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1 **The *B. subtilis* Rok protein compacts and organizes DNA by bridging**

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19 **Abstract**

20

21 Rok from *Bacillus subtilis* is an abundant DNA binding protein similar in function to H-NS-
22 like proteins found in many proteobacteria. Rok binds across the genome with a
23 preference for A/T rich DNA. Such DNA often contains genes of foreign origin that are
24 silenced due to Rok binding. Rok also has been implied in global organization of the *B.*
25 *subtilis* genome. However, how Rok binds to DNA and how it represses transcription is
26 unclear. Also, it is unknown whether Rok-mediated gene repression can be induced or
27 relieved following changes in physico-chemical conditions, as noted for H-NS-like proteins.
28 Here we investigate the DNA binding properties of Rok and determine the effects of
29 physico-chemical conditions on these properties. We demonstrate that Rok is a DNA
30 bridging protein similar to H-NS like proteins from *E. coli* (H-NS), *Pseudomonas sp.* (MvaT)
31 and *Mycobacteria* (Lsr2). Strikingly, unlike these proteins, the ability of Rok to bridge DNA
32 is not affected by changes in physico-chemical conditions. Not being a direct sensor of
33 such changes sets Rok apart from other H-NS like proteins. It implies the existence of
34 other (protein-mediated) mechanisms to relieve Rok-mediated gene silencing in response
35 to changes in environmental conditions.

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37 **Introduction**

38 The bacterial genome, like that of eukaryotic cells, is both functionally organized
39 and compactly folded. Nevertheless, genes need to be accessible for the transcription
40 machinery or need to be made accessible if environmental conditions so require: the
41 nucleoid is dynamically organized and re-organized [1, 2]. Many factors contribute to the
42 compact shape of the nucleoid, including DNA supercoiling, macromolecular crowding and
43 nucleoid-associated proteins (NAPs) [3-5]. The Histone-like nucleoid structuring protein
44 (H-NS), one of the main NAPs in *Escherichia coli*, plays important roles in both genome
45 organization and gene regulation [6]. H-NS non-specifically binds DNA across the genome,
46 but has a preference for AT-rich DNA. DNA acquired via horizontal gene transfer (HGT)
47 is often AT-rich and is recognized as xenogeneic DNA by H-NS [7, 8]. Although genes
48 acquired via HGT are key to the evolution of bacteria by conferring new genetic traits,
49 inappropriate expression of newly acquired genes can lead to loss of competitive fitness
50 of bacteria. H-NS family proteins including H-NS of *E. coli*, MvaT of *Pseudomonas sp.* and
51 Lsr2 of *Mycobacteria*, function as xenogeneic silencers, silencing foreign DNA until an
52 environmental signal leads to relief of repression.

53 Akin to H-NS, MvaT and Lsr2 are also regulators of global gene expression.
54 Although by sequence the proteins are not homologous, they are similar in DNA binding
55 properties and function, due to their similar organization in functional domains. Structural
56 studies have revealed that H-NS, Lsr2 and MvaT have an N-terminal oligomerization
57 domain consisting of two dimerization sites, a C-terminal DNA binding domain and a
58 flexible linker region [7, 9-12]. Due to their ability to dimerize and oligomerize, these
59 proteins can bind along DNA forming a nucleoprotein filament, which stiffens DNA [13-15].
60 Under appropriate physico-chemical conditions the proteins also can bridge remote
61 segments along a DNA duplex [16-18], yielding DNA loops. The bridging activity of H-NS
62 and MvaT can be modulated by both monovalent (Na^+, K^+) and divalent (Mg^{2+}, Ca^{2+}) salt
63 [19-22], while for Lsr2 it remains unclear whether ionic conditions affect its binding
64 properties. The DNA binding activity of H-NS family proteins (either the formation of
65 nucleoprotein filaments or bridged protein-DNA complexes) is sensitive to temperature,
66 pH and salt, which contributes to adaptation of cells to environmental challenges,
67 mediated by the bacterial genome [2, 6, 23]. Both filament formation and DNA bridging
68 activity of H-NS family proteins have been suggested to account for gene regulation [20].
69 However, as only the DNA bridging activity can be switched on or off by small changes in

