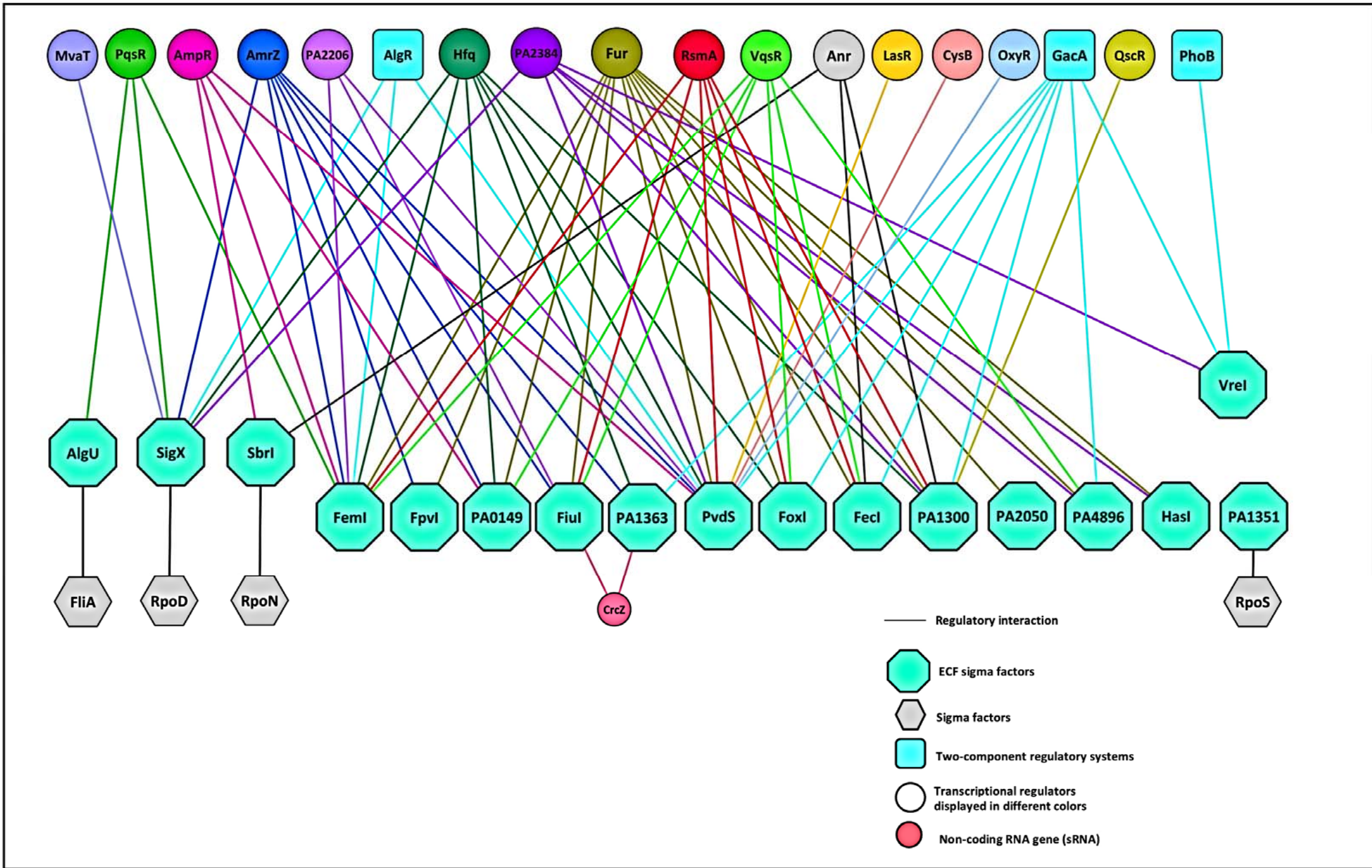


Figure 5. FpvA cell surface signaling

