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## **ApoFnr binds as a monomer to promoters regulating expression of enterotoxin genes of *Bacillus cereus*.**

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1 **ApoFnr binds as a monomer to promoters regulating the expression of**  
2 **enterotoxin genes of *Bacillus cereus***

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6 Running title: Active aerobic apoFnr is monomeric  
7  
8

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## 22 Abstract

23 *Bacillus cereus* Fnr is a member of the Crp/Fnr (cAMP-binding protein/fumarate  
24 nitrate reduction regulatory protein) family of helix-turn-helix transcriptional regulators. It is  
25 essential for the expression of *hbl* and *nhe* enterotoxin genes independently of the oxygen  
26 tension in the environment. We studied aerobic Fnr binding to target sites in promoters  
27 regulating the expression of enterotoxin genes. *B. cereus* Fnr was overexpressed and purified  
28 as either a C-terminal His-tagged (Fnr<sub>His</sub>) or an N-terminal Strep-tagged (StrepFnr) fusion  
29 protein. Both recombinant Fnr<sub>s</sub> were produced as apoforms (clusterless) and occurred as  
30 mixtures of monomer and oligomers in solution. However, apoFnr<sub>His</sub> was mainly monomeric,  
31 while apoStrepFnr was mainly oligomeric, suggesting that the His-tagged C-terminal extremity  
32 may interfere with oligomerization. The oligomeric state of apoStrepFnr was dithiothreitol-  
33 sensitive, underlining the importance of a disulphide bridge for apoFnr oligomerization.  
34 Electrophoretic mobility shift assays showed that monomeric, but not oligomeric apoFnr,  
35 bound to specific sequences located in the promoter regions of the enterotoxin regulators *fnr*,  
36 *resDE* and *plcR* and the structural genes *hbl* and *nhe*. The question of whether apoFnr binding  
37 is regulated *in vivo* by redox-dependent oligomerization is discussed.

38

## 39 INTRODUCTION

40 The facultative anaerobic, spore-forming *Bacillus cereus* has gained notoriety as an  
41 opportunistic human pathogen that can cause a wide range of diseases from periodontitis and  
42 endophthalmitis to meningitis in immunocompromised patients.. However, most of the  
43 reported illnesses involving *B. cereus* are food-borne intoxications, classified as emetic and  
44 diarrheal syndromes (17, 30). Diarrheal syndrome may result from the production in the  
45 human host's small intestine of various extracellular factors including hemolysin BL (Hbl),  
46 non-haemolytic enterotoxin (Nhe) and cytotoxin CytK (17, 26). The genes encoding these  
47 potential virulence factors belong to the PlcR regulon (1, 7, 20, 31).

48 *B. cereus* will grow efficiently by anaerobic glucose fermentation in amino acid-rich  
49 media supplemented with glucose as the major source of carbon and energy (3, 21, 29, 33,  
50 34). The ability of *B. cereus* to grow well under these conditions is controlled by both the  
51 two-component system ResDE (4) and the redox regulator Fnr (33, 34). Unlike ResDE, *B.*  
52 *cereus* Fnr has been shown to be essential for fermentative growth and for enterotoxin  
53 synthesis under both anaerobiosis and aerobiosis (33, 34). Fnr protein is a member of the  
54 large Crp/Fnr superfamily of transcription factors that coordinate physiological changes in  
55 response to a variety of metabolic and environmental stimuli (16). Members of the family are  
56 predicted to be structurally related to the catabolite gene activator protein of *Escherichia coli*,  
57 Crp (also known as the cAMP receptor protein) (10). Like all the members of the Crp/Fnr  
58 family, *B. cereus* Fnr contains an N-terminal region made up of antiparallel  $\beta$ -strands able to  
59 accommodate a nucleotide, and a C-terminal helix-turn-helix (HTH) structural motif. In  
60 addition, it contains a C-terminal extension with four cysteine residues considered, in  
61 *B. subtilis*, to coordinate a  $[4\text{Fe-4S}]^{+2}$  centre that serves as a redox sensor (27). The *B. subtilis*  
62 Fnr forms a stable dimer that is independent of both the oxygen tension in the environment

63 and FeS cluster formation. However, the presence of an intact  $[4\text{Fe-4S}]^{+2}$  cluster is required  
64 for it to bind to a specific DNA-binding site and for subsequent transcriptional activation (27).

65 Structurally, the predicted Fnr of *B. cereus* resembles the *B. subtilis* Fnr (27).  
66 Therefore, the FeS cluster could also be a key component required for the DNA binding  
67 activity of *B. cereus* Fnr under anaerobiosis. However, our previous results suggested that  
68 unlike *B. subtilis* Fnr, *B. cereus* Fnr may also exist in an active state under aerobiosis and thus  
69 conserve some site-specific DNA binding properties. To address this specificity further and  
70 elucidate the mechanism by which Fnr regulates enterotoxin gene expression in aerobically  
71 growing *B. cereus* cells, we characterized the DNA-binding activities of purified aerobic Fnr.  
72 To this end, we overproduced full-length Fnr in *Escherichia coli* with two different tags. We  
73 showed that both recombinant Fnrs were produced in apo forms (devoid of FeS cluster) under  
74 oxic conditions. Recombinant Fnr containing a C-terminal polyhistidine tagged sequence was  
75 shown to be mainly monomeric in solution, while N-terminally Strep-tagged Fnr occurred  
76 mainly as oligomers. Only the monomeric forms of both recombinant apoFnrs were found to  
77 bind to the promoter regions of *fnr* itself, the pleiotropic regulators *resDE* and *plcR* and the  
78 structural enterotoxin genes *hbl* and *nhe*. Finally, our results pointed to some new unusual  
79 properties of Fnr that may have physiological relevance in the redox regulation of enterotoxin  
80 expression, enterotoxin expression being both directly and indirectly (via ResD and PlcR)  
81 regulated by apoFnr under aerobiosis.

82

## 83 MATERIALS AND METHODS

84 **Bacterial strains and growth conditions.** *Escherichia coli* strain TOP 10 (Invitrogen) [ $F^-$   
85 *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\Phi$ 80*lacZ* $\Delta$ *M15*  $\Delta$ *lacX74* *deoR* *recA1* *araD139*  $\Delta$ (*ara-leu*)-7697  
86 *galU* *galK* *rpsL* ( $\text{Str}^r$ ) *endA1* *nupG*] was used as the general cloning host, and strain BL21  
87 CodonPlus(DE3)-RIL (Stratagene) [ $F^-$  *ompT* *hsdS*( $r_B^-$   $m_B$ ) *dcm*<sup>+</sup>  $\text{Tet}^r$  *gal*  $\lambda$  (DE3) *endA*  $\text{Hte}$

88 [argU ileY leuW Cam<sup>r</sup>] was used to overexpress *fnr*. Both *E. coli* strains were routinely grown  
89 in Luria broth with vigorous agitation at 37°C. *B. cereus* F4430/73 wild-type (32) and *fnr*  
90 mutant (34) were grown as previously described.

91  
92 **General molecular methods.** Restriction endonuclease and T4 DNA ligase were obtained  
93 from Promega and used in accordance with the manufacturer's instructions. Genomic DNA of  
94 *B. cereus* was purified as described by Guinebretiere and Nguyen-The (11). Plasmid DNA  
95 was purified using anion-exchange columns (Promega). PCR amplification of DNA was  
96 carried out with *Taq* polymerase using the manufacturer's specifications (Roche Molecular  
97 Biochemicals) for reaction conditions. The 5' end of the *resDE* mRNA was mapped from a 5'  
98 RACE PCR product obtained with the 3'/5' RACE kit (Rapid amplification of cDNA ends,  
99 Roche Molecular Biochemicals). For this purpose, we used total RNA extracted from  
100 *B. cereus* F4430/73 cells harvested at  $\mu_{\max}$ , *i.e.* the maximal expression of the *resDE* operon.  
101 Briefly, the first-strand cDNA was synthesized from total RNA with *fnr*-specific primer SP1  
102 (5'-GCCTGGTAAAGATGGCATTG-3'), avian myeloblastosis virus reverse transcriptase,  
103 and the deoxynucleotide mixture of the 3'/5' RACE kit as recommended by the manufacturer.  
104 After purification and dA tailing of the cDNA, a PCR with the (dT)-anchor oligonucleotide  
105 primer and the specific *fnr* SP2 primer (5'-GATGATGAGGATCGTATTVGTCG-3')  
106 followed by a nested PCR with SP3 primer (5'-GAGAGTGCGCAGCGGGTAGAG-3')  
107 yielded a PCR product of 190 bp, as revealed by 2% agarose gel electrophoresis. This PCR  
108 product was purified and sequenced.

