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Connecting Environment and Genome Plasticity in the Characterization of Transformation-Induced SOS Regulation and Carbon Catabolite Control of the *Vibrio cholerae* Integron Integrase

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► **To cite this version:**

Zeynep Baharoglu, Evelyne Krin, Didier Mazel. Connecting Environment and Genome Plasticity in the Characterization of Transformation-Induced SOS Regulation and Carbon Catabolite Control of the *Vibrio cholerae* Integron Integrase. *Journal of Bacteriology*, 2012, 194 (7), pp.1659-1667. 10.1128/JB.05982-11 . pasteur-03941970

HAL Id: pasteur-03941970

<https://pasteur.hal.science/pasteur-03941970>

Submitted on 16 Jan 2023

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2 **Transformation-induced SOS regulation and carbon catabolite control of the *V.***

3 ***cholerae* integron integrase: connecting environment and genome plasticity.**

4

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12

13 **Running title:** IntIA is induced by SOS during transformation and regulated by CRP.

14

15 **Key words:** integron, tyrosine recombinase, horizontal gene transfer, competence, SOS, CRP

16 catabolite regulation, *Vibrio cholerae*

17

18

19 **Abstract**

20 The human pathogen *V. cholerae* carries a chromosomal superintegron (SI). The SI contains
21 an array of hundreds of gene cassettes organized in tandem which are stable in conditions
22 where no particular stress is applied to bacteria (such as during laboratory growth).
23 Rearrangements of these cassettes are catalyzed by the activity of the associated integron
24 integrase. Understanding the regulation of integrase expression is pivotal to fully comprehend
25 the role played by this genetic reservoir for bacterial adaptation and its connection with the
26 development of antibiotic resistance. Our previous work established that the integrase is
27 regulated by the bacterial SOS response and that it is induced during bacterial conjugation.
28 Here we show that transformation, another horizontal gene transfer (HGT) mechanism, also
29 triggers integrase expression through SOS induction, underlining the importance of HGT in
30 genome plasticity. Moreover, we report a new cAMP-CRP dependent regulation mechanism
31 of the integrase, highlighting the influence of the extracellular environment on chromosomal
32 gene content. Altogether, our data suggest interplay between different stress responses and
33 regulatory pathways for the modulation of the recombinase expression, thus showing how the
34 SI remodeling mechanism is merged into bacterial physiology.

35

36 **Introduction**

37 *Vibrio cholerae* is a marine bacterium that in humans is the causative agent of cholera,
38 a condition that can be deadly if not appropriately treated. *V. cholerae* carries a superintegron
39 (SI) on chromosome 2 that has an array of up to 200 promoterless gene cassettes separated by
40 site-specific recombination sites recognized by the integron integrase IntI4 (formerly called
41 IntI4 (49)). IntI4 catalyses cassette excision and integration, and generates combinations
42 allowing the expression of some cassettes. Unlike the plasmid multi-resistance integrons
43 which carry gene cassettes that code for multiple antibiotic resistance genes, the SI codes
44 mostly for cassettes of unknown function (12, 48). Both types of integrons are extremely
45 stable in laboratory conditions, however, the integron integrases are nevertheless functional
46 (6), suggesting the existence of conditions in nature where the integrons are rearranged. The
47 *V. cholerae* life cycle alternates between planktonic growth, biofilm formation on crustacean
48 shells, and colonization of the intestinal tract during infection. In all cases, it cohabits with a
49 variety of other bacteria, thus offering ample opportunities for genetic exchange.

50 The *V. cholerae* integron integrase expression, like most integron integrases, has been
51 found to be regulated by the bacterial SOS response, which is induced when an abnormal
52 amount of single stranded DNA (ssDNA) is present in the cell (13, 33). Recently, we further
53 showed that ssDNA entry into both *Escherichia coli* and *V. cholerae* during conjugation
54 induces the bacterial SOS response and the expression of IntI4, the SI integrase. Moreover,
55 we showed that SOS induction by the incoming DNA does not require the DNA to be
56 recombined or stabilized in the recipient cell (1). Transformation is a second mechanism of
57 HGT that is based on ssDNA processing (24), and occurs when bacteria reach a competent
58 state where they can take up external DNA and possibly recombine it into their genome (17).

59 *V. cholerae* is a naturally competent bacterium (50) and as DNA is taken up into the
60 competent host cell in a single stranded fashion (24), we hypothesized that transformation

61 could induce the SOS response in this species. Transformation has been widely studied in
62 several γ -proteobacteria. It is well established that a regulator protein called Sxy in
63 *Haemophilus influenzae*, and TfoX in *V. cholerae*, regulates natural competence. TfoX is
64 responsible for the induction of the competence regulon in *V. cholerae* and *H. influenzae* (8,
65 15, 38, 57, 77). In *V. cholerae*, competence is induced in the presence of chitin (50), a
66 component of crustacean shells which are a natural substrate of *V. cholerae* growth. Chitin
67 has been shown to trigger the expression of TfoX in this species (71).

68 Regulation of natural competence also seems to depend on the carbon catabolite
69 repression regulon, also called the CRP (cAMP receptor protein)-regulon in γ -proteobacteria
70 (14). This regulon comprises not only genes involved in carbon metabolism, but also in other
71 functions such as quorum sensing, virulence or competence (32, 44, 74). Carbon catabolite
72 repression is defined as the inhibition of gene expression by the presence of favorite (i.e.
73 rapidly metabolizable) carbon source in the medium (10, 23, 39, 65). Accordingly, when the
74 level of a favorite carbon source (such as glucose) is low in the medium, the bacterial cell
75 starts to use “slow” carbon sources (such as arabinose or succinate), leading to the induction
76 of expression of the CRP-regulated genes through activation of adenylate cyclase.
77 Consequently, rising levels of intracellular cAMP control the CRP regulon through the CRP-
78 cAMP complex that binds to regulatory *crp* boxes on DNA and modulates transcription (21).
79 Notably, an increase in cAMP levels is known to trigger Sxy/TfoX expression in *H.*
80 *influenzae* (14, 57). Indeed, *H. influenzae* Δ *crp* mutants are unable to become competent (57).
81 In *V. cholerae*, Δ *crp* mutants are also affected for transformation, as they produce lower
82 levels of HapR (60). HapR is the major regulator of quorum sensing, biofilm development
83 (35), and virulence (75-76), and is also essential for competence (50). Finally, TfoX, in
84 complex with CRP itself, is involved in the induction of some competence-specific *crp* boxes
85 (14, 62).

