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1 **Two-component systems and toxinogenesis regulation in *Clostridium botulinum***

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9 Key words: *Clostridium botulinum*, toxin, regulation, two-component systems

10

Abstract

Botulinum neurotoxins (BoNTs) are the most potent toxins ever known. BoNTs associate with non-toxic proteins to form complexes of various sizes**.** Toxin production is highly regulated through complex networks of regulatory systems involving an alternative sigma factor, BotR, and at least 6 two-component systems (TCSs). TCSs allow bacteria to sense environmental changes and to respond to various stimuli by regulating the expression of specific genes at a transcriptional level. This review aims to highlight the role of TCSs as a central point in the regulation of toxin production in *C. botulinum*.

I) Introduction

Botulinum neurotoxins (BoNTs) are responsible for human and animal botulism, a rare but severe disease that causes a descendant flaccid paralysis which can be lethal if not treated. BoNT/A is the most potent toxin ever known with an estimated lethal dose of 0.3 ng/kg in mice by intraperitoneal route [1]. BoNTs are mostly produced by *C. botulinum* but also by atypical strains of other *Clostridium* species.

The first descriptions of botulism were reported in Germany in 1793 and this disease was linked to the consumption of blood sausages prepared from pork. Indeed, in its natural form botulism is mostly acquired after absorption of BoNTs from the digestive tract following the ingestion of food contaminated by *C. botulinum* and its toxin. BoNTs are metalloproteases that specifically cleave SNARE (N-ethylmaleimide sensitive factor (NSF) attachment protein receptor) proteins localized at motoneuron nerve endings leading to an inhibition of evoked acetylcholine secretion and thus the characteristic flaccid paralysis.

C. botulinum and the other BoNT-producing clostridia are obligate anaerobic Gram-positive bacteria. Their ability to form spores enables them to survive for long periods of time under stressful environmental conditions. BoNT-producing clostridia are heterogeneous and are divided into 6 groups [2]. Strains from the different groups exhibit specific physiological properties that dictate their main localization in the environment, such as resistance to high NaCl concentration, temperature or extreme pH and nutrient availability [3]. For example, group II strains can grow and produce toxins at temperature as low as 3°C whereas group I strains, which include type A neurotoxin producing *C. botulinum*, do not synthesize toxins at temperatures below 10°C. *C. botulinum* produces 7 BoNT types (A to G) based on their antigenic properties and many subtypes based on amino acid sequence variations [4]. An eighth toxinotype (H) has recently been reported but awaits for further characterization [5].

Considering their physiological differences, limiting the proliferation of *C. botulinum* strains and toxin production has become a major issue in the food-processing industry, especially since the development of modified-atmosphere packaging (MAP) which strongly inhibits aerobic bacterial growth but has only limited effects on anaerobic bacteria [6]. Based on the high potency of BoNTs, understanding the factors that govern the toxinogenesis in *C. botulinum* and control of BoNT production notably in food processing are major challenge in the field.

BoNT production varies between strains, medium and culture conditions; thus it has rapidly become apparent that environmental factors play a central role in toxin production. Two-component systems (TCSs) allow bacteria to sense environmental changes and to adapt in response to various stimuli including chemical (temperature, pH, ions), physical (oxygen

70 pressure, osmolarity, redox state) and nutritional (glucose, tryptophan) stimuli by regulating
71 the expression of specific genes at the transcriptional level. They are widespread among
72 bacterial species and are involved in the control of gene expression of complex regulatory
73 networks. For instance, recent evidence has shown that TCSs regulate cold tolerance in *C.*
74 *botulinum* types E and A [7,8]. It has also been demonstrated that TCSs are major regulatory
75 systems for the control of virulence factor production in numerous intracellular or
76 extracellular pathogenic bacteria such as *Clostridium perfringens*, *Helicobacter pylori* or
77 *Listeria monocytogenes* (for review see [9]).

78 Although the molecular mechanisms of TCSs are well described, most of the stimuli that
79 trigger regulation by TCSs are yet unknown. The involvement of TCSs in toxinogenesis in *C.*
80 *botulinum* is largely underestimated and the aim of this review is to highlight the role of TCSs
81 and additional regulatory genes in the direct or indirect regulation of toxin production in *C.*
82 *botulinum*.

83

84 ## II) Toxinogenesis in *C. botulinum*, a highly regulated process

85 ### a. Genetic organization of the botulinum toxin locus

86 In cultures, food, or digestive tract, BoNTs are produced in combination with associated
87 non-toxic proteins (ANTPs) to form complexes of different sizes that spontaneously associate
88 at low pH and dissociate at pH 7.5 and above (reviewed in[10]). ANTPs include the non-toxic
89 non-hemagglutinin (NTNH) protein and hemagglutinin proteins (HAs) or OrfX proteins
90 whose function is not well defined [11–13]. NTNH has a main role in BoNT protection
91 against low pH and protease degradation [11]. Thus BoNT complexes are stable in the highly
92 acidic environment of the stomach.

93 Genes encoding BoNTs and ANTPs are closely clustered together on the botulinum toxin
94 locus (Fig 1), which is organized into two polycistronic operons. The *ntnh-bont* operon of the
95 botulinum toxin locus is well conserved among BoNT-producing clostridia and consists of the
96 *ntnh* gene, which codes for the NTNH protein, and immediately downstream, a *bont* gene
97 (approximately 3.8 kb). The gene composition of the second operon varies from type to type.
98 This operon lies upstream of the *ntnh-bont* operon and is transcribed in the opposite direction.
99 Indeed, the operon can be formed by *ha* genes (*ha70*, *ha17*, and *ha33* also named *ha1*, *ha2*
100 and *ha3* respectively), which code for HAs proteins and are associated with BoNT/A5, B, C,
101 D and G types, or *orfX* genes (*orfX1*, *orfX2* and *orfX3*) associated with BoNT/A2, A3, A4, E,
102 F types. BoNT/A1 is either associated with HAs or OrfX according to the strains [14–18].

