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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
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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_{His}) or an N-terminal Strep-tagged (StrepFnr) fusion
29 protein. Both recombinant Fnr_s were produced as apoforms (clusterless) and occurred as
30 mixtures of monomer and oligomers in solution. However, apoFnr_{His} 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 β -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 Fnr 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 apoFnr 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* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ *M15* Δ *lacX74* *deoR* *recA1* *araD139* Δ (*ara-leu*)-7697
86 *galU* *galK* *rpsL* (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*⁺ Tet^r *gal* λ (DE3) *endA* Hte

88 [*argU ileY leuW Cam^r*] 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 μ_{\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**ATGACATTATCTCAAGATTTAAAAG
114 AA-3'; *Sma*I restriction site in bold type) and PET52R (5'-
115 **GAGCTCCTAATCAATGCTACAAACAGAAGCA**-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_{His}) 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⁻¹ ampicillin. When OD₆₀₀ 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_{His}**. 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,
128 and 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 Co²⁺ 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⁻¹ 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_{His}. 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 KH₂PO₄ and run through a 30 ml hydroxyapatite agarose column (HA Ultrogel; Pall
148 Corporation) equilibrated with buffer D (50 mM KH₂PO₄ [pH 7], 1 mM DTT). The column
149 was developed with a linear gradient from 50 to 200 mM KH₂PO₄ at a flow rate of 2 ml/min.
150 Fractions containing recombinant Fnr were pooled and concentrated to 48 mg. ml⁻¹ 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_{C18} (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% NaN₃ 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_{His}.** Fnr_{His} 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_{His}, 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_{His}) or anti-Strep
202 antibodies (Strep_{Fnr}), 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 [γ - 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 MgCl₂, 0.5
221 μ g of bovine serum albumin and 1 μ g of poly(dI-dC)/ml in a final volume of 10 μ 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_{His}) 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 (StrepFnr) 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_{His} protein was purified using cobalt affinity chromatography. Because preliminary tests
241 indicated that _{Strep}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_{His} (29 kDa) and _{Strep}Fnr (28 kDa) exhibited the expected
244 molecular masses (See Fig. S2 in supplementary data). The exact average molecular mass of
245 _{Strep}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_{His} 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_{His} and _{Strep}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_{His} 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_{His} 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 _{Strep}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 _{Strep}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 _{Strep}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 _{Strep}Fnr underwent SDS-PAGE in non-reducing conditions (no DTT or
284 β -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 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 Fnr_{His} was found to remain dimeric after 10 mM DTT treatment (Fig. 2B). This
293 suggests that the oligomeric Fnr_{His} population detected by DLS contained mainly non-
294 covalently linked structures. To investigate the ability of monomeric Fnr_{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, Fnr_{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 StrepFnr , Fnr_{His} does not tend to form covalently linked
307 homodimers or, more specifically, intermolecular disulphide bridges.

308

309 **Detection of endogenous 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 REX₃R 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 Fnr_{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. Fnr_{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 μM 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 μM . 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 Fnr_{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 Fnr_{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 Fnr_{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, Fnr_{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 (Fnr_{His}) or an N-terminal Strep-tagged ($_{\text{Strep}}\text{Fnr}$) fusion protein. Unlike Fnr_{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 (Fnr_{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 Fnr_{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 α
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_{His} (Panel
587 A), Fnr_{Strep} (Panel B) and reduced Fnr_{Strep} (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_{Strep} (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 _{Strep}Fnr and Fnr_{His}.** Panels A
596 and B: effect of DTT on _{Strep}Fnr (Panel A) and Fnr_{His} (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_{His} cross-linked with EDC. Fnr_{His} (5 µM) was cross-linked
601 with EDC . Products were visualized by immunoblotting with anti-His antibody. Lane 1,
602 cross-linked Fnr_{His}. Lane 2, untreated Fnr_{His}. Panel D: Non-denaturing SDS-PAGE profile of
603 Fnr_{His} cross-linked with diamide. Lane 1, standard proteins. Lane 2, untreated Fnr_{His}; Lanes 3
604 & 4, disulphide linked Fnr_{His} 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_{Strep} 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_{His} or with reduced _{Strep}Fnr (purified _{Strep}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 μ 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.