86 In this work, we first addressed whether ssDNA entry into competent *V. cholerae* cells
87 (either after chitin treatment or by over-expressing TfoX) triggers the SOS response and
88 integrase expression. Microarray studies previously performed on *V. cholerae* did not show
89 any increase of the expression of the integrase or expression of the SOS regulon following
90 growth on chitin (51). However, these studies were performed on the non-transformable
91 N16961 laboratory strain which naturally carries an inactivated *hapR* allele and is as such
92 deficient for competence induction (7). Moreover, no external DNA was added to the medium
93 in these studies. Redfield and collaborators also performed microarray analysis on *H.*
94 *influenzae* and did not see any up-regulation of SOS regulated genes by Sxy/TfoX, (nor by
95 CRP, which is, as mentioned above, also implicated in natural competence) (57). Here again,
96 no DNA was added during these experiments.

97 We decided to test the effect of competence and transformation in the presence of
98 DNA on the *intIA* gene expression in *V. cholerae* strains N16961 *hapR*⁺ and A1552, which
99 both carry a functional chromosomal *hapR* gene.

100 During our competence induction experiments, we observed that the integrase
101 expression also varied with the carbon source we used. Thus, in a second set of experiments,
102 we addressed the possible CRP regulation of the integrase gene using glucose, succinate or
103 arabinose as a carbon source. As these experiments confirmed the CRP regulation, we pushed
104 the analysis further and identified a CRP-cAMP binding site in the *intIA* promoter region
105 using DNase I footprinting. Our data show that ssDNA uptake induces the SOS response in
106 competent cells and up-regulates the *V. cholerae* integron integrase IntIA. Moreover, we
107 provide evidence showing that IntIA is also independently submitted to CRP-dependent
108 regulation, pointing to an interplay between different regulatory mechanisms that control the
109 expression of a gene implicated in bacterial adaptation through genomic remodeling.

110

111 **Materials and methods**

112 **Bacterial strains and media**

113 Bacterial strains used in this study are listed in table 1. Oligonucleotides are listed in table 3.

114 *Escherichia coli* and *Vibrio cholerae* strains were grown at 37°C in Luria Bertani (LB) or M9
115 minimal medium supplemented with a carbon source (glucose or chitin) and Casamino acids.

116 Antibiotics were used at the following concentrations: ampicillin (Ap), 100 µg/ml,
117 chloramphenicol (Cm), 25 µg/ml, kanamycin (Km), 25 µg/ml, tetracycline (Tc), 15 µg/ml.

118 Thymidine (dT) and diaminopimelic acid (DAP) were supplemented when necessary to a
119 final concentration of 0.3mM. For SOS induction tests, the culture was carried out in LB
120 supplemented with mitomycin C at a final concentration of 0.2µg/ml.

121 **Natural transformation using chitin (7, 46):** An overnight culture was used to inoculate
122 1/100 of bacteria in 5mL LB medium. The culture was grown to OD 0.5 at 30°C. 1mL of cells
123 were centrifuged and resuspended in 1mL M9 minimal medium. Tubes containing 1%
124 glucose or 50-100 mg chitin (chitin flakes from shrimp shells purchased from SIGMA (ref:
125 C9213)) were inoculated with 0.5mL of washed cells and 0.5ml of fresh M9 medium,
126 vortexed and grown overnight at 30°C with shaking. The cultures were then washed and
127 resuspended in an equal volume of fresh M9 medium (supplemented with 1% glucose for
128 chitinless control) and incubated with DNA (2 µg of a mix of 500 to 3500 base long PCR
129 fragment on plasmid DNA or N16961 genomic DNA) for 16 to 24 hours at 30°C with
130 shaking. The bacteria were detached from the chitin surface by vigorous vortexing for 30
131 seconds. 20µl were used for flow cytometry experiments. For the detection of transformation
132 efficiency, the experiment was performed exactly as described but the DNA fragment added
133 to the medium carried 500 to 1000 bp homologies to the *V. cholerae* chromosome framing the
134 *aadA1* spectinomycin resistance marker gene so that transformants can be selected for. In the
135 case of strains marked as "below detection" limit (BD, Figure 1A), we centrifuged and plated

136 4ml of the culture (i.e 10^9 to 10^{10} cells) on LB + antibiotic. Each experiment was repeated on
137 independent cultures at least 3 times.

138 **Natural transformation using TfoX over-expression:** Bacteria containing the pBAD-*tfoX*
139 plasmid was used according to the protocol in (50). 2 μ g of a 500 base long PCR fragment
140 amplified from plasmid DNA was used as extracellular DNA (a PCR fragment of 2400bp
141 was also tested with no significant difference in SOS induction). 1% glucose was used to
142 inhibit TfoX expression from the pBAD promoter. 0,2% arabinose was used for *tfoX*
143 induction.

144 **Carbon source dependent GFP expression measurements:** Bacteria were grown over-night
145 in LB supplemented with 1% glucose, 1 % arabinose or 1% succinate or 1%
146 arabinose/succinate + 1% glucose.

147 **pACYC184 plasmid carrying *Pint* promoter fused to *gfp*:** p4638 was used as template.
148 400bp up to ATG of *intIA* in fusion with *gfp* was amplified using oligonucleotides 1374/1050.
149 The PCR fragment was than cloned into pTOPO-Blunt (Stratagene), extracted with EcoRI
150 restriction and cloned into pACYC184 plasmid (accession number X06403) digested with
151 EcoRI. The plasmid was than transformed into MG1655 *E. coli* cells.

152 **Flow cytometry** protocols are described (1-2). The fluorescence was measured using the
153 FACS-Calibur device.

154 **RNA preparation:** Total RNA was purified as previously described (36), from *V. cholerae*
155 N16961 cultures grown in Marine Broth to an OD_{600} 0.3 and then induced with 0.2 μ g/ml
156 Mytomycine for 1H30. Briefly, bacterial cells were disrupted with a FastPrep apparatus (MP)
157 in the presence of acid phenol. RNAs were then extracted with Trizol (Invitrogen) and
158 chloroform and treated with Turbo-DNase (Ambion).