109  
110 **Cloning and overexpression of recombinant Fnr.** The coding sequence for *B. cereus fnr*  
111 was PCR-amplified from F4430/73 genomic DNA using either primers PET101F ( 5'-  
112 CACCGTGGCAAACAGTATGACATTATCT-3') and PET101R (5'-ATCAATGCTACAAA

113 CAGAAGC-3') or primers PET52F (5'-**CCCGGG**GATGACATTATCTCAAGATTTAAAAG  
114 AA-3'; *Sma*I restriction site in bold type) and PET52R (5'-  
115 **GAGCTC**CTAATCAATGCTACAAACAGAAGCA-3'; *Sac*I restriction site in bold type).  
116 The amplicons were cloned as blunt-end PCR product into pET101/D-TOPO (Invitrogen) and  
117 as a *Sma*I-*Sac*I fragment into the corresponding sites of pET-52b(+) (Novagen), yielding  
118 pET101*fnr* and pET52*fnr*, respectively. *B. cereus* Fnr was produced as a C-terminal fusion  
119 with a His-tag using pET101*fnr* (Fnr<sub>His</sub>) and as an N-terminal fusion with a Strep-tag using  
120 pET52*fnr* (StrepFnr) in *E. coli* BL21 CodonPlus(DE3)-RIL (Stratagene). Recombinant cells  
121 were grown at 37°C in Luria broth with 100 µg ml<sup>-1</sup> ampicillin. When OD<sub>600</sub> reached ~1.0,  
122 protein production was triggered by adding isopropyl-β-D-thiogalactopyranoside (IPTG) with  
123 a final concentration of 0.2 mM (pET101*fnr*) or 0.4 mM (pET52*fnr*). Cells were further  
124 grown for 16 h at 20 °C.

125  
126 **Purification of Fnr<sub>His</sub>**. Cells from a 4.8 L culture were harvested by centrifugation (10,000g,  
127 15 min), resuspended in buffer A (50 mM sodium phosphate buffer [pH 7.0], 300 mM NaCl,) and  
128 incubated with 0.5 mg/ml lysosyme for 30 min under gentle agitation. Cells were lysed  
129 by sonication for 3 min at 80% of maximum amplitude using a Vibra cell ultrasonifier (Fisher  
130 Bioblock Scientific). Cell debris were removed by centrifugation at 20,000g for 20 min. The  
131 supernatant was run through a 5 ml Co<sup>2+</sup> IMAC column (Clontech) equilibrated with buffer  
132 A. The column was washed with 50 ml of buffer A and then with 25 ml of buffer A  
133 containing 10 mM imidazole, and the protein was eluted with 5 ml of buffer A containing 150  
134 mM imidazole. The eluted fraction was desalted on a Sephadex G25 column (Amersham  
135 Pharmacia Biotech) and concentrated using Nanosep 30 kDa molecular-weight-cutoff devices  
136 (Omega disc membrane, Pall Filtron). Concentrated samples were run through a 104 ml  
137 Superdex SD200 column (Amersham Biosciences) equilibrated with buffer B (100 mM Tris-  
138 HCl [pH 8], 150 mM NaCl, 1 mM DTT). Protein was stored as pellets in liquid nitrogen.

139

140 **Purification of *strep*Fnr.** Cells from a 6 L culture were harvested by centrifugation at 10,000g  
141 for 15 min, resuspended in 120 ml of buffer C (25 mM Tris-HCl [pH 8], 1 mM DTT) and  
142 incubated with 0.2 mg.ml<sup>-1</sup> of lysozyme and 0.5 mM EDTA for 10 min at 30 °C. Cells were  
143 lysed by sonication as described above for the purification of Fnr<sub>His</sub>. Cell debris were  
144 removed by centrifugation at 43,000g for 1 h and the resulting supernatant was run through a  
145 30 ml DEAE-cellulose column (DE52; Whatman) equilibrated with buffer C. The column  
146 was then washed with the same buffer. Non-retained fractions were adjusted to pH 7 with 1M  
147 KH<sub>2</sub>PO<sub>4</sub> and run through a 30 ml hydroxyapatite agarose column (HA Ultrogel; Pall  
148 Corporation) equilibrated with buffer D (50 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7], 1 mM DTT). The column  
149 was developed with a linear gradient from 50 to 200 mM KH<sub>2</sub>PO<sub>4</sub> at a flow rate of 2 ml/min.  
150 Fractions containing recombinant Fnr were pooled and concentrated to 48 mg. ml<sup>-1</sup> by  
151 ultrafiltration through an Omega disc membrane (30 kDa cut-off, Ø 43 mm, Pall Filtron). A  
152 polishing step was then carried out with gel filtration on a 104 ml Superdex SD200 column  
153 (Amersham Biosciences) equilibrated with buffer D containing 150 mM NaCl. The purified  
154 protein was stored as pellets in liquid nitrogen.

155 **Protein biochemical analyses.** Protein concentrations were determined by either a BCA  
156 (bicinchoninic acid) assay according to the manufacturer's instructions (Interchim) or a Biuret  
157 method insensitive to thiols (22). Bovine serum albumin (BSA) was used as standard.  
158 Overproduction of Fnr in induced cultures and its purification were monitored by SDS-  
159 PAGE. The Laemmli method was used for SDS-PAGE (18). Proteins were stained with  
160 Coomassie brilliant blue. Reducing agent β-mercaptoethanol was omitted when analysing the  
161 disulphide form of apoFnr. The molecular mass of apoFnr was accurately measured with an  
162 Esquire 3000plus ion trap mass spectrometer equipped with a nanoelectrospray on-line ion



163 source (Bruker Daltonics) essentially as described in (6). Before mass measurement, purified  
164 apoFnr was desalted with a ZipTip<sub>C18</sub> (Millipore), and diluted in acetonitrile/formic acid  
165 (50%/1%, v/v).

166

167 **Dynamic Light Scattering (DLS).** The quaternary structure of purified apoFnr in solution  
168 was measured by DLS. Samples were centrifuged and run through a 24 mL Superdex 200  
169 column (HR10/30) equilibrated and run at a flow rate of 0.5 ml/min with 50 mM TRIS/HCl  
170 pH 8.3 containing 120 mM NaCl and 0.05% NaN<sub>3</sub> filtered at 0.1 μm. The column was  
171 operated with an Agilent 1100 Series reverse-phase high-performance liquid chromatography  
172 (HPLC) system equipped with G1322A degasser, G1311A quaternary pump, and G1313A  
173 autosampler. The elution profile was monitored with a G1315B diode array detector  
174 (Agilent), a miniDawn Tristar Multi-angle laser Static Light Scattering detector (three angles,  
175 45°, 90° and 135°) coupled to a DynaPro Titan Light Scattering Instrument (Wyatt  
176 technology) placed at 90° and an Optilab rEX differential refractometer (Wyatt technology).  
177 The 90° MALS detector was calibrated with pure toluene, and BSA was then used to  
178 normalize the other detector (45° and 135°) in the corresponding buffer.

179

180 **Chemical cross-linking of Fnr<sub>His</sub>.** Fnr<sub>His</sub> in 10 mM 3-(N-morpholino) propanesulfonic acid  
181 (MOPS) buffer (pH 7.75) was treated with protein crosslinking agents, *N*-  
182 hydroxysulfosuccinimide (5 mM) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide  
183 hydrochloride (EDC;12.5 mM). The reaction mixture contained protein at a concentration of 5  
184 μM in a total reaction volume of 20 μl. The reaction was allowed to proceed for 30 min at  
185 room temperature and stopped by adding 25 mM β-mercaptoethanol. The products were  
186 analyzed by 12% non-denaturing SDS-PAGE and detected by Western blot using an anti-His  
187 antibody.

188

189 **ApoFnr antiserum preparation.** Polyclonal antibodies against apoFnr were generated in-  
190 house. Rabbits were immunized with a total of 2 mg of purified Fnr<sub>His</sub>, administered in four  
191 equal doses over a 90-day period, and bled on Day 120. Antisera specificities were checked  
192 by Western blot.

193

194 **Western blot analysis.** *B. cereus* protein extracts were prepared as follows: cells were  
195 harvested by centrifugation, resuspended in buffer containing 8 M urea, 4% (w/v) CHAPS  
196 ([3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonate]), and mechanically disrupted  
197 using a FastPrep instrument (FP120, Bio101, Thermo Electron Corporation). Cell debris were  
198 removed by centrifugation (3,500g, 10 min, 4 °C). Proteins were then filtered and resolved by  
199 SDS-PAGE under non-reducing conditions (18). Resolved proteins were transferred to  
200 nitrocellulose membranes (Amersham Bioscience) in a Biorad liquid/liquid transfer unit. As  
201 appropriate, ApoFnr was detected with either anti-His antibodies (Fnr<sub>His</sub>) or anti-Strep  
202 antibodies (Strep<sub>Fnr</sub>), or with 1:2000 dilution of polyclonal rabbit serum. The blotted  
203 membranes were developed with a 1:2000 dilution of peroxidase-conjugated goat anti-rabbit  
204 IgG (Sigma) and an enhanced chemiluminescence substrate (Immobilon Western, Millipore).