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70 physicochemical condition, this might be the mode of binding essential to regulation of
71 environmentally regulated genes [24].
72 Rok of *Bacillus subtilis* was recently proposed to be a functional homolog of H-NS
73 based on the observation that Rok binds extended regions along the genome, which are
74 preferentially AT-rich and have been acquired via horizontal gene transfer. Rok
75 contributes to silencing of the genes within such regions [25]. The ability of Rok to silence
76 genes on DNA of foreign origin classifies it as a xenogeneic silencer like H-NS, MvaT and
77 Lsr2. Rok is also found associated with a large subset of chromosomal domain boundaries
78 in *B. subtilis* [26], which suggests it contributes to genome organization of *B. subtilis*.
79 However, how Rok binds to DNA and how it silences genes remains unknown. In addition,
80 it is unknown whether gene silencing by Rok can be modulated by changes in physico-
81 chemical growth conditions such as temperature, pH and salt.
82
83 **Rok compacts DNA.** All H-NS family proteins (H-NS, MvaT and Lsr2) exhibit two
84 modes of binding to DNA: filament formation along DNA and DNA bridging [14, 15, 17-19,
85 22]. Both lateral nucleoprotein filament complex formation and DNA bridging are
86 suggested to be important for the function of these proteins in gene silencing. To probe
87 whether Rok, like H-NS family proteins, exhibits DNA stiffening activity (reflecting the
88 formation of a protein filament along DNA) or not, we investigated the effect of Rok binding
89 on the conformation of DNA by using Tethered Particle Motion (TPM) [27, 28]. Here, the
90 Root Mean Square displacement (RMS) of a bead (exhibiting thermal motion) at the
91 extremity of a DNA substrate attached to a glass surface, gives a readout of DNA
92 conformation. If a protein stiffens DNA, the RMS will increase following the binding of
93 proteins. If a protein softens, bends, or bridges DNA, a reduction in RMS will take place.
94 We investigated the interaction between Rok and an AT-rich (32%GC) DNA substrate,
95 which we used earlier in studies of the DNA-binding properties of H-NS [21] and MvaT
96 [22]. We determined the effect of Rok on DNA conformation by titration from 0-10 nM
97 (Figure 1a). Bare DNA has an RMS of 150 ± 2 nm. Upon addition of increasing amounts
98 of Rok, a second unique population at an RMS of about 80 nm appears. Saturation of Rok
99 binding is achieved at 8 nM; at this concentration only the population with reduced RMS
100 is observed (Figure 1b). The observed reduction of RMS is an indication of DNA
101 compaction by binding of Rok; it implies that Rok does not form filaments along DNA as
102 observed for other H-NS-family proteins under similar conditions [14, 15, 19] (Figure 1c).
103 The fact that compaction occurs at low protein concentration and that the structural

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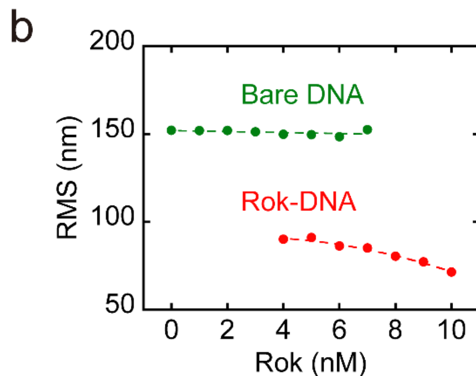
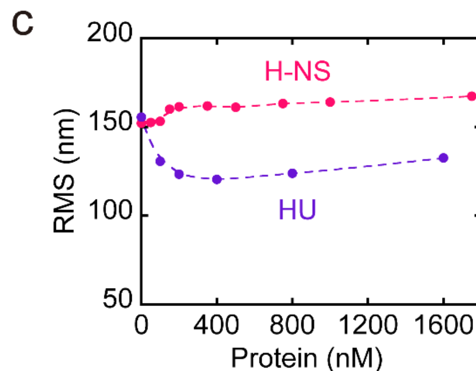
104 transition is abrupt is suggestive of cooperative behavior. The reduction in RMS can be
105 attributed either to DNA bending as observed for HU (Figure 1c) or to DNA bridging.
106