159 ***intIA* 5' RACE-PCR :** 5' RACE (rapid amplification of cDNA ends) assays were performed
160 on 5 μ g total RNA with primer GSP1int 5'-AACTTGATGGGAAGTTGATGCT-3' using the

161 5' RACE System kit (Invitrogen) with a polyC-tail. PCR was then performed using a primer
162 hybridizing with the polyC-tail and primer GSP2int 5'-TTCTAGTTGCGAGCGTTGAA-3'.
163 Purified PCR amplification product was then sequenced.

164 **DNase I footprinting experiments:** A 475-bp DNA fragment corresponding to the promoter
165 region of *intI* was amplified from *V. cholerae* N16961 genomic DNA by PCR using PCR
166 extender system (5 Prime) and synthetic oligonucleotides 736 and 1374 (one of which was
167 labeled with [γ^{32} P]ATP by use of T4 polynucleotide kinase). The PCR product was purified
168 with the High pure PCR product purification kit (Roche).

169 The binding of cAMP-CRP (kindly provided by A. Kolb) to the 475-bp DNA fragment was
170 performed as previously described (40). After 20 min at room temperature, DNase I was
171 added at a final concentration of 0.3 μ g/ml. Reaction mixtures were incubated at room
172 temperature for 20 s. Reactions were stopped and were subjected to electrophoresis after
173 heating at 90°C. Protected bands were identified by comparison with the same fragment
174 treated for A+G sequencing reactions (47).

175 **Plasmid and strain constructions:**

176 Table 2 summarizes the detailed construction of p8348 and p8356.

177

178 **p8348 for *crp* deletion:** The 500 bp regions upstream (*fwd*) and downstream (*rev*) *V. cholerae*
179 *crp* were amplified from N16961 using oligonucleotides 910/911 and 912/913, respectively,
180 and cloned into pTOPO-Blunt vector (Stratagene). The *fwd* region was extracted from
181 pTOPO using EcoRI + SalI digestion and cloned into pSW7848 in the same enzyme sites,
182 yielding plasmid p8342. The *rev* region was extracted from pTOPO using EcoRI + SpeI and
183 cloned into p8342 in the same sites, yielding plasmid p8344. The *aadA1* spectinomycin
184 resistance cassette was extracted with EcoRI from p8168 and cloned into p8344 with the same

185 enzyme to yield p8348 with an insertion of *aadA1* between *fwd* and *rev* regions of the *V.*
186 *cholerae* *crp* gene.

187 **p8356 carrying P_{recN} -*gfp***: The 500 bp region downstream of *E. coli* *sfiA* (*rev-sfiA*) was
188 amplified with oligonucleotides 916/917 from MG1655 and cloned with EcoRI + SpeI in
189 pSW7848, yielding p8169. GFP was amplified from p8264 using oligonucleotides 997/998
190 containing the LexA regulated *recN* promoter and a ribosome binding site (RBS), and cloned
191 in pTOPO-Blunt (Stratagene). P_{recN} -*gfp* was then extracted with EcoRI and cloned into p8169
192 digested with the same enzyme. This plasmid was then digested by Sall + BamHI and the
193 1483 bp insert containing P_{recN} -*gfp*-*rev-sfiA* was cloned into the same restriction sites in
194 pSW23T.

195 **p4640 carrying P_{int} -*int-gfp*** was described in (1). **p4638 carrying P_{int} -*gfp***: A pSW23T-aph
196 derivative (plasmid p4730) (33) was used as the vector. *gfp* from plasmid p8264 was
197 amplified using oligonucleotides 1050/1061. The 700 bp region preceding *intIA* up to and
198 including the ATG was amplified from N16961 using oligonucleotides 1060 (complementary
199 to 1061)/1059. The two fragments were then fused using assembly PCR (1059/1050); the
200 assembled fragment was digested with BamHI + PstI and cloned in p4730 digested with the
201 same enzymes, yielding plasmid p4638.

202 Constructions and mutations were introduced in the chromosomes of *V. cholerae* by
203 homologous recombination as described (1, 33). Allele integration in $\Delta recA$ strains was
204 accomplished through temporary RecA complementation using an exogenous *recA* gene
205 carried on a thermosensitive plasmid (pJJC2791, (18)) as in (1).

206 **Statistical significance.** Each flow cytometry measurement was independently performed on
207 least 3 independent samples. When necessary, in order to test if the mean of two groups were
208 different we first tested the equality of their variance with a Fisher test. When the variances of
209 two groups were found to be significantly different, an unequal variance t-test was performed.

210 Otherwise a simple t-test was performed. The chosen significance threshold was 0.05 for all
211 tests. The corresponding *p-values* are given in Figure 1B.

212

213 **Results**

214 *ssDNA entry during transformation induces the SOS response in Vibrio cholerae*

215 We used the transformation proficient *V. cholerae* strains A1552 and N16961 *hapR+* (as in
216 (7, 46). We also used the wild type N16961 strain, which is *hapR-*, and the A1552 *pilQ*
217 mutant as non-transformable control strains to test the response to extracellular DNA in
218 conditions where no DNA uptake is possible. A secreted nuclease deficient the *hapR+* *V.*
219 *cholerae* (N16961 *hapR+* Δ *dns*) was also tested and gave the same results as nuclease
220 proficient strain (figure 1A).

221 A transcriptional *gfp* fusion containing *intIA* and an *rbs* upstream *gfp* ($P_{int-int-gfp}$) was
222 constructed (*p4640* in the plasmid table) and integrated into the chromosome of *V. cholerae*.
223 We used flow cytometry to measure the expression of the GFP reporter. Mitomycin C is a
224 DNA-intercalating agent that induces SOS and was used to verify that the GFP constructs
225 were indeed responding to SOS induction (not shown)(1, 33).

