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1 *Bacillus subtilis* RarA acts as a positive RecA accessory protein

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14 **Running title:** RarA is a RecA accessory protein

15

16 **Keywords: Mgs1; WRNIP1; replication stress; RecA mediators; RecA modulators.**

17

18 **Abstract**

19 Ubiquitous RarA AAA$^+$ ATPases play crucial roles in the cellular response to blocked replication
20 forks in pro- and eukaryotes. Here, we provide evidence that RarA regulates the activity of the
21 central player in homologous recombination (HR), RecA, in response to DNA damage. During
22 unperturbed growth, absence of RarA reduced the viability of Δ*recA,* Δ*recO* and *recF*15 cells, and
23 during repair of H_2O_2- or MMS-induced DNA damage, *rarA* was epistatic to *recA*, *recO* and *recF.*
24 Conversely, the inactivation of *rarA* partially suppressed the HR defect of mutants lacking end-
25 resection (Δ*addAB*, Δ*recJ*, Δ*recQ*, Δ*recS*) or branch migration (Δ*ruvAB*, Δ*recG*, Δ*radA*) activity.
26 RarA contributes to RecA thread formation, that are thought to be the active forms of RecA during
27 homology search. The absence of RarA reduced RecA accumulation, and the formation of visible
28 RecA threads *in vivo* upon DNA damage. When Δ*rarA* was combined with mutations in genuine
29 RecA accessory genes, RecA accumulation was further reduced in Δ*rarA* Δ*recU* and Δ*rarA* Δ*recX*
30 double mutant cells, and was blocked in Δ*rarA recF*15 cells. These results suggest that RarA
31 contributes to the assembly of RecA nucleoprotein filaments onto single-stranded DNA (ssDNA),
32 in concert with RecF, and possibly antagonizes RecA filament disassembly by RecX or RecU.

33

34 **Introduction**

35 During DNA replication, the forks encounter obstacles that can block their progression, and
36 replication impairment is recognized as an important source of genetic instability (1-3). Maintenance
37 of genome stability is one of the crucial functions in life. As a consequence, numerous and diverse
38 mechanisms have evolved to minimize the frequency or impact of replicative stress (1, 2, 4, 5).
39 Eukaryotic Mgs1/WRNIP1 and prokaryotic RarA, which are evolutionarily conserved AAA^+
40 ATPases associated with a variety of cellular activities, play important but poorly understood roles
41 in cellular responses to stalled or collapsed replication forks (6-17).

42 Previous assays have indicated a poorly understood role for bacterial RarA in homologous
43 recombination (HR). Inactivation of *Bacillus subtilis rarA* renders cells very sensitive to H_2O_2, but
44 not to methyl methane sulfonate (MMS) or the UV radiation-mimetic compound 4-nitroquinoline-
45 1-oxide (17). Similarly, an *Escherichia coli* null *rarA* (Δ*rarA*) mutant strain remains as capable of
46 repairing UV-induced DNA damage as wild-type (*wt* or *rec*$^+$) cells (6, 9). In both bacteria *E. coli*
47 and *B. subtilis* the viability under unperturbed conditions of *E. coli* and *B. subtilis* Δ*rarA* Δ*recA* cells
48 is significantly lower than that of the Δ*recA* control (9, 17). Since the *recA* gene is not epistatic with
49 functions involved in base or nucleotide excision repair, but the *E. coli* or *B. subtilis rarA* gene is
50 epistatic to *recA* in response to DNA damage (9, 17), we assume that RarA is a genuine repair-by-
51 recombination protein. Unless otherwise stated, the indicated genes and products are of *B. subtilis*
52 origin.