205

206 **Electrophoretic Mobility Shift Assay (EMSA).** The 5' untranslated regions (UTR) of *fnr*,  
207 *resDE*, *plcR*, *hbl*, and *nhe* were PCR amplified with the following primer pairs: FnrF (5'-  
208 CGAACACTTCAGCAGGCATA-3') and FnrR (5'-AATGTCATACTGTTTGCCAC-3'),  
209 ResDF (5'-TGGGATCCCAAAGAGGTTTG-3') and ResDR (5'-CGATCC  
210 TCATCATCTACAAT-3'), PlcRF (5'-TATGTTTGTGCAAGGCGAAC-3') and PlcRR (5'-  
211 CCTAATTTTTCTGCGTGCAT-3'), Hbl1F (5'-GGTAAGCAAGTGGGTGAAGC-3') and  
212 Hbl1R (5'-AATCGCAAATGCAGAGCACAA-3'), Hbl2F (5'-

213 TTAACTTAATTCATATAACTT-3') and Hbl2R (5'-TACGCATTA AAAAATTTAAT-3'),  
214 NheF (5'-TGTTATTACGACAGTTCCAT-3') and NheR (5'-  
215 CTGTAACCAATAACCCTGTG-3'), respectively. The forward primers were 5' end labelled  
216 with T4 polynucleotide kinase (Promega) and [ $\gamma$ - $^{32}$ P]ATP (Amersham Biosciences). The 5'-  
217  $^{32}$ P labelled amplicons were purified using High Pure PCR Product Purification columns  
218 (Roche). EMSAs were performed by incubating labelled DNA fragments (1000 cpm per  
219 reaction) with the specified amount of purified Fnr in 50 mM Tris-HCl [pH 7.5] buffer  
220 containing 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 4 mM dithiothreitol, 4 mM MgCl<sub>2</sub>, 0.5  
221  $\mu$ g of bovine serum albumin and 1  $\mu$ g of poly(dI-dC)/ml in a final volume of 10  $\mu$ l. Binding  
222 reactions were incubated for 30 min at 37°C and then loaded onto a 4% or 6% non-denaturing  
223 polyacrylamide gel run with Tris-borate-EDTA buffer at 4 °C and 200 V. Labelled products  
224 were quantified using a Molecular Dynamics PhosphoImager.

225

## 226 **RESULTS**

227

### 228 **Overexpression and purification of two recombinant Fnr proteins**

229 From the sequence alignment of 13 *B. cereus* Fnr homologues (see Fig. S1 in  
230 supplementary data), two possible alternative translation initiation starts could be identified  
231 for *B. cereus* F4430/73: GTG as previously defined (34) or ATG, 12 nucleotides further on.  
232 Taking into account this information, two procedures were developed for the aerobic  
233 production of *B. cereus* F4430/73 Fnr in *E. coli* cells: (i) expression of a His-tag fusion  
234 protein from pET101/D-TOPO to release a Fnr variant (Fnr<sub>His</sub>) that begins with the valine  
235 encoded by the predicted start codon of *B. cereus* (34) and contains 30 additional amino acids  
236 at the C-terminal end, and (ii) expression of a Strep-Tag fusion from pET-52b(+) to release an  
237 Fnr variant (Fnr<sub>Strep</sub>) that contains 22 additional amino acids at the N-terminal end. In the

238 latter variant, the first amino acid of the native Fnr is the methionine located 4 amino acids  
239 after the valine encoded by the predicted start codon (see Fig. S1 in supplementary data). The  
240 Fnr<sub>His</sub> protein was purified using cobalt affinity chromatography. Because preliminary tests  
241 indicated that <sub>Strep</sub>Fnr bound very weakly to Strep-tactin sepharose (IBA), the tagged protein  
242 was purified by means of three successive chromatography runs not based on the tag affinity.  
243 On SDS-PAGE, purified Fnr<sub>His</sub> (29 kDa) and <sub>Strep</sub>Fnr (28 kDa) exhibited the expected  
244 molecular masses (See Fig. S2 in supplementary data). The exact average molecular mass of  
245 <sub>Strep</sub>Fnr determined by mass spectrometry was  $27,913 \pm 2$  Da. This value corresponds almost  
246 perfectly (75 ppm deviation) to the expected polypeptide sequence, except that the initial  
247 formyl-methionine is cleaved (theoretical value: 27,911 Da). This maturation probably also  
248 occurs with the Fnr<sub>His</sub> protein.

249 UV-visible absorption spectrum of both aerobic recombinant forms of Fnr showed a  
250 single peak at 280 nm (data not shown), suggesting that there is no absorbing prosthetic group  
251 (14). This indicates that both recombinant Fnr forms were purified as apoproteins under  
252 aerobiosis.

253

#### 254 **Oligomeric state of both recombinant apoFnr proteins.**

255 DLS was used to examine the oligomeric state of the two recombinant forms of  
256 apoFnr. DLS reveals the homogeneity and oligomeric state of proteins when resolved by gel  
257 filtration based on the scattering of visible light by particles (5). The oligomerization states of  
258 Fnr<sub>His</sub> and <sub>Strep</sub>Fnr were analyzed using a DynaPro Titan DLS instrument and attendant  
259 software ASTRA. Figure 1 shows the elution profile obtained for both proteins and the  
260 molecular mass estimates derived from the light scattering signal. Besides a peak of  
261 aggregates at 16 min, Fnr<sub>His</sub> was resolved into four elution peaks at 22.0 (A1), 26.6 (A2), 28.8  
262 (A3) and 30.7 (A4) min elution time as detected on the UV trace (Fig. 1A). The molar mass

263 across peak A1 could not be determined because of a polydisperse distribution (the molecular  
264 mass varied from 170 to 400 kDa). This strongly suggests that this peak contained aggregates  
265 that interacted with the column, but their proportion was low, as the DLS signal was very  
266 weak. In contrast, the distribution of molar masses across peaks A2, A3 and A4 was constant,  
267 indicating a monodisperse distribution (*i.e.* a homogeneous molecule) for each peak with  
268 molecular masses of 98 (A2), 60 (A3) and 30 (A4) kDa (+/- 3%), respectively. This indicates  
269 that Fnr<sub>His</sub> occurs mainly as a mixture of trimer, dimer and monomer in solution. Considering  
270 the relative mass ratio that can be estimated from the UV trace, the predominant form was the  
271 monomer (70%). The light scattering trace obtained with <sub>Strep</sub>Fnr showed the presence of  
272 aggregates (peak B1) and three peaks with molecular masses of 157 (B2), 106 (B3) and 54  
273 (B4) kDa (+/- 3%), respectively (Fig. 1B). These peaks unambiguously correspond to the  
274 hexameric, tetrameric and dimeric forms of <sub>Strep</sub>Fnr, respectively. In this case, the dimeric  
275 form (33%) formed the largest population. The same DLS experiment was repeated in  
276 reducing conditions with 10 mM DTT in the elution buffer. Complete disappearance of the  
277 hexameric form and almost complete disappearance of the tetrameric form (C1) were  
278 observed (Fig. 1C). The dimeric form (C2) was thus predominant (89%). Hence the addition  
279 of reductant affected the oligomerization state of <sub>Strep</sub>Fnr in solution. Intermolecular  
280 disulphide bridges are involved in the formation of the highest oligomeric forms. In addition,  
281 the absence of monomers in reducing conditions suggests that the dimers observed were  
282 either non-covalently linked structures or DTT-resistant covalently linked structures.

283         When purified <sub>Strep</sub>Fnr underwent SDS-PAGE in non-reducing conditions (no DTT or  
284  $\beta$ -ME) a multiple-band pattern was observed, revealing the presence of a mixed population of  
285 monomer, dimer and higher oligomeric forms in relative ratios compatible with those found in  
286 a DLS experiment (Fig. 2A). In reducing conditions (with DTT), the two major species were  
287 the monomeric and the dimeric forms. Increasing the concentration of DTT from 10 mM to

288 200 mM caused the total reduction of dimeric species to monomeric forms. These data  
289 indicate that most of the protein was reticulated through disulphide bridges, but a significant  
290 amount of dimeric  $\text{StrepFnr}$  could either not be completely reduced by DTT or remained  
291 particularly stable in the electrophoresis conditions used. In contrast, only a very small  
292 fraction of  $\text{Fnr}_{\text{His}}$  was found to remain dimeric after 10 mM DTT treatment (Fig. 2B). This  
293 suggests that the oligomeric  $\text{Fnr}_{\text{His}}$  population detected by DLS contained mainly non-  
294 covalently linked structures. To investigate the ability of monomeric  $\text{Fnr}_{\text{His}}$  to form covalently  
295 linked structures, a fraction of the purified protein was treated with either the chemical cross-  
296 linker EDC or with the divalent thiol-reactive agent diamide. The first cross-linker modifies  
297 an ionic interaction into a covalent link, while the latter mimics disulphide bridge formation.  
298 Figure 2 shows the reaction products analyzed by SDS-PAGE. Formation of oligomers from  
299 monomer could be evidenced using both EDC (Panel C) and diamide (Panel D). Homodimers  
300 and homotrimers were the major products. As expected when using crosslinkers, these entities  
301 migrated at relative molecular weights slightly lower than the exact weights because of their  
302 more rigid structures. Surprisingly, the band corresponding to apoFnr monomer appeared as a  
303 discrete doublet after treatment with diamide, reflecting a possible induced conformational  
304 change trapped by intrapolypeptide crosslinks (Fig. 2D). In conclusion,  $\text{Fnr}_{\text{His}}$  monomers were  
305 able to self-associate and form higher-order covalently linked structures in the presence of  
306 cross-linkers. This suggests that unlike  $\text{StrepFnr}$ ,  $\text{Fnr}_{\text{His}}$  does not tend to form covalently linked  
307 homodimers or, more specifically, intermolecular disulphide bridges.