226 When competence of N16961 *hapR+* was induced by growth on chitin (where
227 transformation frequency is about 10^{-6} (50)), addition of DNA yielded a strong induction of
228 GFP expression compared to cultures grown in minimal medium with glucose as a carbon
229 source (Figure 1A). Both PCR products and *V. cholerae* genomic DNA were used as external
230 DNA and were both found to give similar induction. A1552, an environmental naturally
231 *hapR+* *V. cholerae* strain was also tested and yielded results comparable to the N16961
232 *hapR+* strain. The A1552 Δ *pilQ* mutant has a functional competence regulon but is deficient
233 for DNA uptake as it was shown recently that DNA uptake is dependent on *pil* genes (66). We
234 submitted this mutant as well as the non transformable N16961 strain to the same induction

235 protocols and found that no SOS induction was observed after treatment with chitin and
236 addition of DNA (Figure 1A). This suggested that ssDNA uptake by competent cells up-
237 regulates the integrase expression by inducing the SOS response. As a corollary, integrase
238 induction should be RecA-dependent, because SOS is abolished in *recA*- strains (70). Indeed,
239 when we deleted *recA* in our reporter N16961 *hapR*⁺ strain, integrase induction by DNA
240 addition in chitin containing medium was lost (figure 1A), which is consistent with our
241 interpretation that SOS induction is leading to integrase activation..

242 Another way of inducing competence is by over-expressing the TfoX protein in *V.*
243 *cholerae*. It has been shown that, like during growth on chitin, transformation efficiency
244 under these conditions is about 10⁻⁶ (50), so that we decided to use this approach to verify our
245 results obtained with growth on chitin. We used the plasmid pBAD-*tfoX* where *tfoX* is under
246 arabinose promoter regulation; over-expression is achieved by adding arabinose, whereas the
247 promoter is repressed in the presence of glucose (50). Figure 1B represents the effect of PCR
248 DNA addition in the absence or presence of TfoX over-expression. It can be observed that
249 upon introduction and over-expression of TfoX (using 0.2% arabinose) from plasmid pBAD-
250 *tfoX*, DNA addition induces the *intIA* gene. When we repressed TfoX expression (using 1%
251 glucose), no induction of the *intIA-gfp* fusion was observed upon addition of DNA. These
252 results were consistent with results obtained in the chitin tests described above. We conclude
253 from these data that transformation of DNA induces *intIA* through activation of the SOS
254 response in *V. cholerae* competent cells (Figure 4).

255 *The intIA integrase is also regulated by the CRP-dependent catabolite repression pathway*

256 We observed that growth on chitin (without the addition of external DNA) slightly
257 increased the expression of the *intIA-gfp* fusion in *V. cholerae* cells (Figure 1A, compare plain
258 blue and plain grey bars). This effect of chitin was also observed in the absence of DNA

259 uptake in the *ΔpilQ* et N16961 (hapR-) strains. This led us think that the CRP-cAMP
260 catabolite repression pathway could be involved in the regulation of the *intIA* transcription.

261 In order to determine the localization of the putative regulatory region (internal or
262 upstream of *intIA*), and because of the presence of the whole integrase sequence upstream of
263 *gfp* in our “P_{int}-*int-gfp*” fusion, we constructed a second fusion where *gfp* is directly under the
264 control of the *intIA* promoter (P_{int}-*gfp*). To test the effect of various carbon sources on our
265 strains we used 1% of the selected sugar during growth. Glucose is known to be a repressor of
266 the carbon catabolite control whereas arabinose and succinate are inducers (3, 23, 64). Growth
267 of *V. cholerae* N16961 *hapR*+ on 1% arabinose induced expression of *gfp* in both constructs
268 (Figure 2A). Moreover, addition of glucose repressed *intIA-gfp* induction by arabinose in both
269 constructs, although not always to the lowest level we found with glucose only (compare
270 white and grey bars). This could be due to the fact that in *V. cholerae* glucose is exhausted
271 within 6 hours at this concentration (1%) (72) and the remaining arabinose in the medium
272 may then still induce the CRP regulon.

273 No induction of the SOS-regulated *recN* promoter (P_{recN}-*gfp* fusion) was observed by
274 arabinose in the N16961 *hapR*+ strain, showing that *intIA* regulation by arabinose was
275 independent of SOS. β-galactosidase tests using a *recN-lacZ* fusion confirmed that arabinose
276 did not induce the SOS response in *V. cholerae* (data not shown).

277 The catabolite repression pathway requires the CRP protein. We performed the same
278 experiments in our reporter *V. cholerae* N16961 *hapR*+“P_{int}-*int-gfp*” strain where we deleted
279 the *crp* gene (Figure 2A). Induction of *intIA* by arabinose is no longer observed in this
280 context, while mitomycin C still induces *intIA* (Figure 2A).

281 We used the “P_{int}-*int-gfp*” fusion in different *V. cholerae* strains to further address the
282 effect of another carbon source known to induce the catabolite response, succinate, to confirm
283 our observations with arabinose. We observe that growth in 1% succinate strongly induces

284 *intIA-gfp* in N16961 and A1552 strains. As for arabinose, addition of glucose strongly
285 repressed *intIA-gfp* induction by succinate.

286 Finally, we addressed whether this CRP regulation of *intIA* could take place in *E. coli*
287 (*i.e.* with *E. coli* CRP). In order to do that we cloned our *Pint-gfp* fusion (400bp of the
288 upstream region of *intIA* fused to *gfp*) in the pACYC184 plasmid and introduced this
289 construct in a wild type *E. coli* MG1655 strain. As for *V. cholerae*, we observed that GFP
290 expression was induced by arabinose and repressed by glucose in *E. coli* (Figure 2B),
291 showing that *intIA* regulation by CRP does not require any *Vibrio*-specific co-factor, and
292 rather obeys to "classical" CRP-cAMP regulation.

293 Altogether, these results strongly suggested that *intIA* is also regulated by the
294 catabolite repression pathway and that the carbon-source dependent and the SOS-dependent
295 regulations of *intIA* are independent of each other.

296 *CRP binds the promoter and regulates intIA*

297 Our finding of a CRP-dependent regulation of *intIA* (VCA0291) was intriguing,
298 although another *V. cholerae* integrase, VCA0281 (P4 integrase family), had already been
299 shown to be regulated by CRP (28). We decided to perform a DNA-footprint analysis to find
300 out whether the CRP-cAMP complex binds the *intIA* promoter.

301 We first determined the *intIA* transcriptional start by 5' -RACE-PCR, and identified a
302 unique transcriptional start site 28 bases upstream of the *intIA* initiation codon (figure 3A).
303 We then performed DNase I footprinting to determine the potential CRP-binding site. This
304 experiment revealed that the cAMP and CRP combination modified the *intIA* promoter region
305 footprint in a single region with protected bands flanking one hypersensitive band (figure 3B).
306 Our data show that the cAMP-CRP complex binds specifically between positions -100 and -
307 79 bp upstream the *intIA* transcription start site (figure 3A), and the binding is centered at -
308 89.5 bp, a distance typical of a class I CRP activation site (4). Moreover, the sequence of the

309 DNA site bound by CRP 5'TATGTtgagcaTCACA3', if not identical to the CRP binding
310 sequence consensus 5'T^(G/T)T^(G/T)(^{A/T})N₆TCA(^{C/A})(^{A/T})3', is very similar to it (11, 14).