53 Bacterial RarA shares sequence homology with DnaX, a subunit of the clamp loader complex,
54 and with RuvB, a subunit of the RuvAB branch migration translocase (6), but *B. subtilis* RarA could
55 not substitute for DnaX in the cognate reconstituted *in vitro* DNA replication system (15). Rather,
56 these assays showed that RarA inhibited initiation of PriA-dependent DNA replication, but not chain

57 elongation, suggesting that RarA might impede the assembly of the replicative helicase and prevent
58 that recombination intermediates contribute to pathological DNA replication restart (15). RarA
59 exerts its action through its interaction with the essential SsbA (counterpart of *E. coli* SSB [SSB_{Eco}])
60 and with PriA proteins (15). In addition to RarA, SsbA protein interacts with various recombination
61 (RecQ, RecS, RecJ, RecG, RecO, RecD2, SbcC and SbcE) and replication (PriA, DnaG and DnaE)
62 proteins, of which RecS, RecD2, SbcE and DnaE are absent in *E. coli* cells (18). These data suggest
63 a role of RarA in recombination-dependent DNA replication, although RarA might follow different
64 avenues in distantly related bacteria (14-17). For example, when DNA replication is blocked, upon
65 dNTPs depletion by hydroxyurea, $RarA_{Eco}$ foci disassemble from the replication fork and disappear
66 (19). However, *in vitro* studies suggested that $RarA_{Eco}$ may contribute to replication fork rescue by
67 creating a flap on the lagging strand, so that the replicative helicase and its associated replisome
68 could continue chain elongation without the need for replisome disassembly and replication restart
69 (14). In *B. subtilis* cells, inhibition of the replicative DNA polymerase PolC, by the specific inhibitor
70 *p*-hydroxyphenylazo-uracil (HPUra), confines the RarA molecules towards the collapsed replication
71 forks (17). In this bacterium it was shown that *B. subtilis* RarA-mVenus transiently colocalizes with
72 the DnaX-CFP protein, and it alternates between static and dynamic states. RarA is confined to the
73 replication forks when the preprimosomal DnaB protein is non-functional, but the opposite occurs
74 upon inactivation of the replicative DNA helicase (DnaC) (16, 17), revealing an intricate function
75 related to DNA replication restart.

76 RarA forms mobile foci, usually one per cell containing many molecules, that move in a time
77 scale of minutes in ~50% of total cells, mostly close to replication forks, in which RarA is likely
78 DNA-bound. On a time scale of milliseconds, ~50% of RarA molecules move very slowly or are
79 static, likely within the slowly moving foci, while the remaining fraction was highly dynamic,
80 diffusing throughout the cells (16, 20). DNA damages changed the ratio of static (DNA-bound) and

81 freely diffusive RarA, *e.g*. H_2O_2 decreased the static subpopulation of RarA at the replication forks,
82 and instead, RarA was recruited to areas located away from the replication forks. Exposure to H_2O_2
83 increased the fraction of dynamic molecules, but not treatment with MMS, and this was exacerbated
84 by the absence of end resection or Holliday junction (HJ) processing proteins (16). The number of
85 cells containing slowly moving RarA foci was also affected by several proteins acting in
86 homologous recombination (HR) (16), indicating that the number of molecules acting within the
87 foci, and the positioning of the foci, is affected by interactions with HR proteins.

88 To analyze the role of RarA in repair-by-recombination at the genetic level, the Δ*rarA* deletion
89 was moved into *rec*-deficient strains impaired in DNA end resection (*addAB*, *recQ*, *recS*, *recJ*),
90 RecA mediators (*recO*) and/or modulators (*recF*, *recX*, *recU*), or HJ processing and
91 cleavage/dissolution (*recG*, *ruvAB*, *radA*, *recU*, *recQ*, *recS*). Also, the relation to the DNA repair
92 defect of the poorly characterized *recD*2 mutation (21) was investigated. In this study, we show that
93 lack of RarA reduced cell viability in the Δ*recO* and Δ*recA* and in less extent of the *recF15* context,
94 but these mutant strains were equally sensitive to H_2O_2- or MMS-induced non-bulky DNA lesions
95 of oxidative nature (epistasis). The absence of RarA partially suppressed the DNA repair defect of
96 cells impaired in DNA end resection (*addAB*, *recQ*, *recS*, *recJ*), or HJ processing and
97 cleavage/dissolution (*recG*, *ruvAB*, *radA*, *recU*, *recQ*, *recS*), as well as the DNA repair defect of the
98 *recD*2 mutation. Lack of RarA might reduce the accumulation of the signal (RecA filament
99 formation) that is considered to facilitate LexA self-cleavage as judged by the drop of RecA levels
100 upon exposure to increasing mitomycin C (MMC) concentrations and the reduced number of RecA
101 threads in Δ*rarA* cells. Together, these data suggest that RarA controls RecA filament growth and
102 might counteract negative mediators RecX and/or RecU.