103 Two additional genes (*botR* and *p47*) are also found in the botulinum toxin locus. *botR*
104 codes for an alternative sigma factor which plays an important role in the positive regulation
105 of botulinum toxin synthesis (see part IIc). In most trains, *botR* lies between the two operons,
106 except in botulinum toxin locus type C and D where *botR* is located upstream of *ha* operon
107 (Fig.1). The function of *p47* is still unknown [19]. Most of the strains contain either *botR* or
108 *p47* in their botulinum toxin locus. However, some strains encompass both *botR* and *p47*
109 which are transcribed in the opposite orientation [14,25–30]. This is the case for strains of the
110 *C. botulinum* A and F with an *orfX-bont* locus [20].
111 Depending on the strain, the *ntnh-bont* operon can be transcribed as a monocistronic or
112 bicistronic messenger in *C. botulinum* type A and type C1 strains, whereas the *p47-ntnh-bont*
113 operon of botulinum toxin locus of Kyoto F type A2 strain is transcribed as a tricistronic
114 operon only [15,21,22]. The culture conditions can influence the transcription of the
115 botulinum toxin locus genes. For example, in *C. botulinum* E strain CB11/1-1, distinct
116 expression patterns of the six genes of the botulinum toxin locus at low and high temperatures
117 suggest that type E neurotoxin cluster genes are transcribed as two tricistronic operons at
118 30°C, whereas the locus genes are mainly transcribed as monocistronic operon (*bontE* or
119 *orfx1* alone) and bicistronic (*ntnh-p47* and *orfx2-orfx3*) at 10°C [23]. Transcriptional analysis
120 of *C. botulinum* type A, B and subtype A2 showed that *ha* and *orfX* loci are transcribed as
121 tricistronic messengers [14,16,19,24].
122
123 b. Expression and kinetic production of botulinum complex
124 Expression of botulinum toxin locus genes is a highly controlled process involving
125 various environmental and regulatory factors. Indeed, botulinum toxin gene expression during
126 growth was monitored using real-time PCR and northern blot analyses on *C. botulinum* type
127 A and E [21,32–34]. At 37°C, the botulinum toxin locus genes exhibit the same kinetics of
128 expression including: weak expression during the exponential growth phase, then an increased
129 expression reaching its maximal rate (100-fold increase) at the transition phase between the
130 end of the exponential growth and the beginning of stationary phase. After 24-h of growth, the
131 expression rate of botulinum toxin locus genes returns to the rate observed in the early growth
132 phase [21,32]. These data suggest that toxin gene expression in *C. botulinum* is tightly
133 regulated and is growth phase dependent. BoNT and ANTP accumulation in the supernatant
134 of *C. botulinum* type A and E, as monitored by the lethal activity and biochemical approach,
135 reaches the highest value 12 hours after the peak of *bont* gene expression and remains stable
136 for at least 5 additional days [32].

137 It is not yet defined how the toxin is released in the extracellular medium. One hypothesis
138 suggests that the toxin is freed into the extracellular medium during autolysis. Indeed,
139 comparison of 2 strains from the group I proteolytic stains of *C.botulinum* type A, Hall A and
140 NCTC2916 (A(B)), shows that they exhibit different patterns of growth, toxin expression,
141 production and toxin release [35,36]. Although both strains reach the same maximal amount
142 of toxin after 48h of growth in the extracellular medium, neurotoxin release starts during
143 exponential growth and only a small increase in toxin release is observed during autolysis of
144 *C. botulinum* Hall A, whereas BoNT release and autolysis are concomitant in NCTC916 [35].
145 However, the same strains show similar growth kinetics and patterns of botulinum locus gene
146 expression and BoNT accumulation in the extracellular medium in another study using
147 different culture media [32]. Moreover, BoNT/A release in the extracellular medium starts
148 concomitantly with the exponential growth phase as monitored by ELISA assay and lethality
149 test on mice suggesting that another process than autolysis is involved [32,33]. Thus, the
150 release of BoNT/A in the extracellular medium may differ depending on experimental
151 procedure, growth medium and strains.
152 Interestingly, C2 toxin produced by type C and D strains, which is an additional toxin
153 distinct from the neurotoxin, is only produced during the sporulation phase. Conversely, no
154 direct link between BoNT synthesis and sporulation has been identified at least in *C.*
155 *botulinum* A and E.. [21,32,35]. However, A significant difference in *antp* expression was
156 observed between the two conditions. Thereby, a study performed in our laboratory showed
157 that in non-sporulating cultures of *C. boutlinum* NCTC2916, the relative expression of *bont/A*
158 and *antps* are equivalent, while in sporulating conditions, the relative expression of *bont/A* is
159 twofold higher than that of *ntnh/A* and *ha35*. Moreover, in sporulating conditions, *ha* genes
160 are differentially expressed with *ha35* transcripts being twofold more expressed than *ha17*
161 and *ha70* transcripts [32]. Thus an indirect link between sporulation and BoNT/ANTP
162 production may exist.
163
164 c. BotR, an alternative sigma factor
165 Before 2012, the only known regulator of toxinogenesis in *C. botulinum* was the
166 alternative sigma factor BotR that drives RNA polymerase to transcribe genes of both operons
167 of the botulinum toxin locus [37]. The DNA binding properties of BotR were first described
168 in 1995 in *C.botulinum* type C [15]. Homologs of BotR were subsequently identified in *C.*
169 *botulinum* types A, B, D, F and G but no homologs could be found in *C. botulinum* type E.
170 Homologs of BotR have also been evidenced in other *Clostridium* species like TetR in

Clostridium tetani, TcdR in *Clostridium difficile*, and UviA in *Clostridium perfringens* [19,38–40]. These homologs are positive regulators of their corresponding toxin genes (TetR for tetanus toxin gene (*tent*), TcdR for *toxA* and *toxB* and UviA for *C. perfringens* bacteriocin gene), and they are functionally interchangeable *in vitro* and *in vivo* [41]. For example, an overexpression of *botR*/A and to a lesser extent *botR*/C in *C. tetani*, enhances TeNT synthesis suggesting that these sigma factors have a conserved function in several *Clostridium* and that they probably have evolved from a common ancestor [40]. BotR/A and its homologs have been assigned to a new TcdR sub–family group within the sigma (70) family on the basis of target DNA sequence (Fig.2) [34].□Indeed the members of the TcdR subfamily recognize a conserved promoter region (TTTAXA) which is distinct from the motifs targeted by the sigma factors from other families [42]□. Moreover, the fact that TcdR is not interchangeable by a group 4 ECF (*Bacillus subtilis* σw), a close related □□□□□ factor group, □further supports that TcdR and related proteins belong to a distinct group of □□□□□ factors □[42].

The mechanism of action of BotR/A and TetR was exhaustively studied by S. Raffestin *et al.* [19]. Footprinting experiments on DNA fragments containing the promoter region of the *ntnh-bont/*A operon showed that BotR/A specifically recognizes conserved sequences localized at -10 (GTTATA), and -35 (TTACA) within the promoter binding sites ATGTTATATataa and TagGTTTACAAAA, respectively [19]. These regions are also conserved in the promoter region of *ha* operon of *C. botulinum* types B, C, D and G and in the promoter of *tent* in *C. tetani* [19]. Then, BotR promotes the RNA polymerase-dependent transcription of the *ntnh-bont* and *ha* operons. Overall, BotR/A positively regulates the operons of the botulinum toxin locus in a dose dependent manner, increasing production of BoNT and ANTPs, and it was concluded that BotR/A is a transcriptional activator of botulinum toxin locus genes [40]. BotR/A can also target its own promoter, but no initiation of transcription could be observed *in vitro*, whereas TcdR in *C. difficile* activates its own expression [19,43].