311 The integrase expression is thus shown here to be affected by the carbon source present in the
312 environment.

313

314 **Discussion**

315 We show that like conjugation (1), transformation (defined here as the DNA uptake by
316 naturally competent cells) is a mechanism of HGT that induces the bacterial SOS response,
317 thus the integron integrase expression in *V. cholerae*. The different assays we made, using
318 chitin or overexpressing TfoX, the central competence regulator, demonstrate that
319 competence induction is a prerequisite for the SOS induction during the transformation
320 process, but the *pilQ* mutant results establish that it is the DNA uptake by the competent cells
321 which mediates this cellular response. SOS induction has several implications related to
322 genome stability and mutagenesis in bacterial cells (26, 70). We summarized our findings in
323 the model illustrated in figure 4.

324 In *V. cholerae*, we know that SOS induction leads to cassette rearrangements within the
325 integron (1, 13, 33), regulates other mobile elements such as SXT (5), and is implicated in
326 pathogenicity (56). Interestingly, SOS also regulates some toxin-antitoxin (TA) systems (61);
327 it can thus play a role in the integron maintenance, as there are 13 cassettes carrying TA
328 systems in the *V. cholerae* SI (54). Several *Vibrio* species have been also demonstrated to be
329 naturally competent (34, 53, 55) and it is very likely that many other *Vibrio* species, if not all,
330 can also be transformed, thus that the SOS response induction we describe in *V. cholerae* is a
331 common process for these species. It is very likely that the SOS response is highly conserved
332 in all *Vibrio* species, as LexA and LexA binding boxes are found upstream of the SOS

333 regulon common genes (unpublished data), as well as upstream of the *intIA* gene in all the
334 sequenced *Vibrio* genomes (13).

335 Moreover, this is the first report of a catabolite regulation of the *V. cholerae* integron
336 integrase: we show that the cAMP-CRP complex binds and impacts *intIA* promoter
337 expression. According to our results, the SOS and CRP regulations of *intIA* are most likely
338 fully independent. Like the SOS response, CRP regulation can be considered as a stress
339 response because it modulates the expression of a complex regulon when rapidly
340 metabolizable carbon sources are scarce in the medium (9-10, 31, 39, 65). The cAMP-CRP
341 complex (shortly referred to as CRP in the following lines) controls not only specific
342 catabolic pathways, but also many other genes involved in important aspects of cell
343 physiology and interaction with the environment (31, 58). Many genes related to adaptation
344 and survival are known to be regulated by CRP in the *E. coli* context, such as *cat*, which
345 confers resistance to chloramphenicol, *ccd* toxin, or the *tra* genes in plasmid F (41). CRP is
346 implicated in the regulation of virulence, as in the case of the pathogenicity islands in
347 *Salmonella* (29), or the plasmid and chromosomal virulence genes in *Yersinia pestis* (73). The
348 control of *Listeria monocytogenes* virulence genes by the regulator PrfA, a protein from the
349 CRP family (25, 59) also highlights the influence of carbon and energy sources in the
350 environment on pathogenicity. In *V. cholerae*, CRP represses the cholera toxin and the toxin
351 co-regulated pilus (63), promoting growth in low nutrient environments (outside the host) and
352 favoring virulence in rich media such as the intestine. Moreover, CRP induces the *hapR*
353 quorum sensing regulator in *V. cholerae* (44), and the competence regulon in *H. influenzae*
354 (15, 57). Our data thus underlines the importance of *V. cholerae*'s environment on its genetic
355 adaptability, as the integron integrase is regulated by two different stress responses. It is worth
356 noting that the SI has been reported as the most variable genome part among *V. cholerae*
357 isolates (16, 27, 42).

358 It would be interesting to test whether there is a CRP control of the integron cassettes,
359 as it has been shown that other extracellular stresses can influence cassette expression in the
360 *Vibrio* superintegron (52).

361 The aquatic niche of *V. cholerae* can be considered as a favorable environment for
362 CRP induction, because one of the most abundant carbon sources is chitin. Since integron
363 rearrangements eventually lead to bacterial adaptation and diversification, one can speculate
364 that bacteria induce genetic remodeling during growth outside the human host (*i.e.* in the
365 environment), where virulence is repressed and competence induced, considering that all three
366 functions (integrons, virulence and competence) respond to CRP regulation. In any case, our
367 results point to a link between the external environment (carbon source-dependent CRP
368 induction), competence state, SOS induction, and adaptation.

369 Other examples of genes co-regulated by SOS and CRP exist. Concomitant regulation
370 by these two pathways has been described for the mutator translesional DNA-polymerase
371 DinB in *E. coli* (45, 68), and both pathways influence mutability in *E. coli* cells that have
372 reached stationary phase, suggesting that coordination is achieved in response to
373 environmental stresses (45, 67). The CRP pathway has also been reported to have a role in
374 cell division along with the SOS response (19, 37).

375 Altogether, our previous (Guerin et al., 2009, Cambray *et al.*, 2010, Baharoglu et al.,
376 2010) and present data show how complex the regulation of the *intI* promoter can be, and thus
377 how integrated the SI cassette array remodeling is, in the cell physiology. It is important to
378 note that a large fraction of the integron integrases are regulated by SOS (13), especially those
379 carried in *Vibrio* species genomes, underlining the importance of the induction of this stress
380 response in various conditions. SOS response can be induced in any medium, provided that an
381 external stress or lateral gene transfer mechanisms lead to unusual levels of ssDNA inside the
382 cell. In this line it is worth noting that most antibiotics have been found to induce the SOS

383 response in *V. cholerae* and increase the mutation frequency, even when present in
384 subinhibitory concentrations (2), pointing to these as another source of mutability in this
385 species. Moreover, all *Vibrio* species genomes sequenced so far carry the gene set required for
386 natural transformation (55), and competence has been demonstrated for several of these (34, 53,
387 55), thus, the mechanisms described here could likely be applied to them. It would now be
388 interesting to establish whether SOS is induced in the gut, and if so, to what extent it could
389 induce the integrase.