308

### 309 **Detection of endogeneous apoFnr in *B. cereus* F4430/73 cells.**

310 To determine whether the formation of disulphide-linked homodimers might be of  
311 physiological relevance, we tested the presence of various forms of endogenous apoFnr in  
312 aerobically grown *B. cereus* cells (4). Figure 3 shows the Western blot detection performed

313 with apoFnr antiserum following SDS-PAGE under non-reducing conditions. The antiserum  
314 reacted with two bands of the sizes expected for the monomeric (~30 kDa) and dimeric forms  
315 (~60 kDa) of apoFnr in wild-type cells, but not in *fnr* mutant cells. Two other protein bands of  
316 40 and 80 kDa cross-reacted with apoFnr antiserum in wild-type cells (Fig. 3, lane 3). As  
317 these bands were also observed in the *fnr* mutant cells (Fig. 3, lane 2), they were not related to  
318 Fnr. Finally, these results indicated that the apoFnr antiserum can be used efficiently for the  
319 detection of endogeneous apoFnr in *B. cereus* F4430/73 cells and, more importantly, that  
320 some dimeric apoFnr could be disulphide-linked in *B. cereus*

321

### 322 **Binding of apoFnr to the 5' untranslated regions of *fnr*, *resDE*, *plcR*, *hbl* and *nhe***

323 The amino acid residues forming the REX<sub>3</sub>R motif within the HTH DNA-binding  
324 domain of Crp regulatory proteins are strictly conserved in the potential DNA-binding domain  
325 of *B. cereus* F4430/73 Fnr as in its homologues found in strains belonging to *B. cereus* group  
326 (12). Accordingly, Fnr of *B. cereus* F4430/73 was assumed to bind to DNA motifs similar to  
327 the TGTGA-N6-TCACA consensus defined in previous work (2, 16). Using the Virtual tool  
328 of the ProDoric database and the corresponding *E. coli* Crp position weight matrix, we  
329 scanned the 5'untranslated regions (UTR) of regulatory and structural genes of *B. cereus*  
330 F4430/73 enterotoxins. Figure 4A shows the locations of predicted Fnr binding boxes for *fnr*,  
331 *resDE*, *plcR*, *nhe*, *hbl1* and *hbl2* promoters and their positions relative to the transcriptional  
332 start point of each gene/operon. Except for *resDE* (Fig. 4B), the transcriptional start sites were  
333 identified in previous studies (1, 4). Three putative Fnr binding sites were found in the 5'UTR  
334 of the enterotoxin gene regulators *fnr*, *resDE* and *plcR*. Eight potential Fnr binding sites were  
335 found in the *nhe* promoter region: four were located upstream of the transcriptional start site,  
336 and four downstream. The *hbl* promoter region contained eleven potential Fnr binding sites,  
337 four located upstream of the +1 site and seven downstream.

338 To test whether apoFnr bound to the Fnr boxes predicted from the nucleotide sequence  
339 analysis, EMSAs were performed with both  $_{\text{Strep}}\text{Fnr}$  and  $\text{Fnr}_{\text{His}}$  and DNA fragments containing  
340 5'UTR of *fnr*, *resDE*, *plcR*, *hbl* and *nhe*. In view of its size (1157 bp), the 5'UTR of *hbl* was  
341 first divided into two overlapping fragments of 636 pb (*hbl1*) and 610 pb (*hbl2*), respectively,  
342 as defined in Fig. 4A. Figure 5 shows the EMSA results for the six fragments.  $\text{Fnr}_{\text{His}}$  bound to  
343 all the regions tested, while no DNA-binding activity could be detected with  $_{\text{Strep}}\text{Fnr}$ . The  
344 specificity of the binding was evidenced from the disappearance of complexes in competition  
345 assays using 50-fold excess of homologous unlabelled promoter regions and by the absence of  
346 any competition when an unlabelled heterologous DNA was used (data not shown). EMSAs  
347 in the negative control (Fig. 5G) showed that a shift above 6  $\mu\text{M}$  apoFnr should be considered  
348 as the result of non-specific binding. In addition, the behaviour of apoFnr markedly differed  
349 in the gel-shift titration assay depending on the promoter regions. ApoFnr bound to *fnr* and  
350 *resDE* promoter regions in an ordered fashion giving two retarded species (complex I and II)  
351 below 6  $\mu\text{M}$ . In contrast, an increasing amount of apoFnr resulted in a gradual decrease in the  
352 mobility of the protein-DNA complexes for *plcR*, *hbl* and *nhe* promoter regions, which  
353 appeared to be stabilized at higher protein concentrations. This suggests that, as more protein  
354 was added, the protein complex bound to the DNA increased proportionally in size, with the  
355 added apoFnr being distributed evenly among all the complexes. The smearing of these  
356 species during EMSA also suggested that these high molecular complexes were not stable and  
357 dissociated during electrophoresis. The EMSA data also showed that the *plcR*, *hbl* and *nhe*  
358 5'UTR were bound by apoFnr with a lower affinity ( $K_D \leq 0.4 \mu\text{M}$ ) than the *resDE* and *fnr*  
359 promoter regions ( $K_D = 3$  and  $4.5 \mu\text{M}$ , respectively).

360 To test whether the oligomeric state regulated the DNA-binding activity of both  $\text{Fnr}_{\text{His}}$   
361 and  $_{\text{Strep}}\text{Fnr}$ , the effect of the reducing agent DTT (200 mM) on the binding of  $_{\text{Strep}}\text{Fnr}$  and the  
362 effect of the oxidizing agent diamide (1 mM) on the binding of  $\text{Fnr}_{\text{His}}$  to all promoter regions



363 were investigated. Adding reductant resulted in the generation of  $_{\text{Strep}}\text{Fnr}$  -DNA complex  
364 patterns similar to those obtained with  $\text{Fnr}_{\text{His}}$  (Fig. 5). The effect of DTT was reversible,  
365 addition of diamide (1 mM) abolishing  $_{\text{Strep}}\text{Fnr}$  binding (data not shown). Likewise,  $\text{Fnr}_{\text{His}}$   
366 showed no DNA-binding activity in the presence of diamide (data not shown). Thus the  
367 oligomeric state of apoFnr was found to critically affect its binding activity. The data also  
368 indicate that apoFnr was able to bind the *fnr*, *resDE*, *plcR*, *hbl* and *nhe* 5'UTR regions only  
369 when present predominantly as a monomer.

370

## 371 **Discussion**

372 Our previous studies showed that aerobic enterotoxin expression was regulated by  
373 both the transcriptional regulator Fnr and oxygen availability (or redox state) under aerobiosis  
374 (4, 33). In the present work, we describe experimental evidence for redox regulation of  
375 enterotoxin gene expression mediated by Fnr through its DNA binding properties.

376 *B. cereus* apoFnr was overexpressed in *E. coli* and purified as either a C-terminal His-  
377 tagged ( $\text{Fnr}_{\text{His}}$ ) or an N-terminal Strep-tagged ( $_{\text{Strep}}\text{Fnr}$ ) fusion protein. Unlike  $\text{Fnr}_{\text{His}}$ ,  $_{\text{Strep}}\text{Fnr}$   
378 was purified without affinity chromatography step. The reason was the poor affinity of Strep  
379 tag peptide for streptavidin (strepTactin) due to its fusion to the N-terminus of Fnr (19). No  
380 such problem was encountered in the case of Strep-tagged *B. subtilis* Fnr (27). This different  
381 behavior may be explained by the marked difference in the two N-terminal polypeptide  
382 sequences. Both recombinant Fnr ( $\text{Fnr}_{\text{His}}$  and  $_{\text{Strep}}\text{Fnr}$ ) were produced in multiple oligomeric  
383 apoforms. The distribution of quaternary structures was shown to differ between the two  
384 tagged variants. Purified  $\text{Fnr}_{\text{His}}$  was predominantly monomeric, while  $_{\text{Strep}}\text{Fnr}$  was  
385 predominantly oligomeric, the oligomerization of  $_{\text{Strep}}\text{Fnr}$  appearing to be due to the  
386 formation of disulphide bridges. Data obtained from crystal structure analysis of a member  
387 of the Crp/Fnr family showed that dimerization involved the C-terminal domain (13). This

388 suggests that extension of *B. cereus* Fnr at its C-terminus may introduce steric hindrance that  
389 reduces flexibility and (or) affects interdomain communication. In turn, this would result in  
390 a less permissive, locked conformation, rendering the thiol group less exposed for pairing to  
391 form the disulphide bond.