In comparison with *bont*/A expression, *botR*/A is expressed approximately 100 fold less than the other genes of the botulinum toxin locus [32,33]. Although BotR directly regulates botulinum toxin locus genes, it seems to have multiple targets. Transcriptomic analysis in *C. botulinum* A were performed in isogenic strains which either overexpressed or partially repressed *botR*/A [40]. In these strains, a set of genes are overexpressed (15) and underexpressed (6) of at least 4-fold compared to the wild type strain including botulinum toxin locus genes but also many enzyme genes and to a lower extent, membrane and transporter genes (unpublished data as monitored by a microarray based transcriptomic

205 analysis [44]). However, it is highly improbable that BotR is the only regulatory factor
206 involved in toxinogenesis of *C. botulinum*. Indeed, expression of botulinum toxin genes and
207 toxin production in *C. botulinum* E, which lacks *botR* but has *p47*, show the same kinetics of
208 growth and toxin production as in *C. botulinum* A [32]. The maximal expression of *bont/E* at
209 the transition between the late exponential and early stationary phases strongly suggests that
210 other regulatory pathways are involved.
211
212 d. Environmental stimuli in BoNT production and complex stability
213 It has been known for decades that nutrient availability and environmental stimuli play an
214 important role in toxin production in *C. botulinum* (Fig.3).
215 The source of nitrogen in culture media is critical for BoNT production. Although toxin
216 production by various *C. botulinum* strains has been obtained in diverse culture media ranging
217 from complex media containing meat, yeast hydrolysates, vegetable extracts, to simpler media
218 based on hydrolysates of casein, the complex media with meat extracts yielded the highest
219 levels of toxin [45]. It seems that the size of peptides in complex media is critical
220 (unpublished data).
221 Certain amino acids have been found to be important for toxin synthesis. For example, in
222 minimal defined medium, group I *C. botulinum* strains require high concentrations of
223 phenylalanine (> 1 g/l) and arginine (>3 g/l) for maximal toxin production [46]. However, in
224 *C. botulinum* Okra B and Hall A, an excess of arginine in the culture media (20g/l) represses
225 BoNT/A and BoNT/B synthesis, respectively, without modification of growth or lysis [46]. A
226 similar phenomenon is observed with proline, glutamate or ammonia. Moreover, tryptophan
227 in culture media partially represses neurotoxin production in several *C. botulinum* type E
228 strains without modifying growth kinetics, but this effect seems to be strain dependent [47].
229 Rapidly metabolizable sugars such as glucose are not absolutely required for neurotoxin
230 synthesis but enhance *C.botulinum* growth and subsequently promote increased toxin
231 production [48]. Therefore, rapidly metabolizable sugars are essential factors for maximal
232 toxin production.
233 The control of BoNT synthesis by the general metabolism is probably mediated, at least
234 partially by CodY, which is a global regulator that governs the transition from the exponential
235 growth to stationary phase. CodY senses the level of GTP and controls the expression of
236 numerous genes (>100 in *Bacillus subtilis*) [49]. In *C. botulinum* A, CodY has been reported
237 to regulate positively the expression of *bont* by interacting with the promoter of the *ntnh-bont*
238 operon on a site distinct from that recognized by BotR/A in a GTP-dependent manner [50].

239 Various environmental stimuli such as carbon dioxide, NaCl concentration, pH and
240 temperature have also been shown to regulate botulinum toxin production. CO_2, which is
241 commonly used in food preservation, has a strong antimicrobial activity on aerobic bacteria
242 but is metabolized by numerous anaerobic bacteria and enhances toxin production in many
243 bacteria such as *Staphylococcus aureus*, *Vibrio cholerae* or *Bacillus anthracis* [51–53]. In
244 non-proteolytic *C. botulinum* type B and E strains from group II, CO_2 enhances toxin
245 production but has only minimal effects in *C. botulinum* type A strains [54–56]. An increased
246 CO_2 concentration from 10% to 70% leads to a more than 5-fold increase in BoNT/B
247 neurotoxin synthesis without any growth modification. In *C. botulinum* type E, the increased
248 CO_2 concentration (70%) also enhances toxin production despite a growth rate reduction [54].
249 Thus, in *C. botulinum* type E, toxin production seems to be linked to growth rate as it has
250 been described in stress situations in *C. difficile* [34].

251 Li *et al.* have investigated the effects of pH on *C. botulinum* type A growth, toxin gene
252 expression and production [57]. BoNT/A gene expression is optimal at pH 7 and then
253 decreases at lower or higher pH. Thus, pH is a critical parameter controlling *bont/A*
254 expression and subsequent toxin synthesis and is of great importance in safety of food
255 processing.

256 High temperature (at least until 44°C) which is a common signal involved in the relay of
257 alternative sigma factors (reviewed in [58]), seems not to be an inhibitory signal for toxin
258 production in *C. botulinum* type E and A, but appears to play an important role in complex
259 stability [32]. Even though botulinum toxin locus genes are expressed at the same level
260 between 37°C and 44°C, BoNT/A activity monitored by its lethal activity on mice is strongly
261 reduced after 24-48 h of culture incubated at 44°C suggesting that a protease leading to
262 BoNT/A degradation is activated at 44°C in *C. botulinum* Hall A strain. Indeed, a Ca^{++}
263 dependent protease activated at high temperature is produced by *C. botulinum* A. Unlike *C.*
264 *botulinum* type A, cultures at 44°C has no effect on lethal activity levels of *C. botulinum* type
265 E strains which are not proteolytic bacteria [32]. It has been known for decades that bacterial
266 growth adaptation to changes in temperature implicates two types of protein, those that are
267 induced by cold called Cold Shock Proteins (CspA, CspB, and CspC) and those induced by
268 heat, called Heat Shock Protein (HSPs) [59,60]. Homologs of CspB and HSPs have been
269 identified in *C. botulinum* type A and D to play a major role in temperature tolerance and
270 stressful environmental conditions [61–63]. It is not known whether these proteins also
271 control the toxin synthesis or they play a role in toxin protection.

272 Thus, the composition of culture media and culture conditions differentially affects BoNT
273 synthesis depending on *C. botulinum* strains and one of the major challenges in the
274 understanding of the toxinogenesis in *C. botulinum*, is to identify the molecular mechanisms
275 associated with these environmental regulators.
276

277 III) TCSs play a central role in the regulation of toxinogenesis in *C. botulinum*

278 Thousands of TCSs have been identified among all kingdoms, in Gram-positive,
279 Gram-negative bacteria, Archaea, and to a lesser extent in eukaryotic organisms. TCSs are
280 implicated in the control of various physiological processes including osmoregulation (for
281 example OmpR/EnvZ in *E. coli*), and sporulation (KinA/ KinB/ KinC and Spo0F/ Spo0B/
282 Spo0A in *B. subtilis*), but also in cell-cell communication, and control of virulence factor
283 production [64–68].
284 The number of TCSs can vary from none in *Mycoplasma genitalium* to 80 in
285 *Synochocystis* species, which represents 2.5% of the entire *Synochocystis* genome [69,70].
286 Among these TCSs, several are implicated in the regulation of virulence factors in Gram-
287 positive and Gram-negative bacteria such as *Staphylococcus aureus*, *Salmonella*
288 *thyphimurium*, *S. enterica*, and *Bordetella. pertussis* [71–74]. However, due to their
289 pleiotropic functions and inteconnexions, the individual role played by TCS in toxinogenesis
290 is still unclear and it's highly probable that toxin synthesis is under the control of a complex
291 regulatory network including TCSs and also sigma factors, orphan regulators and/or small
292 regulatory RNAs.
293

294 a. Biochemistry and interconnexions of TCSs

295 TCSs are transduction signal phosphorelay systems that require ATP and at least 2
296 proteins: one transmembrane protein which detects the environmental changes called sensor
297 histidine kinase (SHK) and a regulatory protein (RR for response regulator) which is typically
298 a DNA binding protein (Fig.4). The two TCS genes are often expressed from a single operon
299 which can autoregulate itself [75].
300 The signal pathway is triggered by the SHK protein, which senses the stimuli by its N-
301 terminal region and autophosphorylates a conserved histidine (H) residue localized in the C-
302 terminal region at the expense of ATP. SHKs are structurally complex, homodimeric integral
303 membrane proteins which can vary in size from 40 kDa to 200 kDa. Each protomer is divided
304 into several functionally and structurally dissociable domains: one sensor, two transmembrane
305 helices called linker domains, and one HAMP domain (histidine kinase, adenylyl cyclase,