103

104 **Materials and Methods**

105 **Bacterial strains**

106 *B. subtilis* BG214 and its isogenic derivatives are listed in Table 1. The null *rarA* (Δ*rarA*) mutation
107 was transferred into the other genetic backgrounds by SPP1-mediated chromosomal transduction.
108 The *recF*15 point mutation and a null mutation in *recF* (Δ*recF*) are equally deficient in DNA repair,
109 but the latter showed a reduced cell fitness, because it compromises expression of the downstream
110 essential *gyrB* and *gyrA* genes, thus we worked with the inactive *recF*15 strain (22). In RecF15 the
111 highly conserved negatively charged residue E255 is replaced by a positively charged one K255,
112 RecF E255K (22). The accuracy of the double mutations was analyzed by PCR amplification and
113 nucleotide sequence analyses.

114 **Survival studies**

115 H_2O_2, MMS and MMC were obtained from Sigma Aldrich (Germany). The sensitivity of cells to
116 acute exposure to MMS or H_2O_2 was determined by growing *rec*$^+$ and its isogenic derivative strains
117 (see Table 1) in NB to an OD_{560} = 0.4 at 37 ºC with agitation. Then, cells were incubated with
118 increasing concentrations of MMS or H_2O_2 for *15 min. Treated cells were diluted and plated on NB*
119 *agar plates,* incubated overnight (ON) at 37 ºC, *and the* colonies forming units/ml (CFUs/ml) were
120 counted. The large majority of cells were one and two non-separated with an average of ~1.6
121 cells/CFU, thus we have assumed an acceptable correlation of OD_{560} with CFUs.

122 **Cell staining**

123 The LIVE/DEAD BacLight bacterial viability kit was purchased from Fisher Scientific was used.
124 Cells were exponentially grown in NB to an OD_{560} = 0.4 at 37 ºC with agitation for *30 min*.
125 Appropriate dilutions were stained with membrane-permeant SYTO 9, which labels living bacteria
126 with green fluorescence, and then with membrane-impermeant propidium iodide (PI), which stains

127 cells with a membrane compromised defect with red fluorescence, and subjected to conventional
128 direct count of total cells using a fluorescence microscope and appropriate filters (470 ± 20 nm
129 excitation filter and 515 ± 20 nm emission filter for both SYTO 9 and PI), as reported (21). When
130 cells are permeant to PI, its counterstaining activity competes with SYTO 9 for binding to DNA,
131 and SYTO 9 staining signal is not detected. In each experiment >1000 CFUs were counted.

132 ***RecA protein quantification***

133 For quantification of RecA induction of the *recA* gene expressed from its native locus and promoter,
134 cells were grown in NB to an OD_{560} = 0.4 at 37 ºC with agitation and treated with increasing MMC
135 concentrations (0.07 to 1.5 µM) for 30 min. Cells (2 ml) were centrifuged, resuspended in 100 µl of
136 buffer A (50 mM Tris HCl, pH 7.5, 1 mM DTT, 5% glycerol) containing 300 mM NaCl and lysed
137 by sonication. Extracts from each experimental condition, containing similar concentrations of total
138 and housekeeping proteins, were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide
139 gel electrophoresis (PAGE) alongside the purified RecA protein standard (10 to 500 ng) as reported
140 (23). Gels were transferred, and Western blots were developed with rabbit polyclonal anti-RecA
141 antibodies (23). This antibody showed no signal in the absence of RecA, suggesting that no cross-
142 reactive signal interferred in our studies.

143 RecA protein bands on developed immunoblots were quantified with a scanning densitometer
144 (ImageLab software, BioRad). Purified RecA protein standard yielded a linear relationship between
145 antibody signal and the RecA protein concentration. The amount of RecA protein in each induced
146 sample was interpolated from the standard curve performed with known amounts of purified protein,
147 as described previously (23). The *in vivo* concentration of RecA was estimated considering the cell
148 volume of 1.2 femtoliters, and the amounts of cells loaded in the gel, based on the total number of
149 CFUs.