392 Our results showed that the active DNA-binding form of both recombinant apoFnrs  
393 was the monomer. Diamide treatment inactivated monomeric apoFnr in a DTT-reversible  
394 manner, suggesting that it was subject to redox regulation. In addition, we detected the  
395 presence of disulfide-linked endogeneous dimers in *B. cereus* cells. Taken together, these  
396 findings suggest that formation of stabilized dimeric apoFnr by means of one or more SS  
397 bonds may be a regulatory mechanism that controls Fnr binding under exposure to oxidizing  
398 conditions. Figure 6 shows the scheme we propose for the reversible activation/inactivation  
399 of *B. cereus* apoFnr. It implies that this protein mediates a response to oxygen concentration  
400 and (or) redox state causing the repression or activation of relevant genes. Such a thiol-based  
401 redox switch has been observed with *Desulfitobacterium dehalogenans* CrpK, a member of  
402 the Crp/Fnr family (24, 25). In this bacterium, the redox switch involves formation of an  
403 intermolecular disulphide bond that links two CprK subunits in an inactive dimer. Although  
404 it belongs to the same family, *B. cereus* Fnr contains three more cysteines than CprK and  
405 should have the capacity to bind a FeS cluster like *B. subtilis* Fnr (27). For this reason, our  
406 findings are original. Additional work is now required to determine which of the seven  
407 cysteine residues are involved in this redox state sensing.

408 Many transcription factors bind DNA to form dimeric protein-DNA complexes. For  
409 these proteins, there are two limiting pathways that can describe the route of complex  
410 assembly. The protein can dimerize first, and then associate with DNA (dimer pathway), or  
411 can follow a pathway in which two monomers bind DNA sequentially and assemble their  
412 dimerization interface while bound to DNA (monomer pathway) (15). Many regulators bind

413 DNA by the dimer pathway, and this is the case for Fnr of *B. subtilis* and *E. coli* under  
414 anaerobiosis (27). Under aerobiosis, apoFnr is produced as an inactive monomer in *E. coli*  
415 (28) and as an inactive dimer in *B. subtilis* (27). Because only the monomeric form of  
416 *B. cereus* apoFnr binds to DNA, we propose that Fnr binding in *B. cereus* occurs via the  
417 monomer pathway under aerobiosis (Fig. 6). Binding through the monomer pathway allows a  
418 dimeric transcription factor to respond rapidly to stimuli and to locate its target site quickly  
419 without becoming entrapped kinetically at a non-specific site (23). Therefore, in addition to a  
420 faster assembly of apoFnr-DNA complexes in response to oxygen tension in the environment  
421 allowed by the monomer pathway, an efficient way to discriminate between specific and non-  
422 specific target sites is also provided.

423         Since apoFnr bound to the promoter regions of *fnr* itself, the two-component system  
424 *resDE*, the virulence regulator *plcR* and the enterotoxin genes *hbl* and *nhe*, we concluded that  
425 apoFnr directly controlled both its own expression and that of *resDE*, *plcR*, *hbl* and *nhe* (34).  
426 The relatively low DNA binding affinity observed for apoFnr suggests that other factors may  
427 be involved in DNA recognition as well as in protein-DNA complex stabilization (16). For  
428 example, it is conceivable that apoFnr operates with a specific oxidoreductase system or that  
429 for some other reason the cytoplasmic environment provided by *B. cereus* enhances its site-  
430 specific DNA binding ability. In addition, interaction of apoFnr with one or more other  
431 regulatory proteins may facilitate its interaction with DNA. High affinity binding to 5'UTR  
432 regions of enterotoxin genes may require apoFnr-PlcR interaction insofar as PlcR (1)  
433 possesses binding sites close to the predicted Fnr binding sites (Fig. 4A). Another possible  
434 interaction partner of apoFnr is the redox regulator ResD (4).

435         Transcriptional regulators such as members of the Crp/Fnr family interact with the  $\alpha$   
436 subunit of RNA polymerase (RNAP) (10). It has been shown that the protein-protein  
437 interaction increases the affinity of both partners to the promoter site (2). The contacts

438 established between a Crp/Fnr protein and RNAP involve three patches of surface-exposed  
439 amino acids (called activating regions 1, 2, and 3) of Crp/Fnr protein. These contacts depend  
440 on the specific architecture of each promoters. The Crp/Fnr -dependent promoters can be  
441 grouped into three classes (labelled I, II, and III) based on the number and position of the  
442 Crp/Fnr binding sites relative to the start of transcription, and on the mechanism for  
443 transcription activation (2). The upstream DNA binding site in class I promoters is centred  
444 either at position  $-61.5$  (*i.e.*, its axis of symmetry is between positions  $-61$  and  $-62$ ) or one to  
445 three helical turns further upstream (*i.e.*,  $-71.5$ ,  $-82.5$ , or  $-92.5$ ). In class II promoters, the  
446 symmetry axis of the binding site is located at position  $-41.5$  relative to the transcription start  
447 site, thus overlapping with the  $-35$  region. Class III promoters comprise two or more DNA-  
448 binding sites for Crp/Fnr and have various architectures according to both the spacing  
449 between the DNA binding sites and the distance between the Crp/Fnr and the RNAP-DNA  
450 binding sites. In the case of *B. cereus*, the location of predicted Crp/Fnr binding sites  
451 upstream of the transcriptional start site suggests that the *B. cereus fnr* promoter region is a  
452 class I activating promoter, while *resDE* and *plcR* promoter regions are class II promoters.  
453 The *nhe* and *hbl* promoters are different and may be considered as class III Crp/Fnr-dependent  
454 activated promoters. However, *nhe*, *hbl*, and to a lesser extent *fnr*, *resDE* and *plcR* promoter  
455 regions, also contain predicted Crp/Fnr boxes located close to the  $-10$  region and (or)  
456 downstream of the transcriptional start site *i.e* at positions different from those found in  
457 classical Crp/Fnr activated promoters. Comparable results were found for *E. coli* and *B.*  
458 *subtilis* Fnr (9, 27), where repression of transcription is mediated by Fnr binding to sites  
459 differently located compared with activate sites. Thus we hypothesize that the regulation of  
460 enterotoxin gene expression involves an interplay of transcriptional activation and repression  
461 by Fnr. Repression may be mediated by occupancy of sites located downstream of the  $+1$  site.  
462 In conclusion, the mechanism of Fnr-dependent regulation of enterotoxin in *B. cereus* is

463 undoubtedly complex, and further extensive studies are required to examine the essential role  
464 of the downstream binding sites. Importantly, both *hbl* and *nhe* promoters have a long UTR  
465 (Fig. 4A), making it likely that mechanisms at the post-transcriptional level also control their  
466 expression. Such regulation could involve interaction between transcriptional regulator and  
467 ribosomal proteins (8). Finally, deciphering the complexities of this Fnr-dependent regulation  
468 is necessary to fully understand the mechanisms employed by *B. cereus* to ensure optimal  
469 virulence gene expression in response to changes in oxygen tension such as those encountered  
470 during infection in a human host.

471         In conclusion, this work shows that unlike its homolog in *B. subtilis* (12, 27), *B. cereus*  
472 Fnr is able to function as a transcriptional factor independently of the integrity of the FeS  
473 cluster. Thus *B. cereus* Fnr illustrates the great versatility of the archetypal Crp/Fnr structure  
474 for transducing environmental signals to the transcriptional apparatus. More importantly, this  
475 study expands our knowledge of the molecular mechanisms used in *B. cereus* to modulate the  
476 transcriptional level of enterotoxin genes in response to redox variations.

477

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484

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582

583

584 FIGURE LEGEND

585

586 **Figure 1: Gel filtration and DLS chromatograms of purified Fnr proteins.** Fnr<sub>His</sub> (Panel  
587 A), Fnr<sub>Strep</sub> (Panel B) and reduced Fnr<sub>Strep</sub> (Panel C) were injected (~300 µg in 100 µl) into a  
588 Superdex 200 column (HR 10/30) with TRIS-HCl 50 mM (pH 8.3), NaCl 120 mM as eluant  
589 at a flow rate of 0.5 ml/min. DTT (10 mM) was added to the elution buffer to determine the  
590 oligomeric state of reduced Fnr<sub>Strep</sub> (Panel C). The black and grey lines correspond to the light  
591 scattering (LS) signal and the UV signal recorded at 280 nm, respectively. These signals were  
592 normalized as 0-1 ratio for comparison (left axis). The molecular weight estimates of the  
593 major peaks are also indicated in dashed lines (right axis).