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108 **Figure 1. *B. subtilis* Rok compacts DNA.** a) Histograms of Root Mean Square (RMS) obtained
109 for 32%GC DNA as a function of Rok at concentrations of 0, 1, 3, 4, 5, 6, 7, 8, and 10 nM as
110 measured by TPM in the presence of 50 mM KCl. The histograms were fitted to Gaussian
111 distributions, in which the RMS value at around 150 nm represents bare DNA and the population
112 of RMS at around 80 nM represents DNA bound by Rok. The bare DNA and Rok-DNA complex
113 populations are highlighted with a light green and red box, respectively. The data for each
114 concentration is the combination of three independent measurements and the RMS for each
115 concentration was obtained by fitting the combined data to a Gaussian distribution. b) RMS values
116 obtained for 32%GC DNA as a function of Rok at concentrations from 0 nM to 10 nM. Green and
117 red dots represent the average RMS resulted from fitting with a Gaussian distribution, where green
118 dots and red dots represent bare DNA and Rok-DNA complexes, respectively. Error bars represent
119 the standard error of the mean; due to their small size they are hidden behind the data points. How
120 the RMS values are distributed among the two populations is not taken into account in this
121 representation. c) RMS as a function of protein concentration of *E. coli* H-NS and HU. Error bars
122 represent the standard error of the mean; due to their small size they are hidden behind the data
123 points. The Rok coding gene from *B. subtilis* was cloned into pET30b using Gibson Assembly [29]
124 resulting in vector pRD231. Following overproduction in Rosetta™ (DE3) pLysS, cells were lysed
125 and the lysate was centrifuged for 30 min at 37000 rpm. The supernatant was filtered with a 0.22
126 µm Millex-GP Syringe Filter. Next, the protein was purified using a HiTrap Heparin HP 1 mL affinity
127 column (GE Healthcare), a HiTrap SP HP 1 mL column (GE Healthcare) and a GE Superdex 75
128 10/300 GL column. The purified protein was checked by mass spectrometry. The concentration
129 was determined using a Pierce™ BCA Protein Assay Kit (Thermo Scientific). The DNA used for
130 Tethered Particle Motion (TPM) experiments is a random, AT-rich (32% GC), 685 bp DNA substrate
131 [21, 30]. Measurements were performed as previously described [21] with minor modifications.
132 Briefly, the flow cell was washed with 100 µL experimental buffer (10 mM Tris-HCl pH 8, 10 mM
133 EDTA, 5% glycerol, 50 mM KCl) to remove excess beads and 100 µL protein diluted in experimental
134 buffer is flowed in and incubated for 5 minutes. Next, the flow cell was washed with protein solution
135 one more time, sealed with nail polish and incubated for 5 minutes. After incubation, the flow cell
136 was directly transferred to the holder and incubated for 5 more minutes to stabilize the temperature
137 at 25°C for the measurement. For each flow cell more than 200 beads were measured and
138 measurements for each concentration were performed in triplicate.

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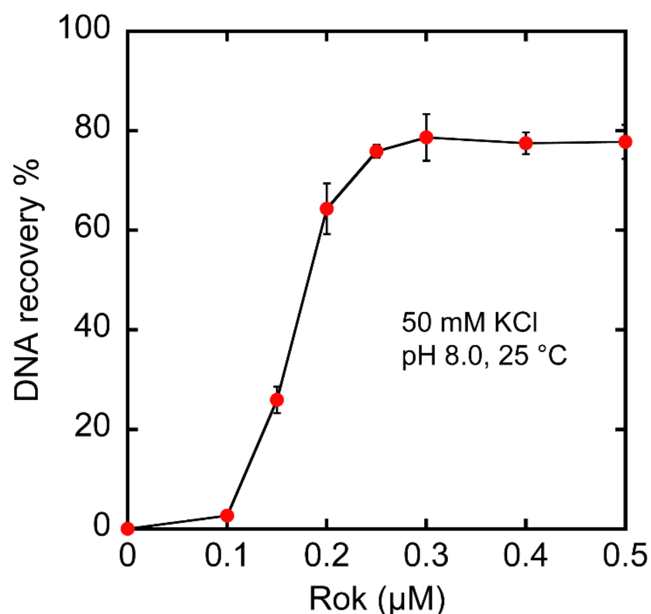
144 **Rok is able to bridge DNA.** In order to determine the structural basis of the
145 observed DNA compaction, we next investigated the ability of Rok to bridge DNA in a
146 quantitative biochemical DNA bridging assay, which we used earlier to evaluate the impact
147 of buffer conditions on the DNA bridging efficiency of H-NS and MvaT [21, 22]. In this
148 assay biotinylated DNA is bound to streptavidin-coated beads and radioactively labeled
149 DNA offered in trans can be recovered by magnetic pull-down of beads when bridged by
150 protein. The radioactive signal of the DNA pulled down is a proxy of DNA bridging
151 efficiency. To determine whether Rok bridges DNA we carried out a titration with Rok from
152 0 - 0.5 μM. The DNA (685 bp, 32% GC) used in the bridging assay is same as the DNA
153 used in TPM experiments. In the absence of Rok, no radioactive DNA was recovered.
154 DNA recovery increases upon addition of increasing amounts of Rok. Saturation of DNA
155 recovery occurs at a Rok concentration of 0.3 μM (figure 2). These data unambiguously
156 show that Rok is a DNA bridging protein. Rok has a similar DNA bridging efficiency as H-
157 NS, yet reaches this efficiency at 10 times lower concentration (0.3 μM vs 3 μM) [21],
158 which is attributed to the high cooperativity in DNA binding by Rok.