390

391

392

393 **Table 1: Bacterial strains and Plasmids**

| Strain Genotype | Strain number | Phenotype of interest | AB-Res (*) | Construction or reference |
|--|---------------|---|-----------------|--|
| <i>V.cholerae</i> N16961 | 7805 | <i>Vibrio cholerae</i> El Tor non transformable strain | | Laboratory collection |
| N16961 <i>hapR</i> ⁺ | 8637 | <i>Vibrio cholerae</i> El Tor transformable strain | str | |
| N16961 Δ <i>dns hapR</i> ⁺ | 8638 | <i>Vibrio cholerae</i> El Tor transformable strain lacking extracellular nuclease | str | M. Blokesch. (7) |
| N16961 Δ <i>dns hapR</i> ⁺ Δ <i>recA::km</i> | 7955 | Defective for SOS induction | str km | Deletion of <i>recA</i> from strain 7093 with plasmid pMEV97 |
| N16961 Δ <i>dns hapR</i> ⁺ Δ <i>crp::spec</i> | 8349 | Defective for CRP pathway | sp km str | Deletion of <i>crp</i> from strain 7093 with plasmid p8348 |
| A1552 | 8635 | <i>Vibrio cholerae</i> El Tor transformable strain | rif | M. Blokesch (50) |
| A1552 Δ <i>dns ΔpilQ</i> | 9545 | Defective for DNA uptake | rif | M. Blokesch (66) |
| Plasmids | | | | |
| pBAD- <i>tfox</i> | | TfoX expression under arabinose control | ap | M. Blokesch (50) |
| pSW23T-PrecNGFP-af-sfiA | 8356 | <i>gfp</i> in transcriptional fusion with <i>recN</i> promoter integrates by homologous recombination into <i>sfiA</i> in <i>E. coli</i> and into <i>recN</i> in <i>V. cholerae</i> . | cm km | This study |
| p8348 | 8348 | Crp deletion vector derived from pSW7848 with insertion of <i>aadA7</i> in forward and reverse regions of the <i>V. cholerae crp</i> gene | sp cm | This study |
| pSW23T | 970 | <i>oriT</i> _{RP4} , <i>oriV</i> _{R6K} Pir dependent replication | cm km | (22) |
| pSW7848 | 7848 | A <i>ccdB</i> containing derivative of pSW4426T R6K <i>oriV</i> , RP4 <i>oriT</i> , <i>ccdB-araC</i> Pir dependent replication | cm | (43, 69) |
| pMEV97 | 6993 | Δ <i>recA::km</i> - Counter-selection <i>sacB</i> For <i>recA</i> deletion in <i>V.cholerae</i> | cm km | (69) |
| p4638 | 4638 | <i>gfp</i> in fusion with <i>int1A</i> promoter. "Pint- <i>gfp</i> " | cm km | This study |
| p4640 | 4640 | <i>gfp</i> in transcriptional fusion with <i>int1A</i> . "Pint- <i>int-gfp</i> " | cm km | (1) |
| p8168 | 8168 | pTOPO (Stratagene) carrying <i>aadA1</i> amplified from pAM34 (30) with oligonucleotides 918/919 | sp km | This study |
| pZE1-GFP | 8264 | Carries GFP | km | C. Beloin (20) |

394 (*) Antibiotic resistance : str streptomycin, km kanamycin, sp spectinomycin, rif rifampicin, cm chloramphenicol,
 395 ap ampicillin
 396

397 **Table 2 : detailed construction of p8348 for *crp* deletion and p8356 carrying P_{*recN*}-*gfp***
 398

| PCR template or plasmid | Primer or enzyme | Amplifies or extracts | Cloned in | Digested with | Cloned in | Final plasmid |
|-------------------------|------------------|-------------------------------|-----------|---------------|-----------|---------------|
| N16961 | 910/911 | <i>Fwd crp</i> | pTOPO | EcoRI+Sall | pSW7848 | P8342 |
| N16961 | 912/913 | <i>Rev crp</i> | pTOPO | EcoRI+SpeI | P8342 | P8344 |
| P8368 | EcoRI | <i>aadA1</i> | | | P8344 | P8348 |
| MG1655 | 916/917 | <i>Rev sfiA</i> | | EcoRI+SpeI | pSW7848 | P8169 |
| P8264 | 997/998 | <i>PrecN-rbs-gfp</i> | pTOPO | EcoRI | | P8169 |
| P8169 | Sall+BamHI | <i>PrecN-rbs-gfp_Rev sfiA</i> | | | pSW23T | P8356 |

399
 400

401 **Table 3: Oligonucleotides**

| Oligonucleotide number | Sequence 5' 3' |
|------------------------|--|
| 736 | CATTAAACCATTGATAAAATGCTTTTTATTACG |
| 910 | TTGCGTCGACATCACCCCGCAATTTTCACG |
| 911 | GTCAGAATTCAATAATCTCACTTCCTCTGC |
| 912 | CGTAGAATTCGTGCCCGATAACCCGTCTCG |
| 913 | GCTAACTAGTACAAGCATTAGCCAGTGAGG |
| 916 | GTCTGAATTCCTGTGAGTTACTGTATGG |
| 917 | GCTCACTAGTTGCATCGAGAGAATATTTTCG |
| 918 | GTGACGCACACCGTGGAAACGG |
| 919 | CCGCGAAGCGGCGTCGGCTTGAACG |
| 1050 | AACTGCAGTCATTATTTGTAGAGCTCATCCATGCC |
| 1059 | TTGGATCCAAACCCGGGAGCAGATACTGT |
| 1060 | AGTTCTTCTCCTTTGCTAGCCATATAGTTCTCACTGAATATTTAACTG |
| 1061 | CAGTTAAATATTCAGTGAGAACTATATGGCTAGCAAAGGAGAAGAACT |
| 1372 | TCTTACCTTCCAACAGGTAGGCTACC |
| 1373 | GGATAAATTTGAGTTGATTATCAATCATTACAAGAAGTTGTTC |
| 1374 | CATTAAACCATTGATAAAATGCTTTTTATTACG |
| 1401 | GATGGCAACATATTTAACTCATCTTGAGG |
| 1402 | GATGGCAACATATTTAACTCATCTTGAGGATAAATTTTAATTTATTATCAATTAT TACAAGAAGTTGTTCTAAGGG |