594

595 **Figure 2: SDS-PAGE analysis of the oligomeric nature of <sub>Strep</sub>Fnr and Fnr<sub>His</sub>.** Panels A  
596 and B: effect of DTT on <sub>Strep</sub>Fnr (Panel A) and Fnr<sub>His</sub> (Panel B) oligomerization. Purified  
597 proteins were incubated with 0, 10, 50, 100 or 200 mM DTT (lanes 2-6, respectively).  
598 Recombinant proteins were then subjected to non-reducing SDS-PAGE. The arrows indicate  
599 monomers (m), dimers (d) and higher oligomers (o). Standard proteins (Lane 1) are shown.  
600 Panel C SDS-PAGE profile of Fnr<sub>His</sub> cross-linked with EDC. Fnr<sub>His</sub> (5 µM) was cross-linked  
601 with EDC . Products were visualized by immunoblotting with anti-His antibody. Lane 1,  
602 cross-linked Fnr<sub>His</sub>. Lane 2, untreated Fnr<sub>His</sub>. Panel D: Non-denaturing SDS-PAGE profile of  
603 Fnr<sub>His</sub> cross-linked with diamide. Lane 1, standard proteins. Lane 2, untreated Fnr<sub>His</sub>; Lanes 3  
604 & 4, disulphide linked Fnr<sub>His</sub> with 1 mM and 10 mM diamide, respectively. The arrows  
605 indicate monomers (m), dimers (d), trimers (t) and higher oligomers (o).

606

607 **Figure 3: Western blot detection of endogeneous Fnr species from *B. cereus* cells.** Lysates  
608 of *B. cereus* F4430/73 wild-type (wt) and *fnr* mutant were probed with polyclonal Fnr

609 antiserum. Both strains were grown in regulated batch culture (pH 7.2) under aerobiosis (4).  
610 Proteins were separated by non-reducing SDS-PAGE. Lane: 1, Fnr<sub>Strep</sub> purified from *E. coli*.  
611 Lane 2, *fnr* mutant. Lane 3, Wild-type strain. Putative identities shown on the right were  
612 determined for the wild-type strain on the basis of results obtained with both recombinant Fnr  
613 and *fnr* mutant strains. The arrows indicate monomer (m) and dimer (d) forms. The position  
614 and mass (kDa) of molecular weight marker are given in the left.

615

616 **Figure 4: Potential Fnr binding sites in the 5' untranslated regions of *fnr*, *resDE*, *plcR*,**  
617 ***hbl*, and *nhe*.** All numbering is relative to the transcription start site at position +1. Panel A:  
618 potential Fnr binding sites are shown relative to the transcription start site as grey boxes. PlcR  
619 boxes are highlighted by dark boxes. Panel B: genetic organization of the *resDE* promoter  
620 region. The transcriptional start site (+1) determined by 5'-RACE PCR is in bold. The  
621 putative -35 and -10 motifs are underlined. Putative Crp/Fnr boxes are indicated by a grey  
622 background.

623

624 **Figure 5: Binding of apoFnr to 5'UTR regions of *fnr*, *resDE*, *plcR*, *hbl*, and *nhe* genes**  
625 **determined by EMSA.** DNA corresponding to *fnr* (A), *resDE* (B), *plcR* (C), *hbl1* (D), *hbl2*  
626 (E), *nhe* (F) and a negative control (G) were bound with increasing concentrations of apoFnr  
627 as indicated. The results presented are representative examples of an experiment performed in  
628 triplicate with either purified Fnr<sub>His</sub> or with reduced <sub>Strep</sub>Fnr (purified <sub>Strep</sub>Fnr + 200 mM DTT).  
629 Lanes 1 to 10: 0, 0.2, 0.4, 0.6, 0.8, 1, 2, 3, 4, 5, and 6  $\mu$ M of protein, respectively.

630

631 **Figure 6: Proposal for the regulation of apoFnr activity by a thiol-disulphide redox**  
632 **switch.** Brackets indicate that one or more disulphide bonds may be involved.