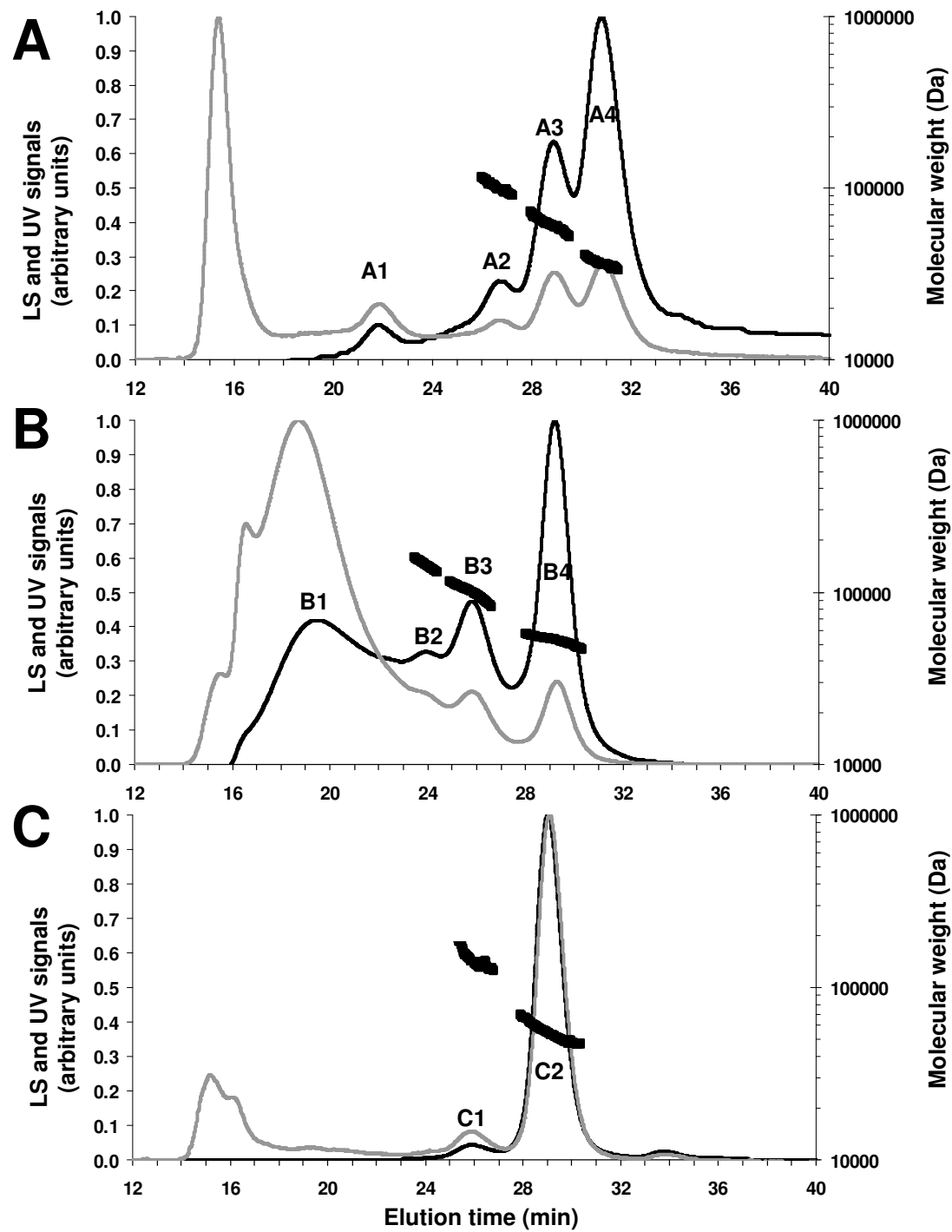


Figure 1