402

403

Figure Legends

404 **Figure 1: Uptake of ssDNA induces integrase expression through SOS response**
405 **activation. A:** N16961 *hapR*⁺ or A1552 (*hapR*⁺) and derivative strains carrying the *P_{int}-int-*
406 *gfp* reporter fusion were grown with chitin or glucose. The Y-axis represents the ratio of
407 fluorescence induction compared to growth on glucose in specified cultures. Transformation
408 efficiencies obtained in the laboratory are shown as transformants/total *cfu* above histogram
409 bars. BD (Below Detection threshold) means that no transformants were obtained ($<10^{-9}$). **B:**
410 The N16961 *hapR*⁺ Δ *dns* *P_{int}-int-gfp* strain containing pBAD-*tfoX* were grown on LB
411 supplemented with glucose or arabinose. "TfoX⁻" represents inhibition of the pBAD promoter
412 with 1% glucose. "TfoX⁺" represents over-expression of TfoX with 0.2% arabinose. **C:** P
413 values. Statistical analyses were performed using the GraphPad InStat software. Statistical
414 tests are described in the materials and methods section. **A, B:** Each experiment was repeated
415 on independent cultures at least 3 times. The error bars represent standard deviation. See
416 Figure 1C for *P-values*.

417

418 **Figure 2: Arabinose and succinate induce integrase expression in a CRP-dependent**
419 **manner.** Bacteria were grown overnight in LB supplemented with 1% glucose, 1% arabinose,
420 1% succinate or both glucose and the other carbon source. The Y-axis represents the ratio of
421 fluorescence induction in specified cultures compared to growth on glucose. Each experiment
422 was repeated on independent cultures at least 3 times. The error bars represent standard
423 deviation. **A:** *V. cholerae*. **B:** *E. coli*. 400bp of the region preceding the integrase was
424 amplified, fused to *gfp* and cloned into plasmid pACYC184. The same experiment as above
425 was performed on *E. coli* carrying plasmid with the *P_{int}-gfp* fusion.

426

427 **Figure 3: Regulatory region of *intI* and identification of the CRP-binding site in the *intI***
428 **promoter region by DNase I footprinting assay. A:** The *attI* site, CRP and LexA binding
429 sites are indicated. The transcriptional start site (+1) is indicated with an arrow. The -10 and -
430 35 boxes are indicated in bold. The coding sequence is indicated with capital letters. **B:** The
431 labeled complementary strand of *intI* was incubated with 0 (-), 125, 250 or 500 nM cAMP-
432 CRP complex. Protected regions are indicated by solid lines. The coordinates were
433 determined with the G+A technique developed by Maxam and Gilbert (47) and correspond to
434 the numbering used in Figure 3A.

435 **Figure 4: Model of *intIA* regulation and implications on genome plasticity.** Grey boxes
436 represent mechanisms involved in IntIA regulation. Horizontal gene transfer (conjugation,
437 transformation) induces SOS through ssDNA uptake by recipient cells, which triggers *intIA*
438 transcription. Carbon sources present in the environment also regulate IntIA expression
439 through carbon catabolite control.

440

441 **Acknowledgments**

442 We thank M. Jason Bland for helpful reading of the manuscript. We thank Melanie Blokesch
443 for the gift of competence proficient *V. cholerae* strains and the $\Delta pilQ$ derivative and Annie
444 Kolb for the gift of purified CRP. We also thank Myriam Gominet and Olga Soutourina for
445 their help setting up the footprint experiment. The setting up of flow cytometry experiments
446 was done with the help of the cytometry platform of the Institut Pasteur. This study was
447 funded by the Institut Pasteur, the Centre National de la Recherche Scientifique (CNRS-URA
448 2171), the French National Research Agency (ANR-08-MIE-016), the EU (NoE
449 EuroPathoGenomics, LSHB-CT-2005-512061) and the Fondation pour la Recherche
450 Médicale (équipe FRM 2007). ZB is supported by a postdoctoral fellowship from the Roux
451 foundation and a DIM Malinf fellowship (Conseil régional d'Île-de-France).