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160 **Figure 2. Rok bridges DNA.** A) DNA recovery (as a percentage of the input DNA) as a function of
161 Rok concentration from 0 to 0.5 μM as measured by the DNA bridging assay in the presence of 50
162 mM KCl at 25 ^{o}C. Data are plotted as mean values and the error bars represent the standard
163 deviation from independent triplicate measurements. The DNA used for the bridging assay, the
164 same as that used for TPM, was ^{32}P-labeled as described previously [31]. The DNA bridging assay
165 was performed as previously described [21, 32] with minor modifications. Streptavidin-coated
166 Magnetic M-280 Dynabeads (Thermo Fisher) were resuspended in buffer (20 mM Tris-HCl pH 8.0,

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167 2 mM EDTA, 2 M NaCl, 2 mg/mL BSA (ac), 0.04% Tween 20) containing 100 fmol biotinylated 32%
168 GC DNA (685 bp) and incubated at 1000 rpm for 20 min at 25°C. The beads with associated DNA
169 were washed twice before resuspension in buffer (10 mM Tris-HCl, pH 8.0, 5% v/v Glycerol, 1 mM
170 Spermidine). Radioactive ^{32}P-labeled DNA and unlabeled DNA were combined to maintain a
171 constant (2 fmol/µL) concentration and a radioactive signal around 8000 cpm, and then added to
172 each sample. Next, Rok was added (concentration as indicated) to initiate formation of bridged
173 protein-DNA complexes. The samples were incubated for 20 min at 1000 rpm at 25°C. After the
174 incubation, the beads were washed with the same experimental buffers before resuspension in
175 counting buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl, 0.2% SDS). The radioactive
176 signal of DNA was quantified by liquid scintillation and was used for the calculation of protein DNA
177 bridging efficiency (%) based on a reference sample containing the same amount of labeled ^{32}P
178 685 bp DNA used in each sample. All DNA bridging experiments were performed at least in
179 triplicate.
180
181 **DNA bridging activity of Rok is not sensitive to environmental conditions**
182 **(temperature, pH and salt)**. Bacteria adapt to environmental changes and environmental
183 cues have a direct effect on the function of H-NS-like proteins. This is suggestive of
184 environment-sensory activity of these proteins at H-NS-regulated environment-responsive
185 genes. The DNA bridging activity of H-NS is sensitive to environmental conditions, such
186 as salt [19]. Adaptation of *B. subtilis* to osmotic up- and downshift is a frequent challenge,
187 which is also essential for growth and survival in its natural living environment [33]. To
188 determine whether Rok's DNA bridging activity is also sensitive to salt condition, we
189 investigated the effect of changing monovalent and divalent salt concentration. An
190 increase in concentration of KCl from 50 mM to 300 mM has no significant effect on the
191 DNA bridging activity of Rok. Nevertheless, at higher concentration of KCl the formation
192 of bridged complexes is abolished (figure 3a). A similar result was obtained for titration
193 with $MgCl_2$ in the range from 0 mM to 90 mM (figure 3b). Only beyond a certain ionic
194 strength, the bridged Rok-DNA complexes start disintegrating and this ionic strength is
195 likely not physiologically relevant anymore. It has been reported that the bridging activity
196 of H-NS and MvaT can be modulated by both monovalent (K^+) and divalent (Mg^{2+}) salt
197 [19-22]. However, unlike H-NS and MvaT, the formation of bridged Rok-DNA complexes
198 requires neither Mg^{2+} nor a high concentration of K^+. These results indicate that DNA
199 bridging activity of Rok neither requires nor is inhibited by salt at biologically relevant
200 concentrations.