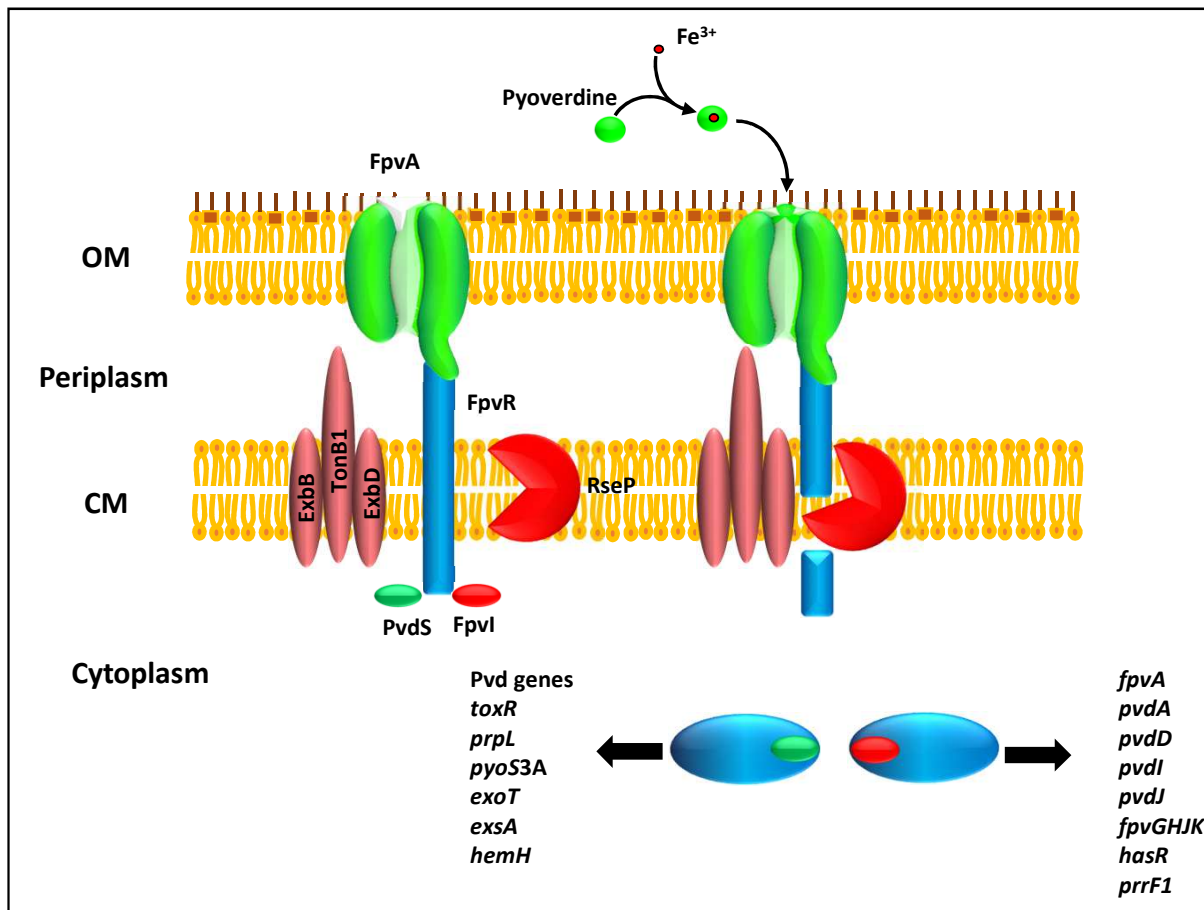
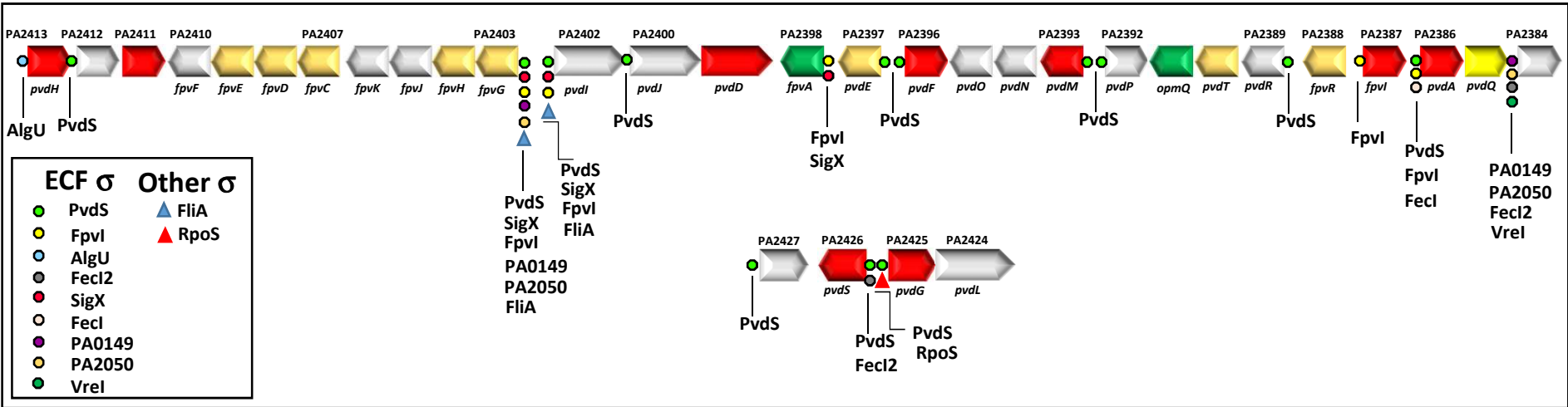
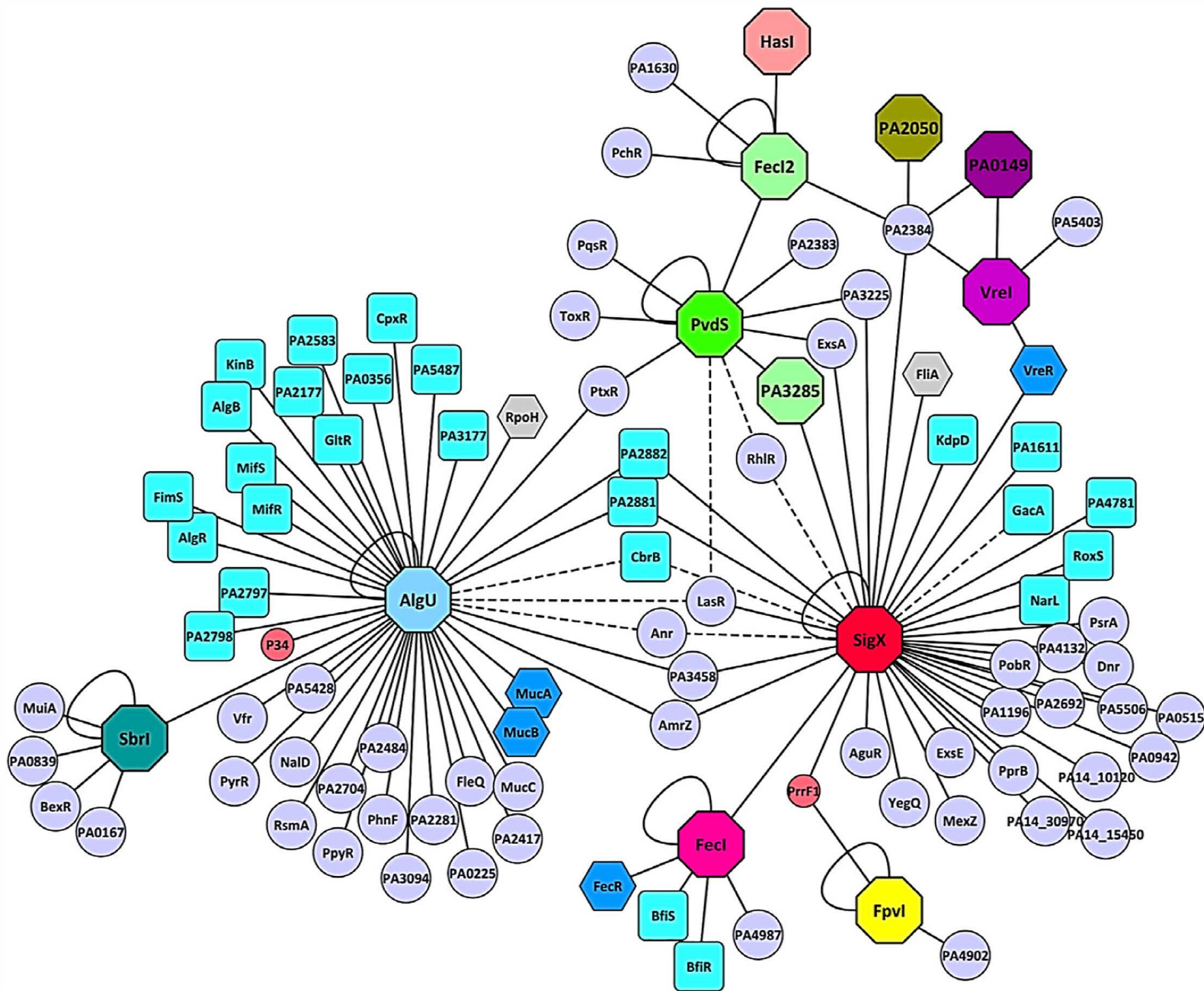


Figure 6: Pyoverdine cluster





- 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)