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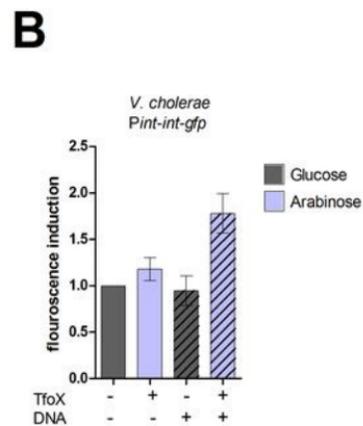
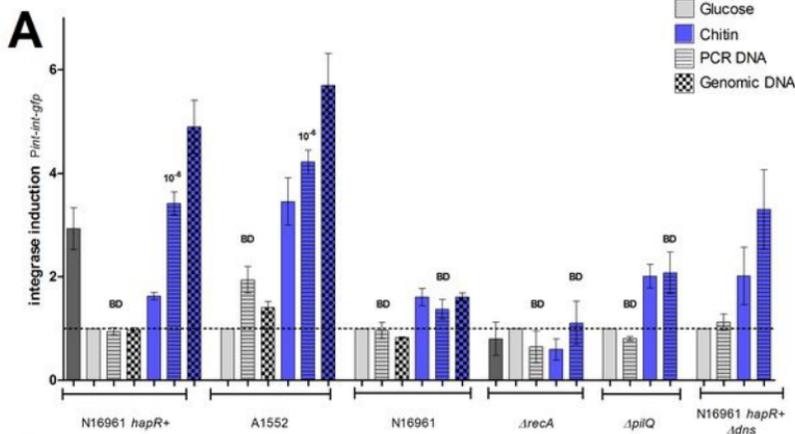
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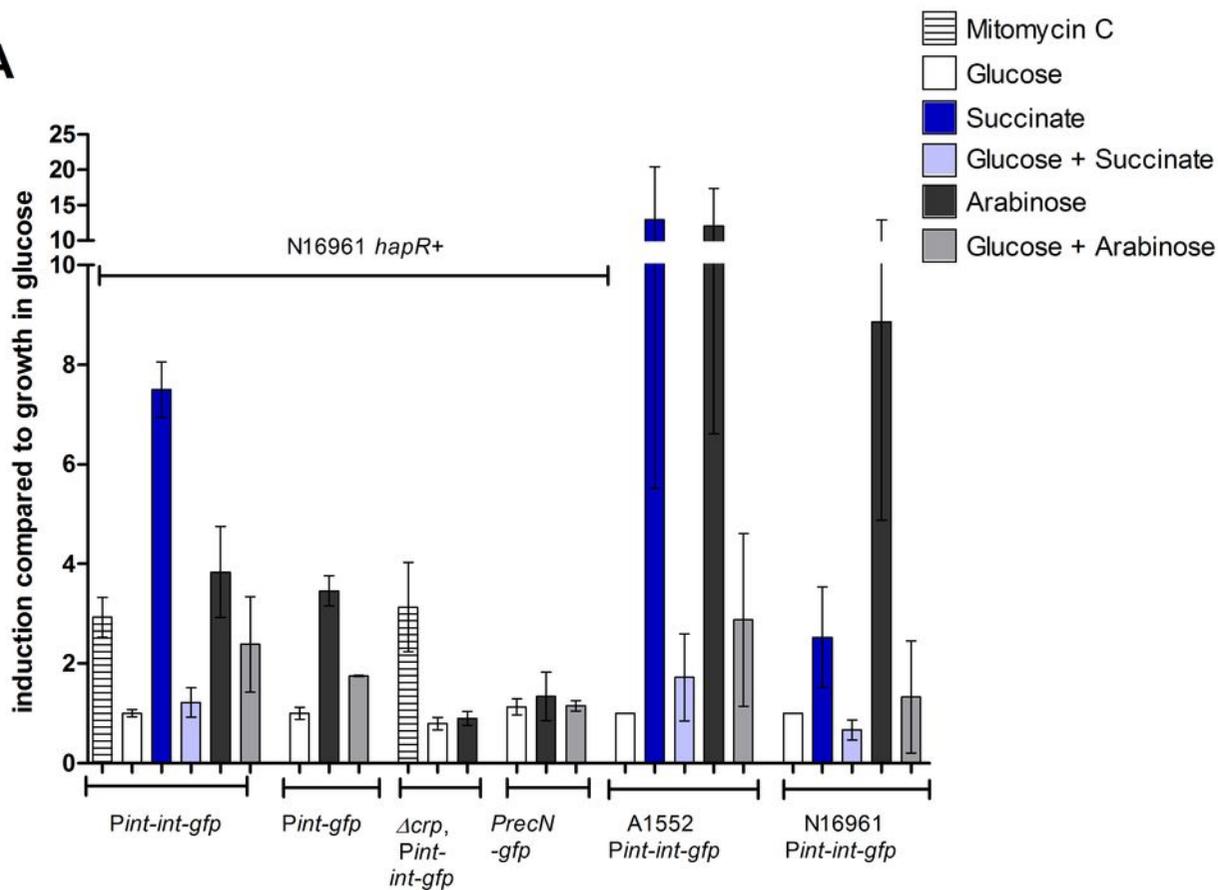
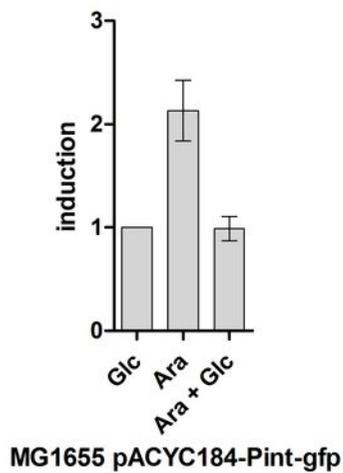
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658



C

| Strain | Condition 1 | Condition 2 | <i>P</i> value | Conclusion |
|---|---------------|-----------------|------------------|--|
| N16961 <i>hapR+</i> | Glucose | Glucose + DNA | 0.4716 | Not different |
| | Glucose | Chitin | 0.0045 | Very significantly different |
| | Chitin | Chitin + DNA | <0.001 | Extremely significantly different |
| <i>ApilQ</i> | Glucose | Glucose + DNA | 0.5008 | Not different |
| | Glucose | Chitin | 0.01 | Very significantly different |
| | Chitin | Chitin + DNA | 0.892 | Not different |
| <i>Adns</i> | Glucose | Glucose + DNA | 0.38 | Not different |
| | Glucose | Chitin | 0.0213 | Significantly different |
| | Chitin | Chitin + DNA | 0.0248 | Significantly different |
| <i>AreA</i> | Glucose | Glucose + DNA | 0.8955 | Not different |
| | Glucose | Chitin | 0.2837 | Not different |
| | Chitin | Chitin + DNA | 0.1369 | Not different |
| Figure 1B N16961 <i>hapR+</i> pBAD- <i>tfoX</i> | Glucose | Arabinose 0.2% | 0.5196 | Not different |
| | Glucose | Glucose + DNA | 0.8372 | Not different |
| | Glucose + DNA | Arabinose + DNA | 0.0195 | Significantly different |
| | Arabinose | Arabinose + DNA | 0.0381 | Significantly different |

A**B**

A*Cassette 1 (VCA0292)**attI* site

agttgattat caatcattac aagaagttgt tctaagggat tgatctaaca ttagcaagaa agcgcctaac | gctttctaga 80
 tcaactaata gttagtaatg ttcttcaaca agattcccta actagattgt aatcgttctt tcgcggattg | cgaaagatct

cagcttctta cctttccaac aggtaggcta ccgctatcta attaatagac cactgggtgc atactacat aacaaccttc 160
 gtcgaagaat ggaaaggttg tccatccgat ggcgatagat taattatctg gtgaccacg tatgatggta ttggttgaag

CRP box

taaatcagta tgttgagcat cacaccattc aaaaatctca gttatagat tgctgatgtc tgtatagcct **ttgaca**ataa 240
 atttagtcat acaactcgta gtgtggtaag tttttagagt cgaatatcta acgactacag acatatcgga gaactgtatt

aaacaaccaa caaa **tactgt** acaaatAacc agttaaatat tcagtgagaa ctatATGAAA TCCAGTTTT TGTTAAGTGT 320
 tttgttggtt gtttatgaca tgtttattgg tcaatttata agtcactctt gataACTTT AGGGTCAAAA ACAATTCACA

LexA box*intI*A**B**