306 methyl accepting chemotaxis protein and phosphatase). The HAMP domain connects the
307 second transmembrane domain to the dimerization and histidine phosphorylation domain
308 (DH) where the conserved H is localized, and finally to a catalytic and ATP-binding domain
309 (CA) (reviewed in [76]). The sensor domain can be highly variable, allowing the detection of
310 a wide range of stimuli. SHKs can sense environmental stimuli when bound to the periplasmic
311 membrane like EnvZ which is involved in the osmoregualtion pathway in *E. coli*. But SHKs
312 can also detect intracellular stimuli through enzymatic behavior (for example CheA involved
313 in the control of chemotaxis in *E. coli*) or by interacting with other cytoplasmic protein which
314 promote an adaptive response (reviewed in [77]). Some SHKs exist as hybrids that contain
315 multiple phosphodonor and phosphoacceptor sites, adding steps to the phosphorelay system as
316 in the case of BarA which is implicated in growth stationary phase and ArcB which is
317 involved in the anoxic redox control [69].

318 Once SHK is phosphorylated, the phosphate group is transferred to a conserved aspartate
319 residue (D) on the cognate RR receptor domain which regulates the expression of specific
320 target genes. RR proteins are structurally less complex than SHKs but their multitude of
321 targets make them difficult to classify [78]. They are divided into one receiver domain (REC)
322 which is well conserved among RRs and one variable effector output domain [79]. RR
323 proteins catalyze phosphate group transfer from SHK H to the conserved aspartate (D) residue
324 on the receiver domain of RR protein, and then to the effector output domain. Most of the
325 RRs can also catalyze autodephosphorylation to limit the time lapse of activation. The
326 effector domains are variable. They are either DNA-binding, RNA-binding, ligand binding,
327 transporter output domain or core enzyme and can act at a transcriptional post-transcriptional
328 or post-translational level. However most of them are DNA-binding proteins and are
329 transcriptional regulators. They contain only one output domain as in the case of LytR/AgrA,
330 OmpR/PhoB and Spo0A domain families or two output domains as in the case of Ntrc/DctD
331 family (reviewed in [76,77,80]).

332 SHKs and RRs share sequence and structural similarities permitting cross-phosphorylation
333 or cross-communication between them. As for RRs, most SHKs are bifunctional and can
334 catalyze phosphorylation and dephosphorylation of their cognate RR. Since RRs are more
335 abundant than SHKs, SHK phosphorylation is competitive depending on their interaction with
336 RRs. Three types of cross-communication have been described: cross-talk, cross-regulation
337 and one-to-many or many-to-one interaction. However in most cases, the cross-talks have
338 only been described after important genetic modifications like overexpression of SHKs (see

339 [81]). These data highlight the fact that TCSs should not be studied individually, but in
340 interaction with other TCSs and bacterial regulatory systems.
341

342 b. TCSs in *C. botulinum*

343 In 2007, the first genome of *C. botulinum* Hall A was sequenced and its analysis allowed
344 the prediction of the presence of several regulatory systems including 15 sigma factors, 16
345 orphan genes and 28 putative TCSs [82]. Another analysis showed that this genome contains
346 39 proteins which exhibit TCS characteristics including a Rec domain and a DNA binding
347 domain [33].

348 Analysis of RRs show that numerous *C. botulinum* Hall A TCSs share more than 60%
349 identity with homologs in other clostridia [33]. Among the 39 *C. botulinum* Hall A TCSs, the
350 majority (12/39) are homologous to those found in *C. carboxidivorans* and *C. ljungdahlii*.
351 Eight RRs also exhibit high TCS similarity to the closely related *C. tetani* RRs. However,
352 some RRs (12) have no similarity with proteins in other clostridia and thus seem specific of *C.*
353 *botulinum*. Interestingly, 2 RRs, of which one is an apparent orphan regulator, show high
354 protein identity (38%) with the VirR protein of the well-known VirR-VirS *C. perfringens*
355 TCS. The VirR-VirS TCS regulates the synthesis of numerous toxins in *C. perfringens* at the
356 transcriptional level, either directly by interacting with the toxin gene promoter like in the
357 case of theta toxin or perfringolysin O, or indirectly via a regulatory RNA (VR-RNA) like in
358 the case of alpha toxin and collagenase A [83–85]. However, the VirR-VirS related TCS in *C.*
359 *botulinum* Hall A seems not to be involved in the regulation of toxinogenesis (see below)

360 Two-third of TCS RR proteins identified in *C. botulinum* Hall A by genome analysis
361 belong to the OmpR family. Regulator proteins of the OmpR family have been described to
362 control multiple functions including bacterial surface organization, polysaccharide synthesis
363 and/or assembly. One of the best understood TCSs is OmpR/EnvZ. In *E. coli*, OmpR/EnvZ
364 TCS is involved in the adaptive response to extracellular osmolarity changes by regulating the
365 transcription rate of 2 porin proteins. Moreover, it has also been demonstrated that regulation
366 by the OmpR/EnvZ TCS is critical for curli formation and thus plays an important role in
367 biofilm formation [86].

368 Four *C. botulinum* RRs belongs to the LytR family, which is widespread among bacteria
369 and control the synthesis of several virulence factors. The LytR family has been characterized
370 in *S. aureus*. Indeed, the *S. aureus* infection potency is linked to the synthesis of more than 50
371 virulence factors including toxins which are mostly regulated by cellular density (quorum
372 sensing) via the accessory gene regulator (Agr) system including the TCS AgrA/LytR

373 [80,87,88]. Other *C. botulinum* predicted TCSs have RRs that belong to the NarL family
374 (2/39) which regulates nitrite- and nitrate-dependent gene expression or CheB (1/39) for
375 chemotaxis in *E. coli*, and WspR (1/39) which has been found to stimulate biofilm formation
376 in *Pseudomonas aeruginosa* or Pas_4 (1/39) [33].

377 c. TCSs and BoNTs production

378 Different techniques are available to investigate the regulatory pathway linked to a TCS.
379 One approach is to modulate an environmental signal already known to trigger the TCS of
380 interest by either supplementing or depleting the culture media with this specific signal
381 (nutrient or chemical compounds). However, TCSs function as a saturating process leading to
382 variable results depending on the amplitude of the environmental signal variation, as is the
383 case of the phosphate regulon [89]. Another way is to modulate the TCS expression in
384 bacteria at the transcriptional or translational level. As discussed above, SHK domain
385 overexpression may lead to cross-talk, rendering the involvement of one TCS in a pathway of
386 interest difficult to analyze (see [81]). In contrast, silencing or knocking out TCS gene
387 expression can be a relevant experiment to identify the role of a TCS [33,90–92].