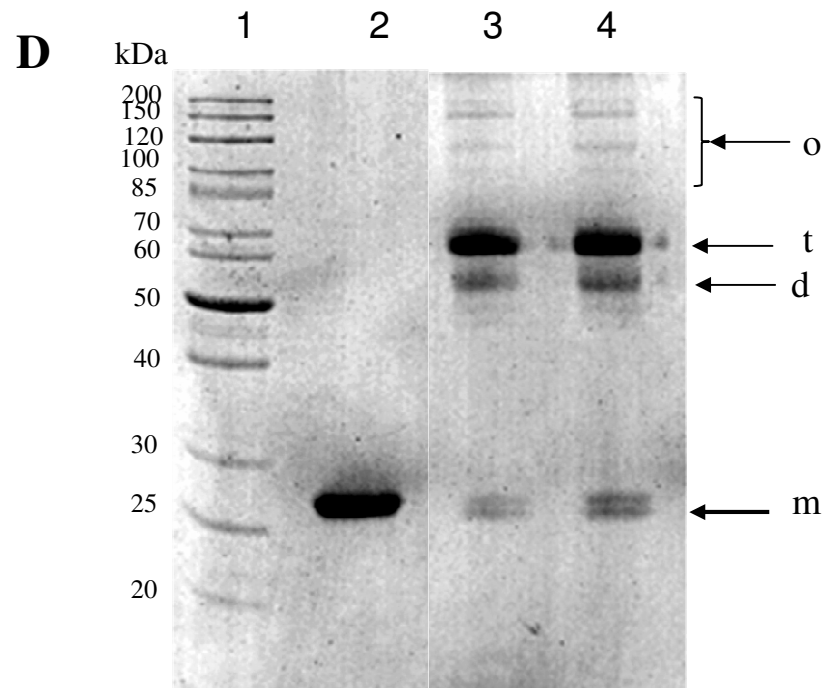
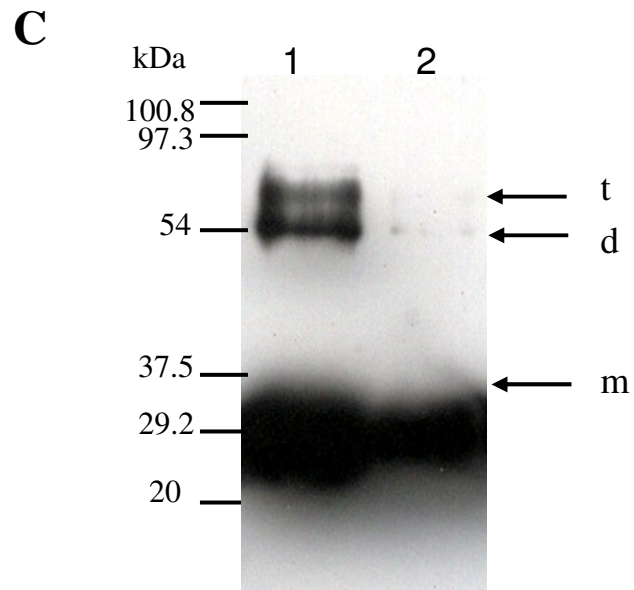