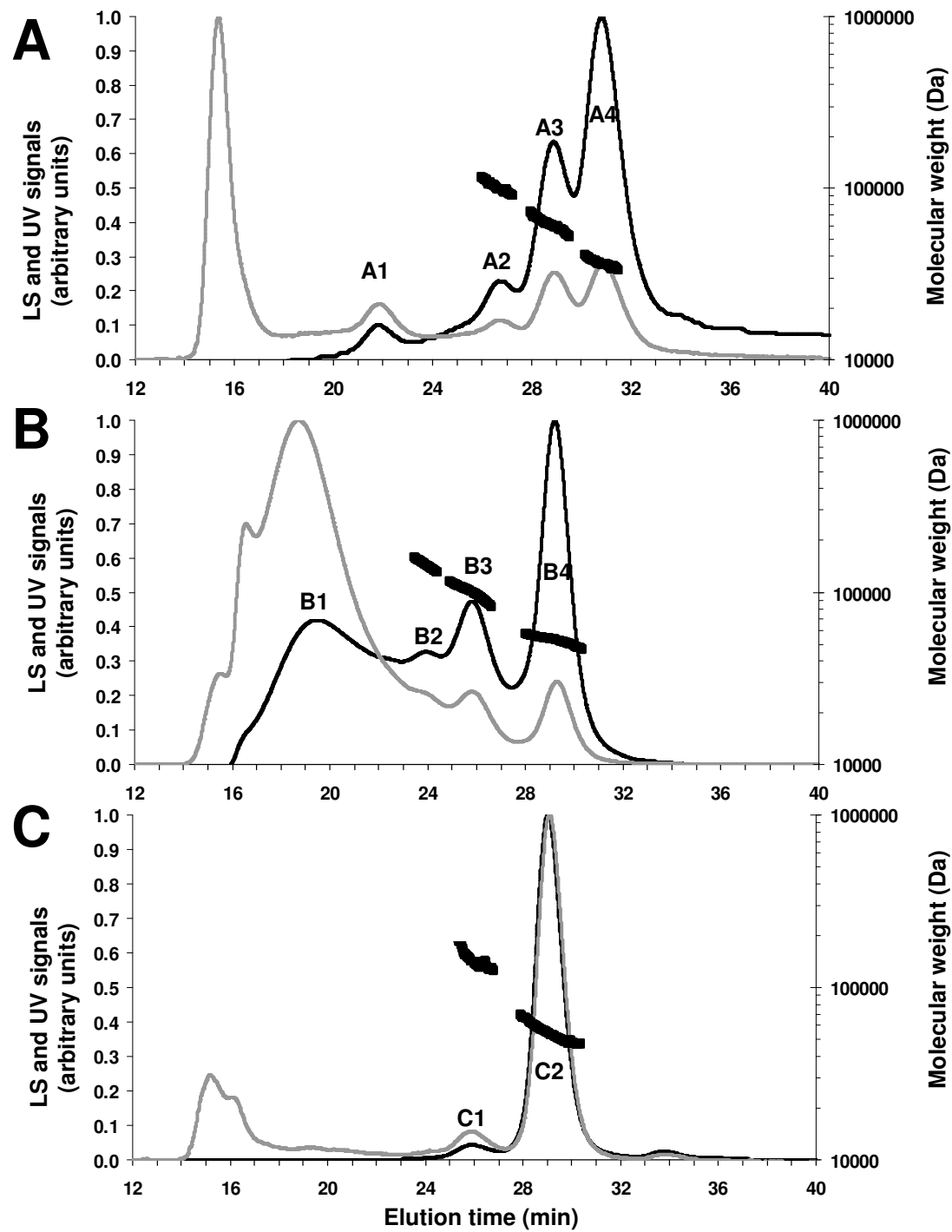


Figure 1

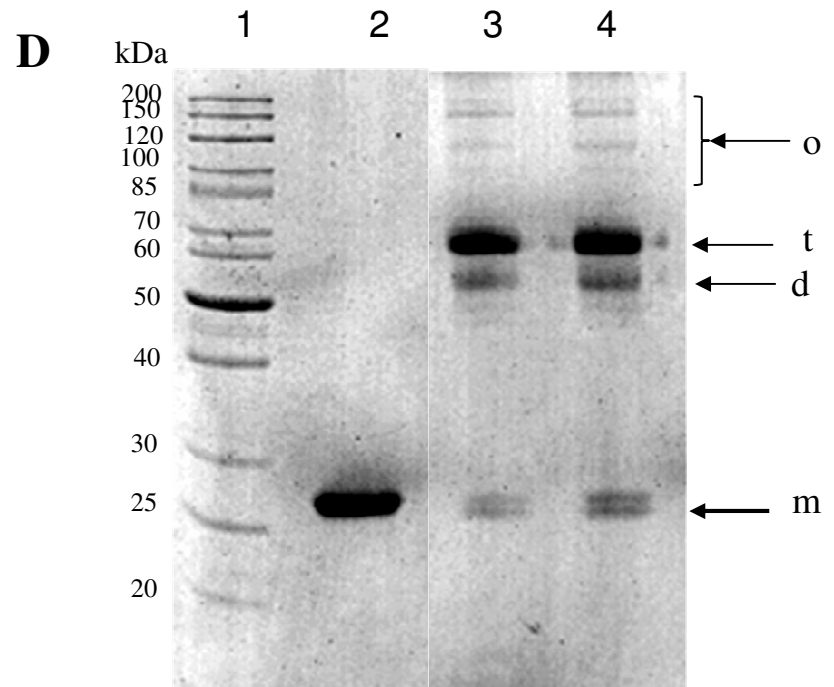
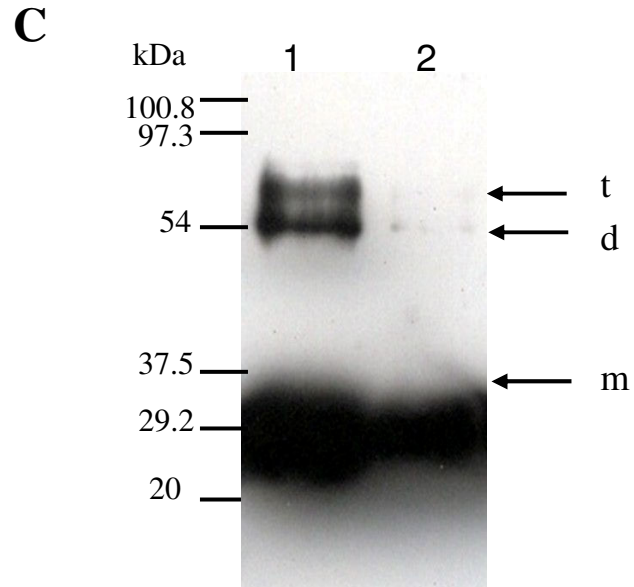
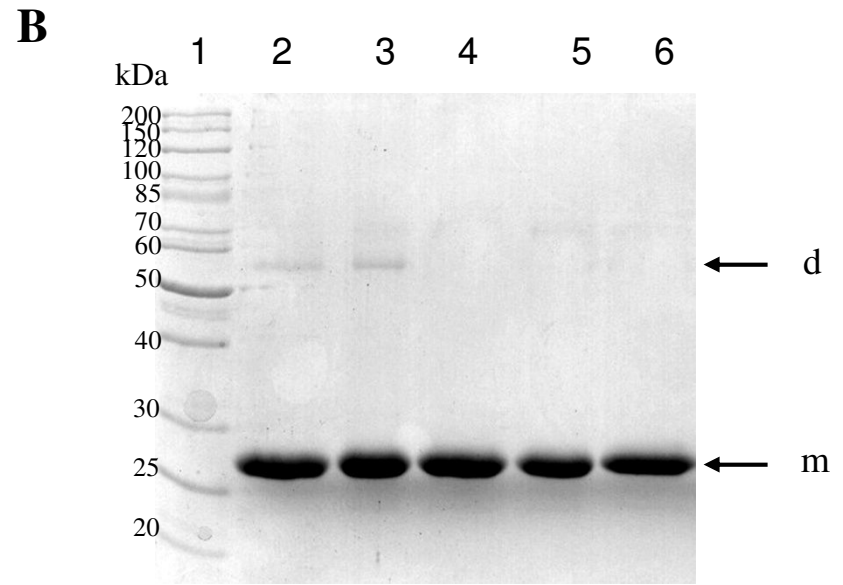
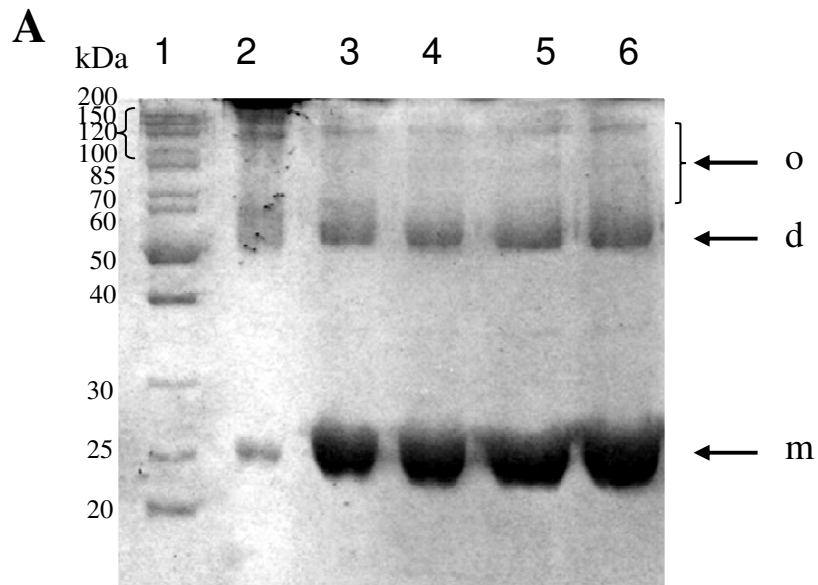


Figure 2

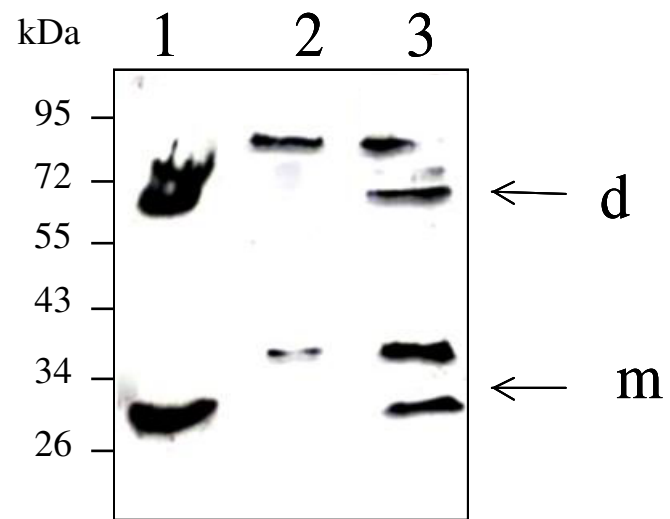
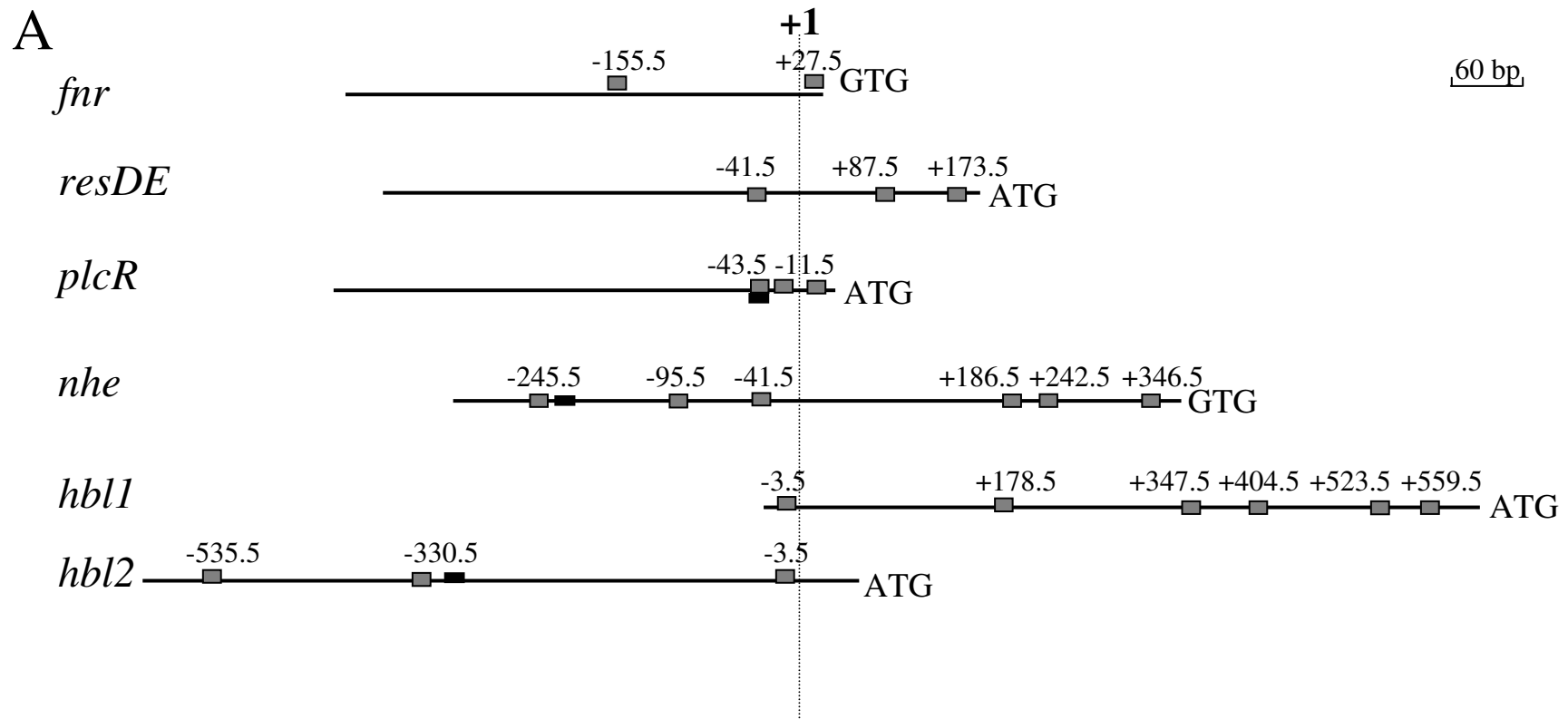