388 The potential role in toxin production of 34 TCSs has been investigated in the laboratory
389 using the antisense mRNA strategy. Compared to gene knock-outs, the mRNA antisense
390 methodology only partially silences gene expression but allows the detection of very fine
391 regulation. The ability of isogenic *C. botulinum* strains to produce BoNTs and ANTPs was
392 investigated by western blot analysis and ELISA assay in the culture supernatant, and the
393 botulinum locus gene expression by qRT-PCR [37]. This method allowed the identification of
394 6 TCSs involved in toxinogenesis in *C. botulinum* Hall A [33,92].

395 Isogenic strains repressed in 5 RRs showed a drastic decrease in toxin and ANTP
396 production, with, for some strains, a more important decrease than that observed in the
397 isogenic strain repressed in *botR/A*. However, 2 of these isogenic antisense strains
398 corresponding to CLC_0411 and CLC_3293 TCSs present a more rapid growth and lysis
399 explained by drastic changes in cell wall or surface structure [33]. The corresponding RRs
400 belong to the OmpR family and thus seem to be involved in the regulation of cell surface
401 properties, e.g. surface polysaccharide synthesis and integrity and only indirectly in toxin
402 production and/or secretion. Isogenic strains of the 2 RRs sharing sequence similarity with
403 VirS/ VirR RR were also obtained. The corresponding orphan RR (CLC_0632) showed a
404 drastic delay in growth compared to the wild type strain but produces a similar level of toxin.
405 Thus, this TCS seems to be involved in global metabolism and not in the direct control of
406 toxin synthesis. In this case, the other homolog (CLC_1105) of VirR did not show any

407 difference in growth or toxin production. Thus VirR/VirS TCS homologs seem to regulate
408 different processes in *C. perfringens* and in *C. botulinum* [33]. Concerning the three other
409 positive regulatory TCSs (CLC_1093/CLC_1094, CLC_1914/CLC_1913 and
410 CLC_0661/CLC_0663) no difference in growth or lysis was observed in the isogenic strains
411 containing antisense mRNA construction targeting each of these three TCS RRs. Expression
412 of *bont/A* and *antp*s in isogenic strains containing antisense mRNA construction targeting the
413 3 TCSs is repressed at the same level as in the isogenic antisense strain repressed for *botR/A*.
414 But the production of BoNT/A and ANTPs is slightly lower than in the control strain,
415 indicating that these three TCSs may have a more important effect on toxin production than
416 BotR/A. Moreover qRT-PCR analysis showed no decrease in *botR/A* expression in these
417 isogenic strains, indicating that the three TCSs control directly or indirectly the expression of
418 the botulinum locus genes independently of BotR/A [33].

419 The first TCS downregulating BoNT synthesis was described recently [92]. In this study,
420 the methodology used is a shutdown of TCS gene of interest by the Clostron technology
421 which is a group II intron-based insertional mutagenesis [93]. Unlike the mRNA antisense
422 method, Clostron engineering totally represses gene expression. The TCS
423 CBO0787/CBO0786, equivalent to CLC_0842/CLC_0843 is located approximately 11 kb
424 upstream of the botulinum toxin locus in contrast to the other TCSs involved in positive
425 regulation of *bont* which lie at distance of the botulinum. This TCS binds to the consensus
426 core promoter region of both operons of the botulinum toxin locus, thus inhibiting the
427 transcription of the botulinum locus genes enhanced by BotR/A. Thus, this TCS probably acts
428 by preventing BotR/A binding to the promoters of the botulinum toxin locus operons and thus
429 inhibiting the transcription of botulinum toxin locus genes. Additionally, TCS
430 CBO0787/CBO0786 could be also an anti-sigma factor similarly to TcdC in *C. difficile* by
431 impairing the transcription and/ or translation of *botR/A*. But, this TCS has not been found to
432 bind to *botR/A* promoter [94]. Isogenic strain silenced for TCS CBO0787/ 0786 by the mRNA
433 antisense method has been investigated [33]. No difference in growth or toxin production
434 compared to wild type strain was evidenced suggesting that the regulation of toxin synthesis
435 by this TCS is not a tight process.

436 Interestingly, the five TCSs which directly or indirectly control positively BoNT
437 production are conserved among *C. botulinum* strains from group I (subtype A1, A(B), A2,
438 A3, Ba4, B1 and F) but they are not detected in *C. botulinum* group II strains. Similarly, the
439 negative regulator TCS has homologs (more than 90% identity) in other *C. botulinum* group I
440 strains including subtypes (A2 strain Kyoto, A5 and F strain Langeland) [33,92]. No

441 significantly related genes were found in other *C. botulinum* groups. These data strongly
442 suggest that *C. botulinum* from group I share a common regulatory network of toxin
443 production distinct from that of the other *C. botulinum* groups.

444 Overall, certain TCSs control toxin production either in a direct way by regulating
445 botulinum locus gene transcription or indirectly via regulating general metabolism pathways.

446 d. Quorum sensing, TCS and toxin production

447 One of the most interesting systems that bacteria have developed to adapt their
448 physiological behaviors and pathogenesis (biofilm formation, virulence, antibiotic resistance)
449 to local population density is quorum sensing. Quorum sensing controls multiple metabolic
450 processes but also virulence factors in several Gram-positive and Gram-negative bacteria. For
451 example, it has been shown that quorum sensing is implicated in the production of the type III
452 secretion system and the regulation of motility and flagella in *E. coli* EHEC
453 (enterohemorrhagic *E.* coli) and EPEC (enteropathogenic *E. coli*), or in the production of
454 virulence factors in *V. cholerae* and biofilm formation in *C. perfringens* or *P. aeruginosa* [95–
455 97]. Thus quorum sensing may be a potential antimicrobial target.

456 For decades, several teams have been trying to evidence quorum sensing in *C. botulinum*.
457 It was shown in *C. botulinum* 56A that spores in contact with latency phase bacteria
458 germinate more rapidly than the control spores [98] demonstrating that quorum sensing in *C.*
459 *botulinum* exists and may influence spore germination.

460 Quorum sensing molecular mechanisms differ between Gram-positive and Gram-
461 negative bacteria. In the case of Gram-negative bacteria, the stimuli are small molecules
462 called AutoInducers (AIs) that are passively transported through the bacterial cell wall,
463 whereas in Gram-positive bacteria, the AIs are secreted by specific transport systems and
464 detected by TCSs (Fig. 5).

465 One well known peptide detected by this system is AI-2 (AutoInducer-2) produced by
466 LuxS from SAM (S-adenosylmethionine). Numerous bacterial species carry *luxS* in their
467 genomes and thus AI-2 can be produced and detected by different species allowing inter-
468 species communication [99]. In order to determine the involvement of LuxS in toxinogenesis,
469 an isogenic strain silenced for the homologs of *luxS* identified in the genome of *C. botulinum*
470 Hall A was generated, but only a small delay in growth and toxin production could be
471 observed (unpublished data). Even though the implication of LuxS in the *C. botulinum*
472 toxinogenesis is still unclear, it has been demonstrated that the TCS VirR/VirS of *C.*
473 *perfringens* has a pleiotropic action and regulates toxin production but also *luxS* transcription.
474 LuxS enhances toxin production in the mid-exponential growth phase, demonstrating that

475 TCS and quorum sensing are tightly linked in maximal toxin production in *C. perfringens*
476 [83,100]. Furthermore, AI-2 positively regulates virulence factors in *C. difficile* such as toxin
477 A and B, and also the holine encoded by *tcdE* which plays an important role in biofilm
478 formation [101–103].