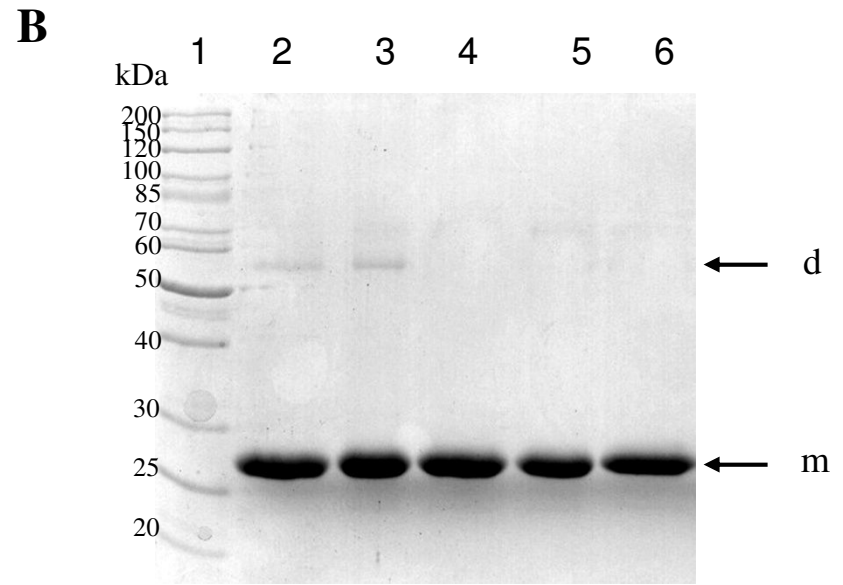
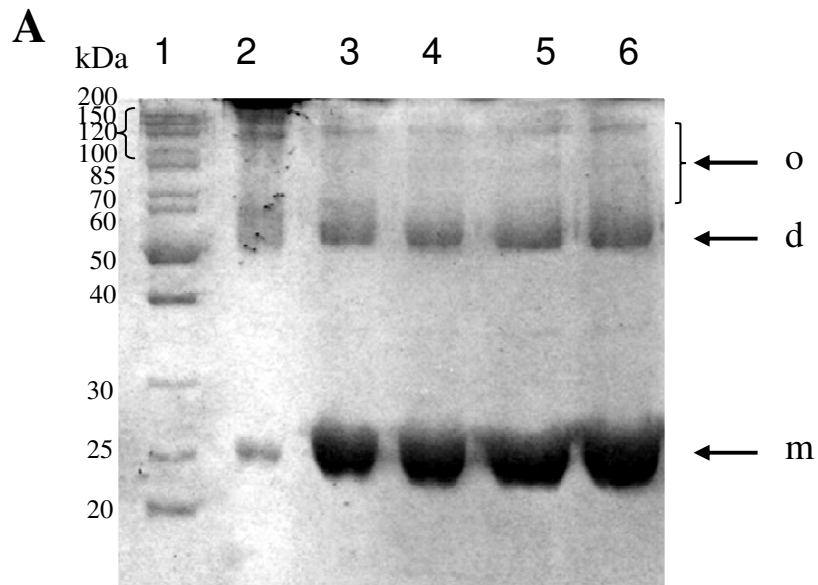