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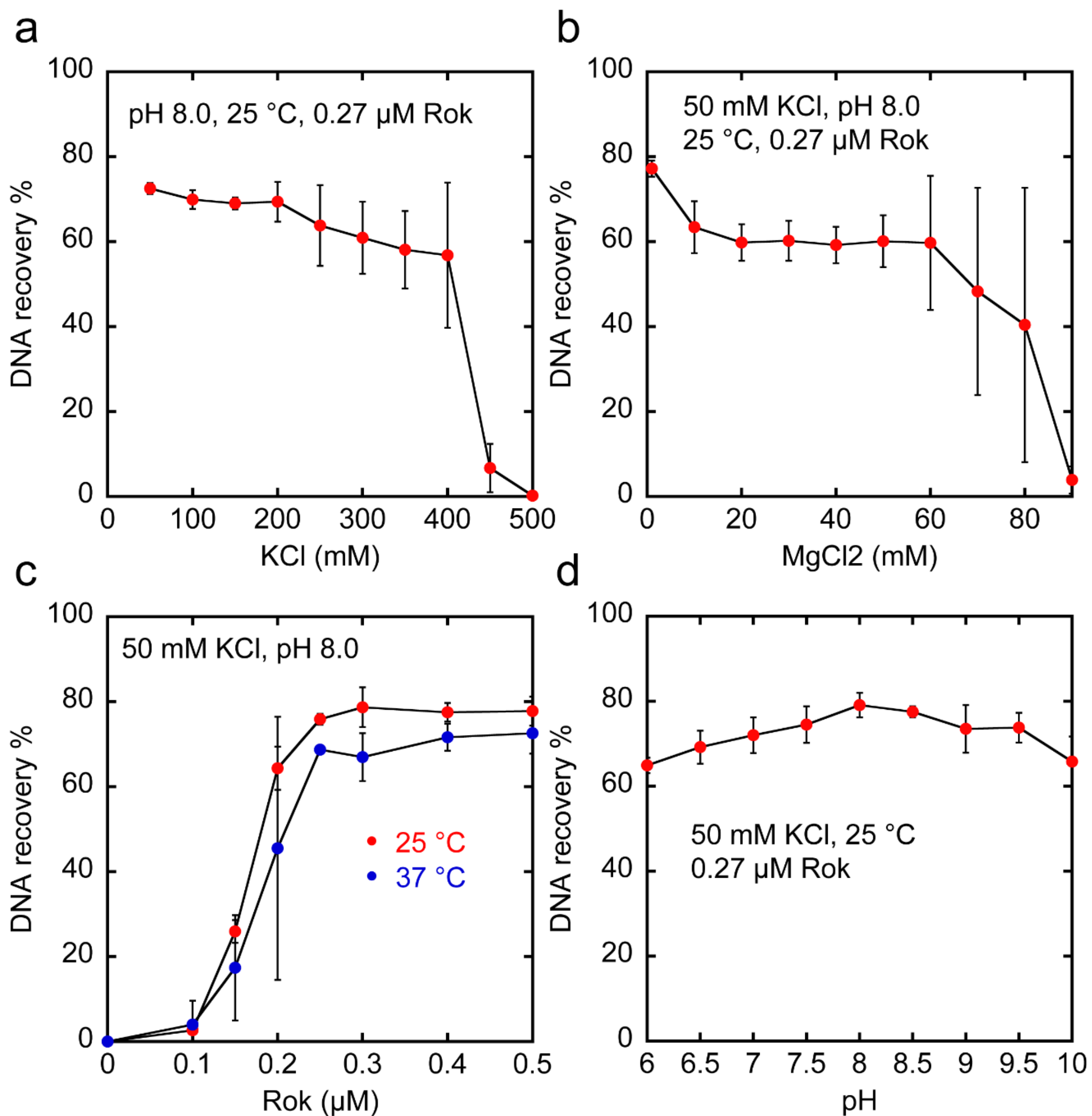
201 *B. subtilis*, found in soil and the gastrointestinal tract of ruminants and humans,
202 can live at different temperatures and pH conditions. Therefore, we investigated the effect
203 of temperature on DNA bridging efficiency. An increase in temperature from 25 ºC to 37
204 ºC has no significant effect on the DNA bridging activity of Rok (figure 3c). Next, we also
205 investigated the effect of pH. Across a range from 6 to 10, pH has no effect on the DNA
206 bridging activity of Rok (figure 3d). Strikingly, even crossing the pI of Rok (9.31) did not
207 interfere with its capacity of DNA bridging. Taken together, all these results indicate that
208 the DNA bridging activity of Rok is not affected by changes in physico-chemical conditions,
209 which is unexpectedly different from H-NS, MvaT and Lsr2.
210