479 Another type of AIs is the AIPs (AutoInducers Peptide). AIPs are small peptides that
480 require a highly specific detection by appropriate TCSs and that only mediate intra-species
481 communication [104] (Fig. 5). One of the most well known systems of intra-species
482 communication is the Agr system of *S. aureus* which is responsible for 50% of iatrogenic
483 infection. In this system the AIP is produced from a peptide encoded by *agrD* which is then
484 hydrolyzed by AgrB, an integral membrane endopeptidase. Once AIP reaches a threshold
485 level in the environment, it is detected by the TCS AgrA/ AgrC which upregulates the
486 production of a small RNA (sRNA) called ARN III and subsequently enhances toxin
487 production [105]. A putative *agr*BD signaling system has been identified in proteolytic *C.*
488 *botulinum* group I strains and homologs of *agrB* and *agrD* called *agr*-1 and *agr*-2 were
489 identified but they seem to play different roles than in *S. aureus*. Indeed, *agr*-1 is suggested to
490 regulate the sporulation state while *agr*-2 seems to be implicated in BoNT/A production
491 [106]. However the isogenic antisense strain silenced for the homologous AgrA/ AgrC TCS in
492 *C. botulinum* Hall A was not impaired in BoNT production or growth in the tested
493 experimental process [33]. Thus the effect and the mode of action of *agr-2* homologs in toxin
494 production in *C. botulinum* remain to be characterized.

495 In addition, quorum sensing plays a major role in the transition between growth phase
496 and stationary phase and regulates virulence factor production at high cellular density. This is
497 the case in *P. aeruginosa*, which is a major opportunistic pathogen responsible for pulmonary
498 infections as well as burn wound and other infection. Its multiple virulence factors are
499 expressed at high cellular density and are under the control of quorum sensing (reviewed in
500 [107]). Thus quorum sensing is evidenced only under certain conditions, such as growth in
501 nutrient-limitation, very high population density, or in response to specific stresses and it is
502 conceivable that the experimental conditions used in *C. botulinum* studies are not appropriate
503 to evidence a quorum sensing-mediated regulation in this bacterium.

504

505 **IV) Discussion and conclusion**

506 Data collected in recent decades has shown that TCSs are highly involved in several
507 processes in bacteria including the regulation of virulence factor production such as toxins.
508 However, few links have been made between environmental stimuli and bacterial processes,

509 underestimating the capacity of bacteria to adapt to their environment. Thanks to the
510 structural understanding of TCSs, the mode of action of TCSs is well understood, but a
511 correlation between SHK domain and stimuli is still elusive. Even though techniques based on
512 phenotypic microarray approaches are available to characterize phenotypes under different
513 environmental conditions, the number and combination of variable parameters are too
514 numerous and complex to effectively carry out a systematic screening of all stimuli and all
515 putative genes [108].

516 As discussed above, only a few regulatory systems are known to participate in
517 botulinum toxin production in *C. botulinum*. The alternative sigma factor BotR was the first
518 positive regulator identified in this process. Botulinum locus genes reach their maximum
519 expression at the transition phase between exponential and stationary growth [21,32]. The
520 transition between these 2 phases is a highly regulated process in *E. coli* and is under the
521 control of RpoS, a sigma factor which replaces the vegetative sigma factor σ70 in stationary
522 phase and under numerous stress conditions (reviewed in [109]). This mechanism is very
523 complex and is partly regulated by a TCS (BarA/ UvrY). Even if no TCS has been described
524 to regulate the transcription of BotR, *botR/A* has the same kinetic of expression as the other
525 genes of the botulinum toxin locus suggesting that *botR/A* regulation of expression is under
526 the control of environmental stimuli [32].

527 Small RNAs (sRNAs) are another regulatory system described to be involved in the
528 regulation of virulence factor production in bacteria and in quorum sensing (reviewed in
529 [110]). Numerous sRNAs have been identified in the genomes of 21 *Clostridium* species and
530 most of them are not found in bacteria of other phyla [111]. Although the synthesis of toxins
531 in *C. perfringens* is under the control of a TCS and involves a sRNA called VR-RNA, the role
532 of sRNAs in botulinum toxin synthesis is still unknown [83].

533 Overall, only a few regulatory systems of toxinogenesis are described in *C. botulinum*
534 including the positive regulatory sigma factor BoTR and 6 TCSs [33,40,92]. Some small
535 regulatory RNAs may also be involved but no such regulation has yet been described [111].
536 The environmental factors triggering BoNTs synthesis are so far poorly understood but new
537 technologies associated with a better understanding of the structural and molecular
538 mechanism of TCSs should help to unravel the mechanism of toxin regulation and eventually
539 lead to the development of new strategies to control botulism. Due to their absence in
540 mammals and their wide range of action, TCSs constitute interesting therapeutic targets.

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FIGURE LEGENDS

Figure 1: Schematic cartoon of the different steps of transcription initiated by RNA polymerase in association with the s70 family.□ factor RNA polymerase is formed by several subunits: two □ subunits which link the two □-subunits and permit the recognition of the activation elements in cis and trans. The two □ subunits allow the linkage to the DNA, RNA synthesis and terminator recognition. The □ factor allows the specific linkage to the promoter region.

Figure 2: Schematic representation of the regulation by Two Component System (TCS). The signal is detected by the SHK (sensor histidine kinase) via its transmembrane domain which leads to a conformational change and an ATP-dependent autophosphorylation on the conserved Histidine (H) residue.

Figure 3: Regulation of the botulinum toxin locus in *C. botulinum* Hall A by environmental factors and regulatory factors. Factors identified in other *C. botulinum* strains are written in italics. Red arrows correspond to a positive regulation and green arrows correspond to a negative regulation (adapted from [19]).

Figure 4: Schematic representation of the two kinds of quorum sensing communication. Inter-species communication is represented in the left panel. The peptide signal precursor locus is transcribed and translated into a precursor peptide which is cut enzymatically to form small peptides which are the AIPs. AIPs are then transported through the bacterial cell wall by an ABC transporter. AIPs are detected by specific TCSs which regulate target genes. The right panel representes the inter-species communication where AI-2s are produced by LuxS from

872 SAM and are then passively released into the extracellular medium. AIs are detected by a
873 TCS which regulates specific target genes.