Figure 2

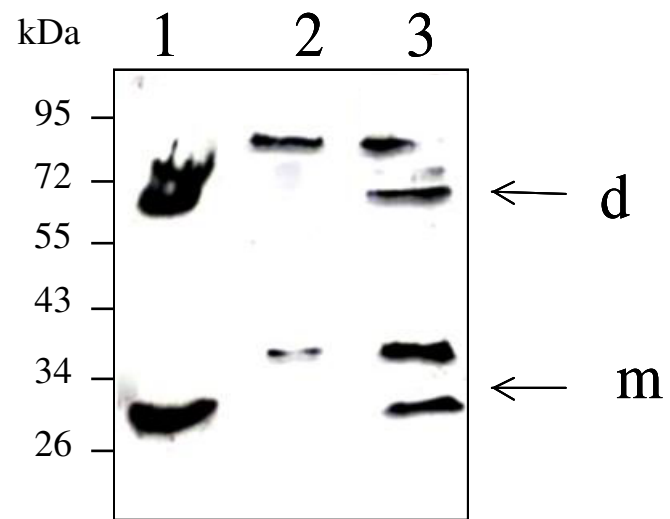
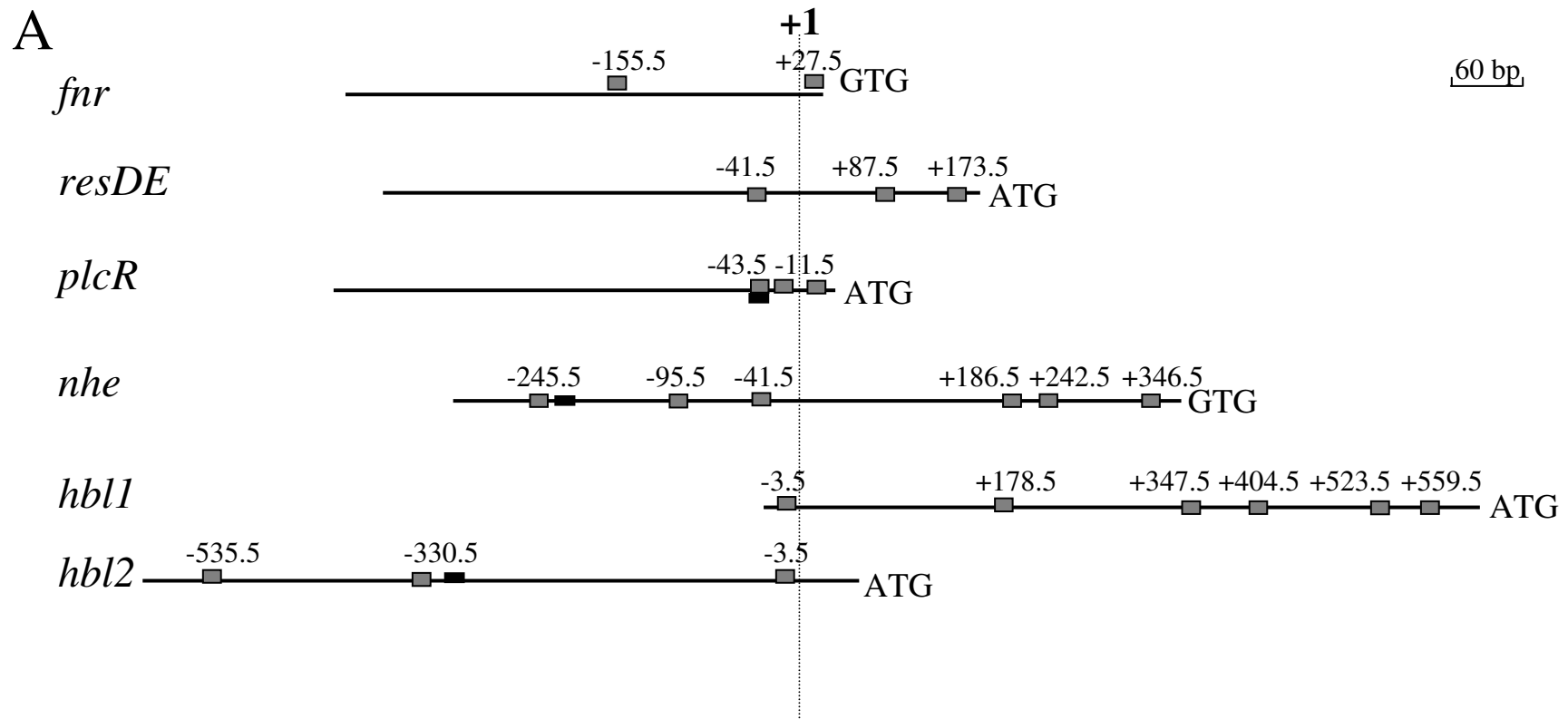