Figure 3



**B**

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-62 TTTTCTTCTTT TTTGAGAAAAACATGACA AAGTAGCGGTTGATTTTGTA AAAATGATGTCGAGGACATTATATGCT +13
GTTCAAAAAAATACATAGTTTTGGAAAAAATTGAACAAAATTTATTATTCTTGTGAAGCATGATA TGGGAAGT +88
GAAACATTTA GAAGATTGCTTAAATAAACGAGAATAGCGCAACATAATAGTTAAAGAAGGGTAGG TGTGAACCGC +163
TGAGGTGAGAGTGCGCAGCGGGTAGAGATG +193

```

Figure 4

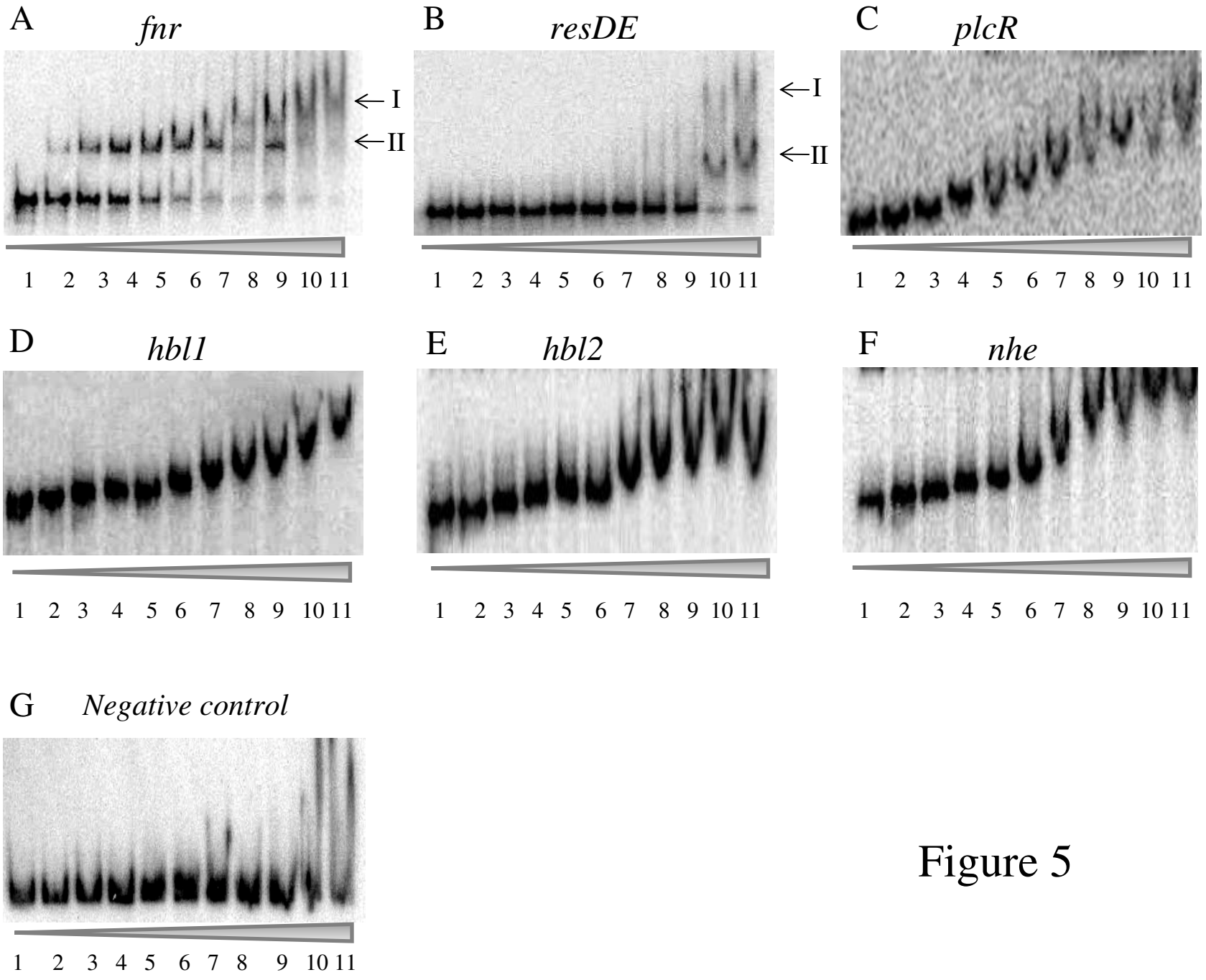


Figure 5



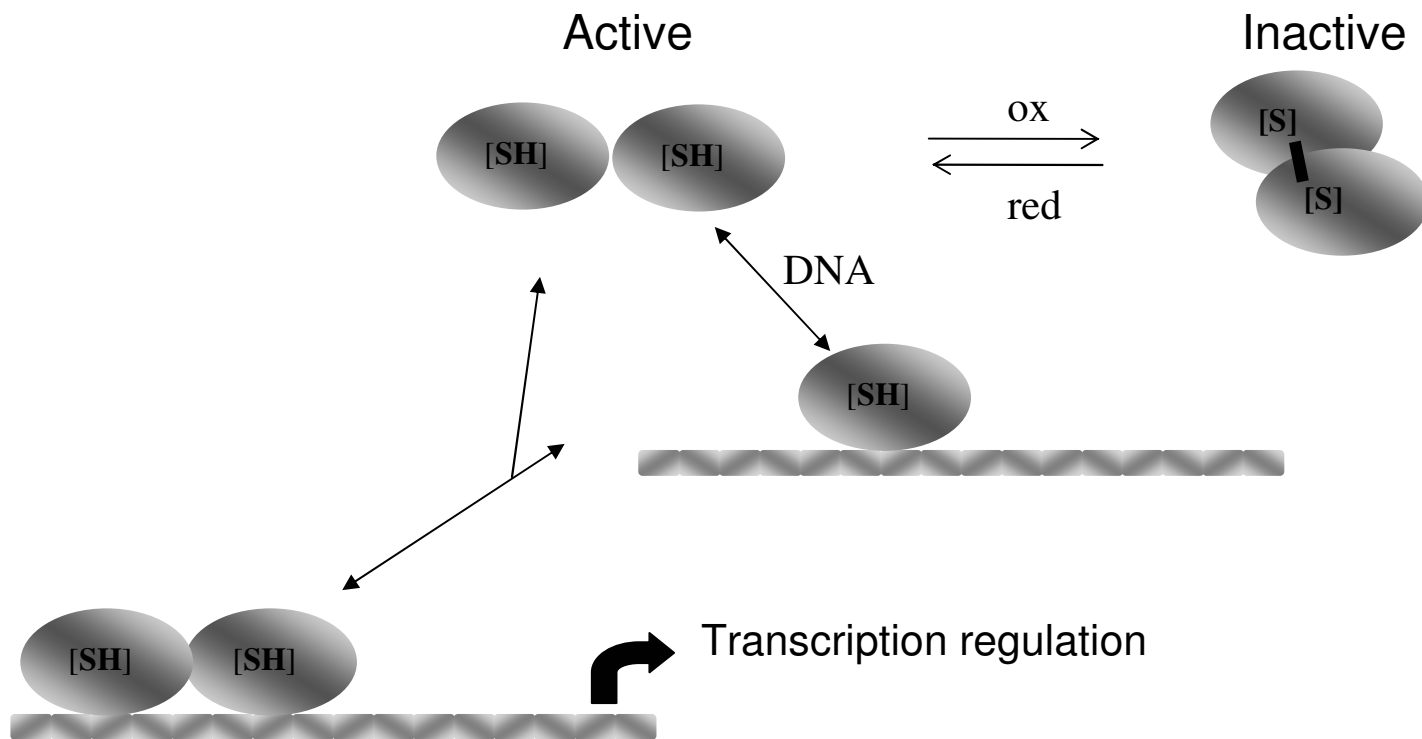


Figure 6