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Figure

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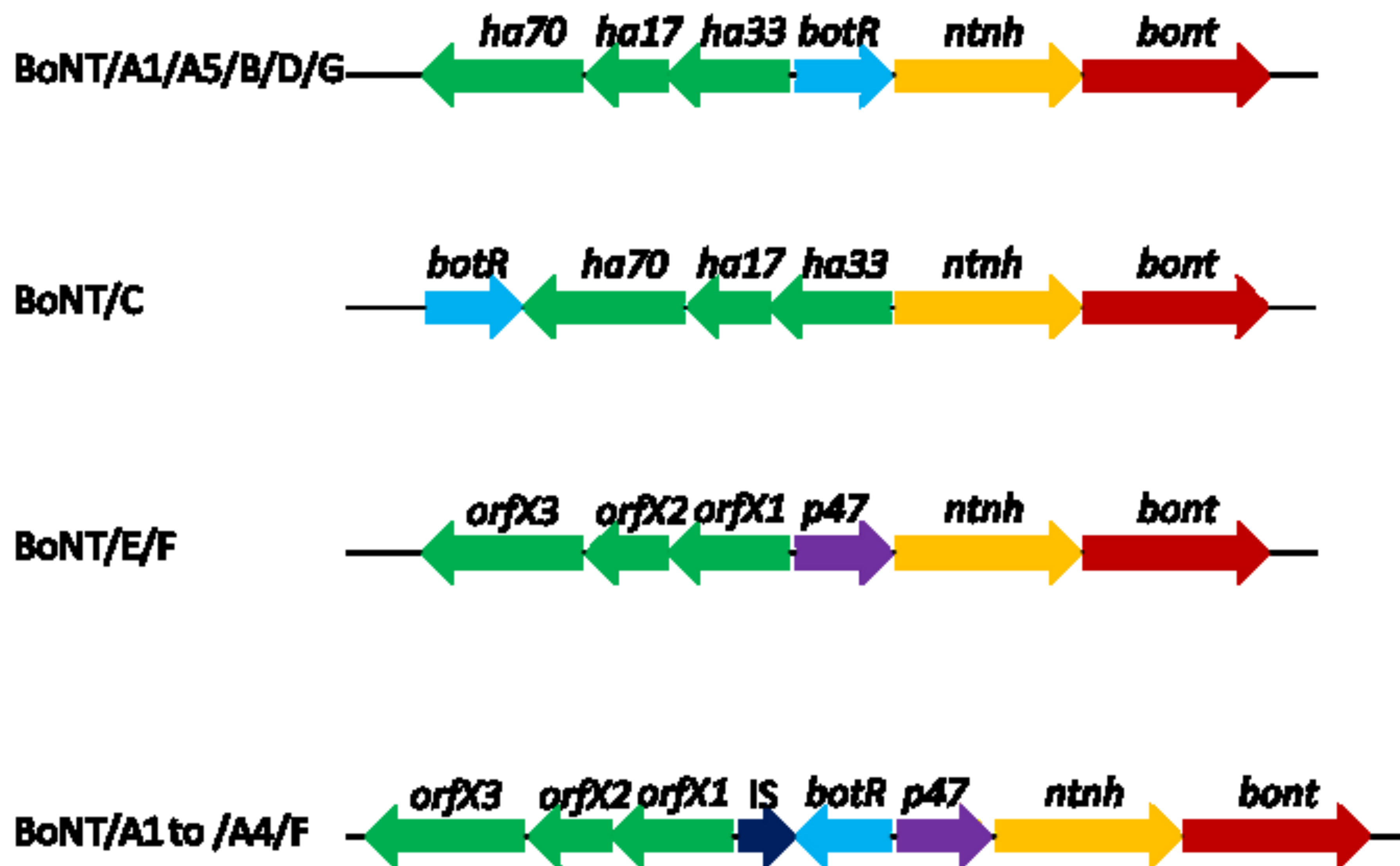

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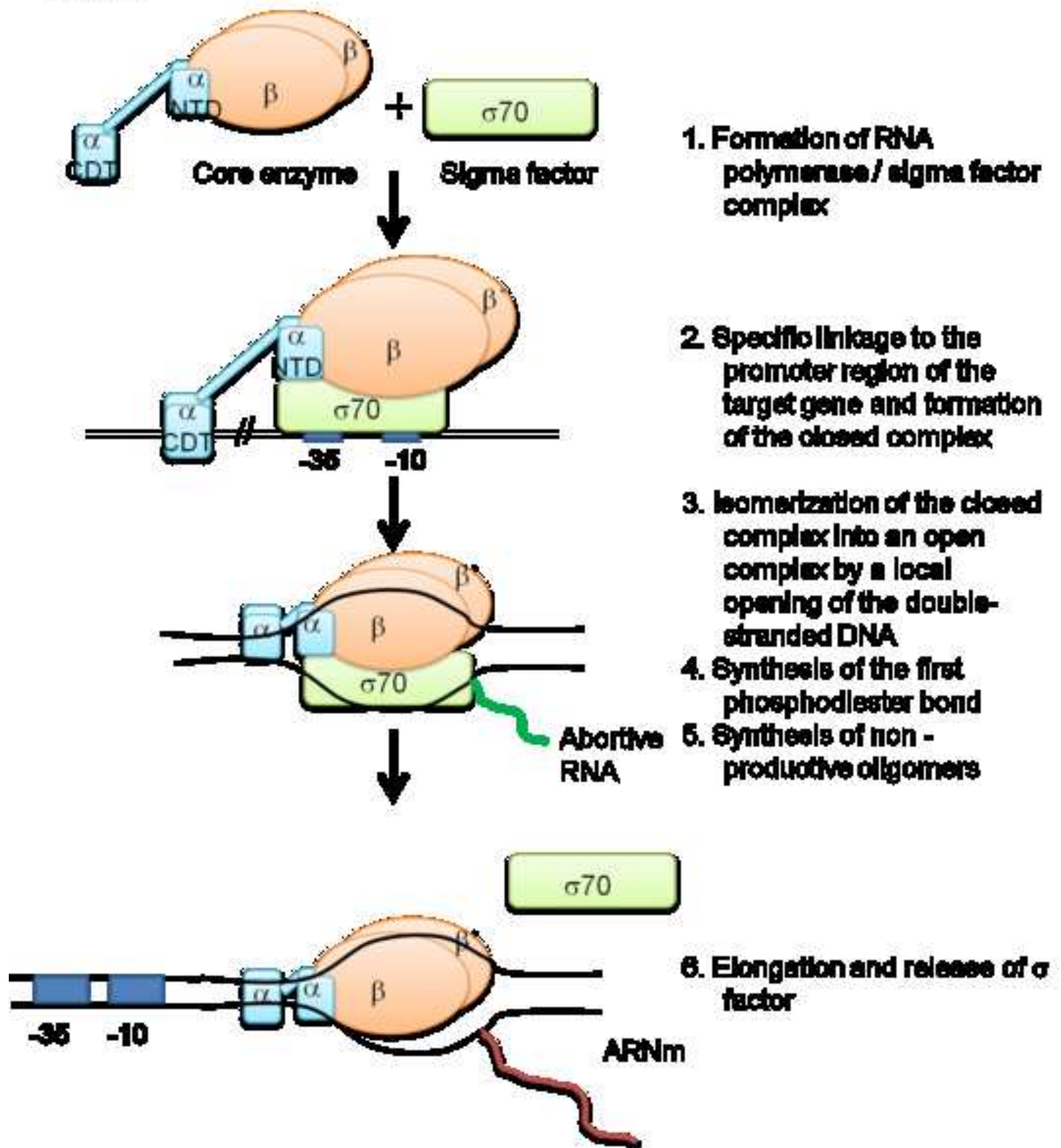