Figure 3



B

```

-62 TTTTCTTCTTT TTTGAGAAAAACATGACA AAGTAGCGGTTGATTTTG TAAAAATGATGTCGAG GACATTATATGCT +13
GTTCAAAAAAATACATAGTTTTGGAAAAAATTGAACAAAATTTATTATTCTTGTGAAGCATGATA TGGGAAGT +88
GAAACATTTA GAAGATTGCTTAAATAAACGAGAATAGCGCAACATAATAGTTAAAGAAGGGTAGG TGTGAACCGC +163
TGAGGTGAGAGTGCGCAGCGGGTAGAGATG +193

```

Figure 4

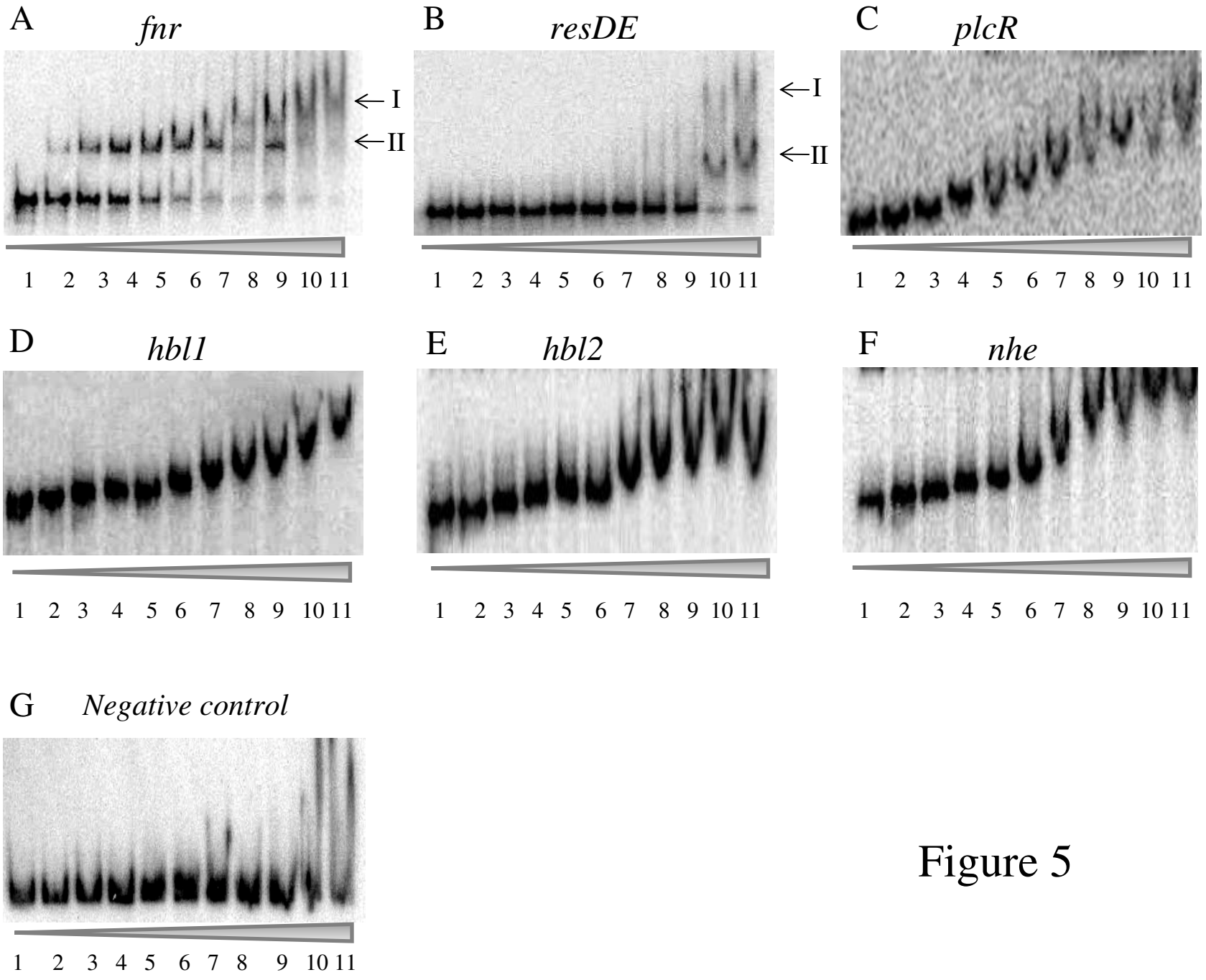


Figure 5

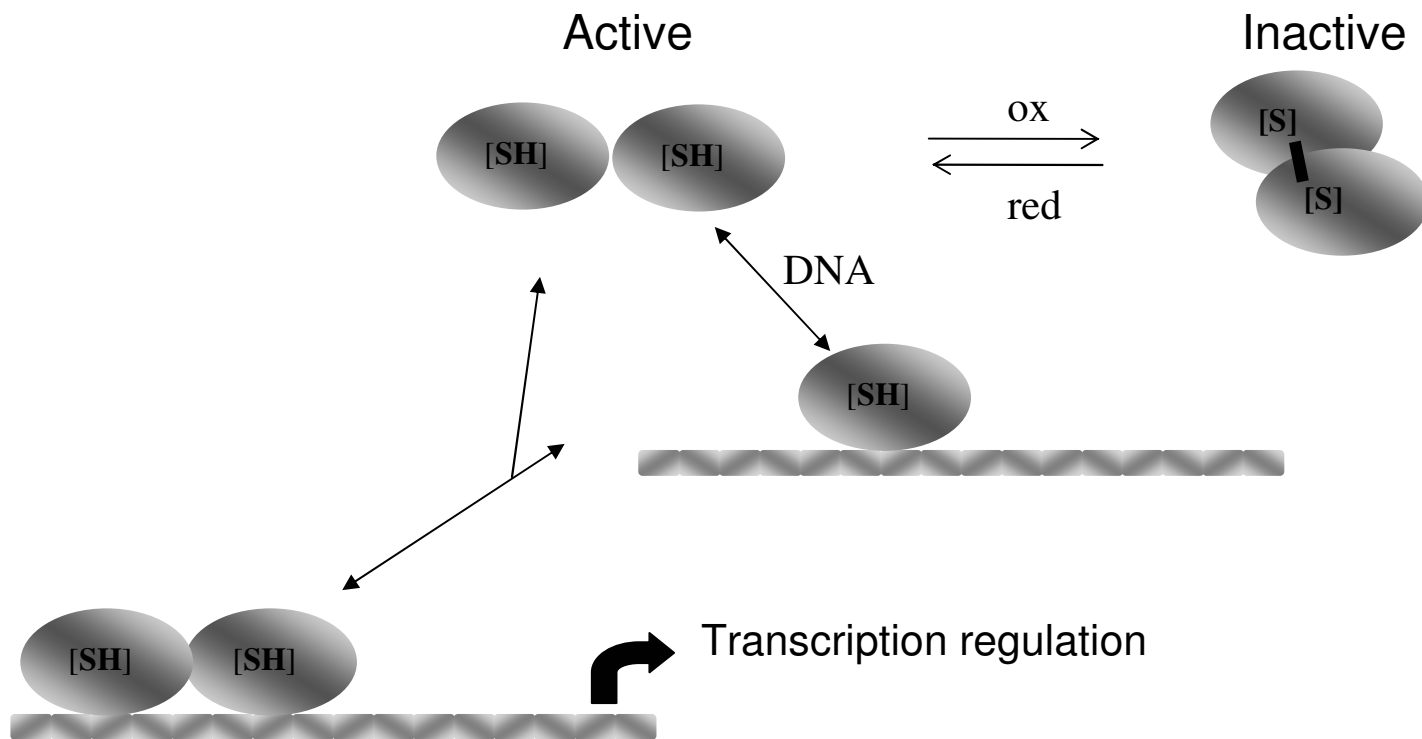


Figure 6