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212 **Figure 3. DNA bridging activity of Rok is not sensitive to temperature, pH and salt.** A) DNA
213 recovery (as a percentage of the input DNA) as a function of KCl concentration from 50 to 500 mM
214 measured by the DNA bridging assay in the presence of 0.27 μM Rok at 25 ºC. b) DNA recovery
215 as a function of $MgCl_2$ concentration from 1 to 90 mM in the presence of 0.27 μM Rok at 25 ºC. c)
216 DNA recovery as a function of Rok concentration from 0 to 0.5 μM at 25 ºC (red) and 37 ºC (blue),
217 respectively. d) DNA recovery as a function of pH from 6 to 10 in the presence of 0.27 μM Rok at
218 25 ºC. Data are plotted as mean values and the error bars represent the standard deviation from
219 three independent measurements. The experiment was carried out in the same way as described
220 for figure 2. Salt concentration (KCl or $MgCl_2$), protein concentration, temperature and pH were
221 varied in line with the experiments. For pH 6 and 6.5 MES (2-morpholinoethanesulfonic acid) was
222 used instead of Tris-HCl and for pH 9, 9.5 and 10 CHES (*N*-Cyclohexyl-2-aminoethanesulfonic acid)
223 was used.

224

225 We have studied the DNA binding properties of Rok. We found that Rok is a DNA
226 bridging protein and evaluated its DNA bridging capacity under various physiologically
227 relevant conditions. The protein is remarkably insensitive to changes in physico-chemical
228 conditions. Although Rok has low sequence similarity with H-NS, MvaT or Lsr2, they have
229 similar domain organization: the N-terminal dimerization and oligomerization domain, a C-
230 terminal DNA binding domain and a flexible linker in between. H-NS, MvaT and Lsr2 can
231 oligomerize along DNA, which causes DNA stiffening. Such a stiffening effect was not
232 observed for Rok. Instead, only DNA compaction was observed in our study. Because we
233 demonstrated that Rok is a DNA bridging protein, we attribute the observed DNA
234 compaction to bridging by Rok. Based on our results and the known properties of Rok, we
235 propose that Rok bridges DNA, without associating into nucleoprotein filaments, but
236 employing dimeric Rok as bridging units (Figure 4). The Rok dimers cluster cooperatively
237 due to high local DNA concentration upon initiation of bridging, but earlier studies suggest
238 that the protein forms oligomers in solution [34], while our study does not provide evidence
239 for Rok oligomers along DNA. Unexpectedly, Rok-DNA bridging activity is not affected by
240 changes in physico-chemical conditions, which sets the protein apart from H-NS, MvaT
241 and Lsr2. It implies the presence of other (protein-mediated) mechanisms to relieve Rok-
242 mediated gene silencing in response to changes in environmental conditions. An example
243 of a Rok antagonist is ComK which can relieve gene repression mediated by Rok at the
244 *comK* promotor [35]. Based on the robustness of Rok in binding to DNA we expect the
245 existence of similar antagonistic factors operating at other sites across the genome.