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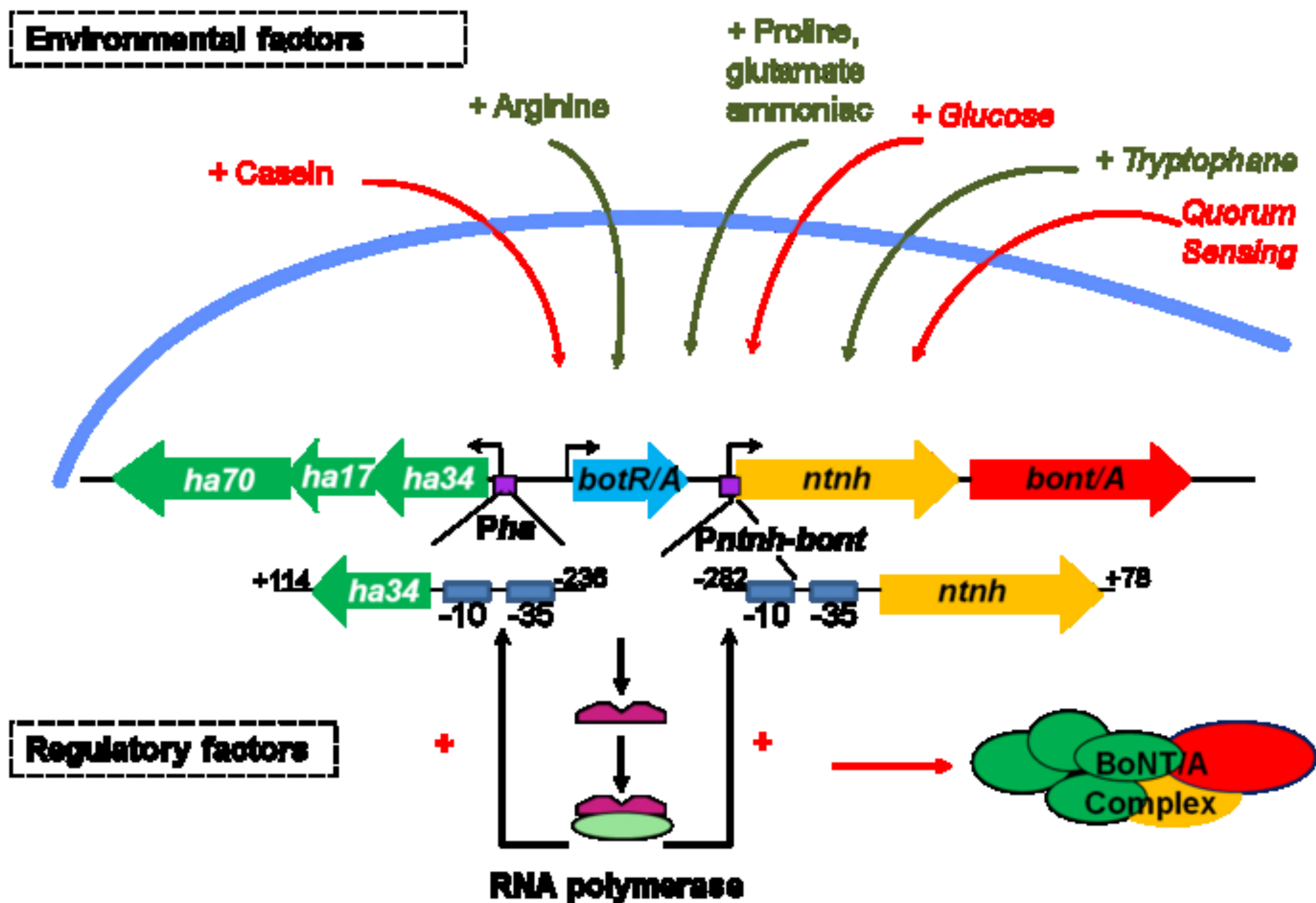

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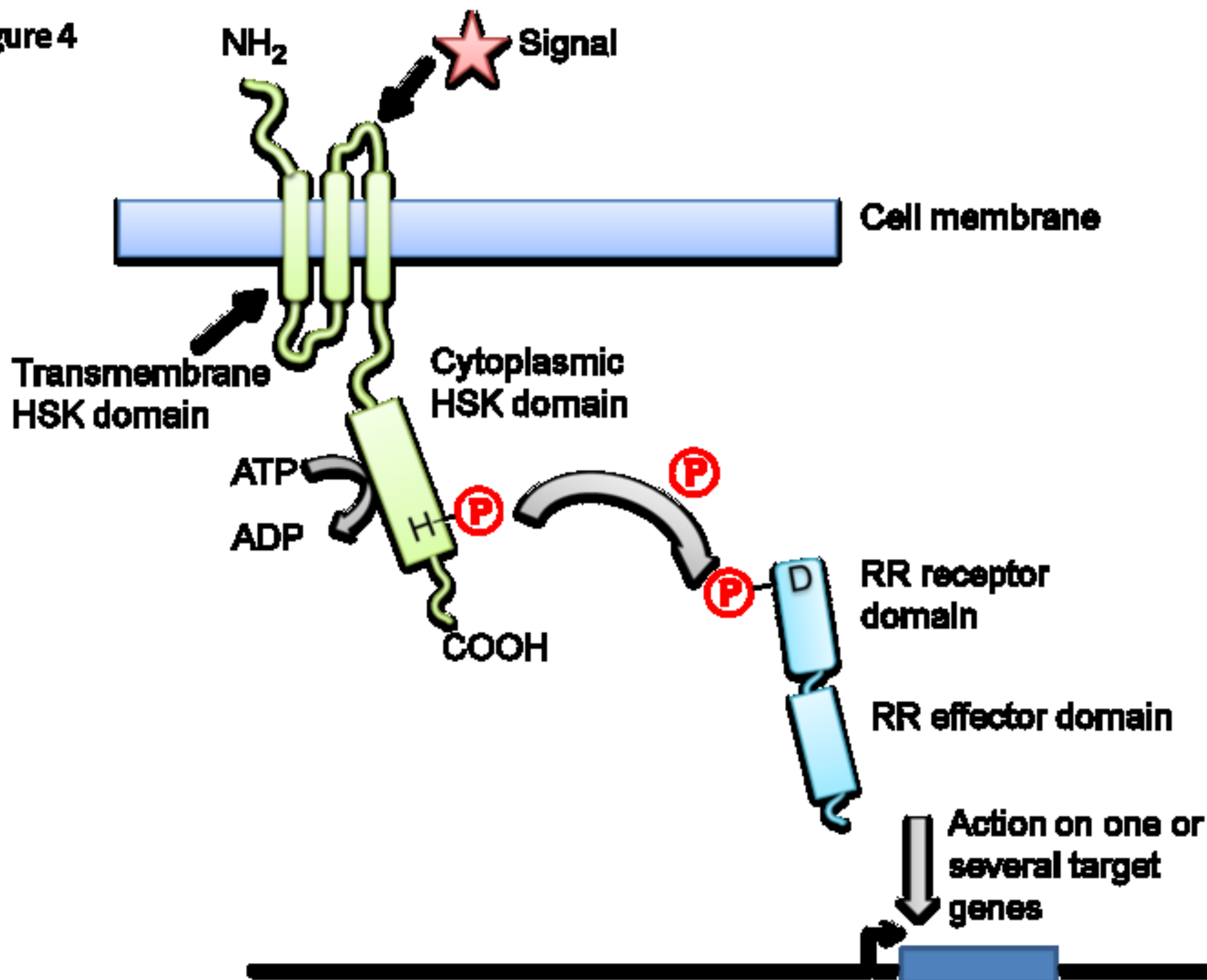

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