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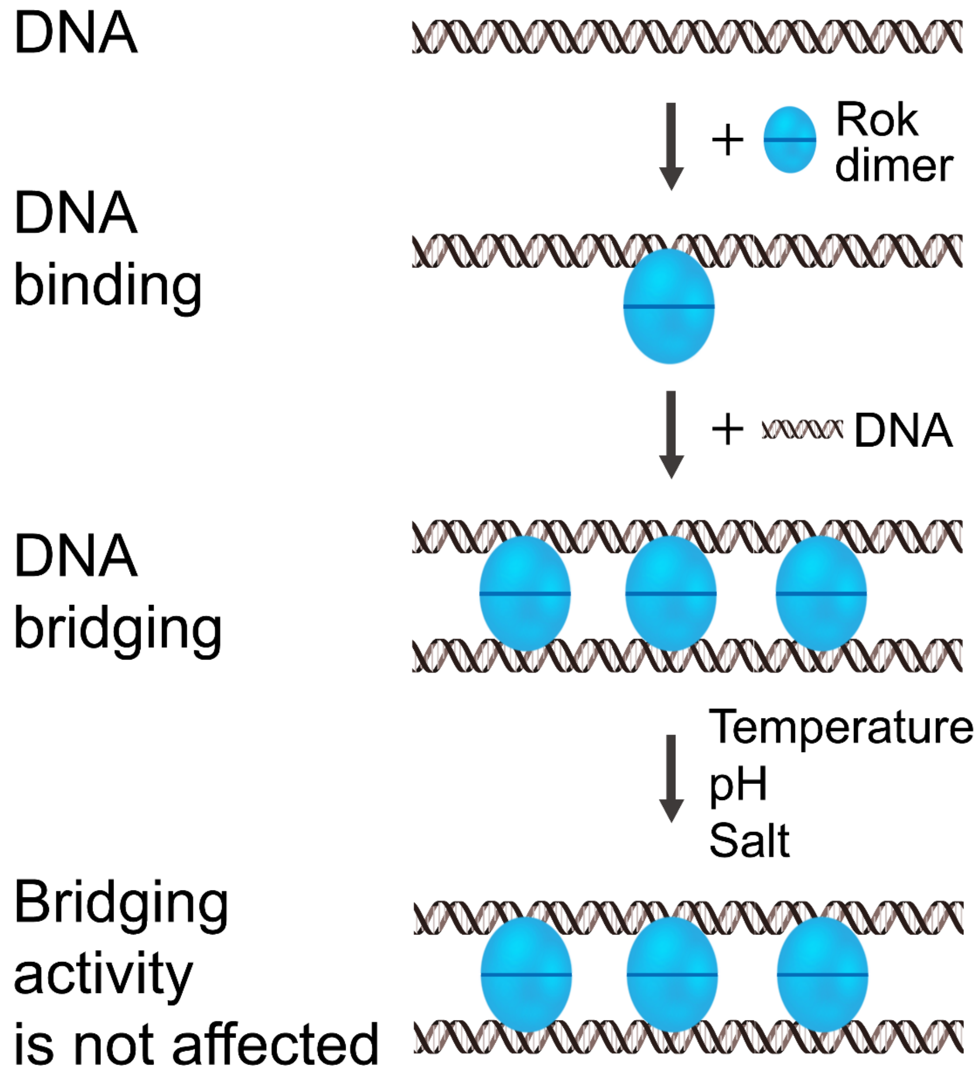

246
247
248 **Figure 4. The proposed mechanisms of DNA binding by Rok.** Rok binds DNA as a dimer
249 without associating into nucleoprotein filaments. Dimeric Rok acts as bridging unit. Rok dimers
250 cluster cooperatively in between two DNA duplexes, not due to dimer-dimer interactions, but due
251 to high local DNA concentration which drives association and bridging by additional dimers. The
252 DNA bridging activity of Rok is not sensitive to changes in physico-chemical conditions
253 (temperature, pH and salt).
254

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255 **Acknowledgments**

256

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261

262

263

264 **Contributions**

265

266 L.Q., A.M.E. and D.M. performed the experiments. L.Q., A.M.E., D.M. and R.T.D.
267 contributed to data analysis and discussion. L.Q. and R.T.D. supervised the project. L.Q.,
268 A.M.E., and R.T.D. wrote the manuscript. L.Q., A.M.E. and R.T.D. reviewed and corrected
269 the manuscript.

270

271

272 **Competing interests**

273

274 The authors declare no competing interests.

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