150

151 **Fluorescence microscopy and data analysis**

152 A C-terminal fusion of the fluorescent protein mVenus to RecA was generated by cloning the 3′-
153 end 500-bp of *recA* (excluding the stop codon) into plasmid pSG1164 mVenus (24), which was
154 integrated into the *recA* gene locus on the *B. subtilis* chromosome by single crossover
155 recombination. Epifluorescence microscopy was used to monitor filament formation and dynamics
156 of RecA before and after stress conditions at 30 °C (OD_{600} = ~ 0.3). Cells were treated with 0.5 mM
157 H_2O_2 (obtained from Sigma Aldrich) or were not treated. For fluorescence microscopy, *B. subtilis*
158 cells were grown in $S7_{50}$ minimal medium at 30 °C under shaking conditions until exponential
159 growth, using a Zeiss Observer Z1 (Carl Zeiss) with an oil immersion objective (100× magnification,
160 NA 1.45 alpha Plan-FLUAR) and a CCD camera (CoolSNAP EZ, Photometrics). Electronic data
161 were processed using Metamorph 7.5.5.0 software (Molecular Devices, Sunnyvale, CA, USA),
162 which also allows the calibration of the fluorescence intensity and pixel size to determine the cell
163 length and BacStalk (25), time-lapse epifluorescence microscopy of RecA-mV were collected every
164 5 min.
165

166 **Results and Discussion**

167 **Δ*rarA* reduces viability in Δ*recO* and Δ*recA***

168 To better understand the role of RarA in repair-by-recombination, a *rarA* deletion (Δ*rarA*) was
169 combined with *rec*-mutations in DNA end resection (*addAB*, *recQ*, *recS*, *recJ*), RecA mediators
170 (*recO*) and/or modulators (*recF*, *recX*, *recU*), and HJ processing and cleavage/dissolution (*recG*,
171 *ruvAB*, *radA*, *recU*, *recQ*, *recS*), as well as the DNA repair defect of the poorly characterized *recD*2
172 mutation (Table 1). The *recA*, *recF*, *recO*, *recG*, *recJ*, *recQ, recR*, *ruvA*, *ruvB*, *radA* and *rarA* genes
173 have their counterpart in *E. coli* genes with identical name, the *addAB* and *recU* genes have their
174 counterpart in the $recBCD_{Eco}$ and $ruvC_{Eco}$ and the *recS* and *recD*2 genes are absent in *E. coli* (26,
175 27). In contrast, *B. subtilis* cells lack the RecA modulators *dinI* and *rdgC* (28).

176 If a DNA damage is not removed, DNA replication is not able to continue to completion and
177 the cell will not survive. It has recently been reported that *B. subtilis* replisome dissociation occurs
178 at a frequency of ~5-fold events per replisome, per cell cycle (29). Interestingly, even in the absence
179 of any external DNA damage the viability of the constructed strains listed in Table 1 was quite
180 different. The combination of Δ*rarA* with Δ*addAB*, Δ*recS*, Δ*recQ* or Δ*recJ* (impaired in alternative
181 pathways of end-processing), Δ*recX* (negative modulator), Δ*radA* (impaired in branch migration
182 translocase) or with Δ*recD2* (yet unclassified) (23, 30-32) yielded similar or only slightly reduced
183 (<1.4-fold) viability relative to rec^+ cells (Figure 1A). Thus, additional deletion of RarA in these
184 mutant backgrounds having similar viability as *wt* cells (21, 32, 33) appeared to be associated with
185 a low fitness cost.

186 At mid-exponential phase the number of CFUs was reduced 5- to 7-fold in Δ*recU*, Δ*ruvAB* or
187 Δ*recG* (impaired in translocation of branched structures and HJ resolutution) cells, compared to the
188 rec^+ control (21, 33), and the viability of the double (Δ*recU* Δ*rarA*, Δ*recG* Δ*rarA*) or triple (Δ*ruvAB*
189 Δ*rarA*) mutant strains was marginally affected, as shown in Fig. 1A. These findings suggest that the

190 deletion of *rarA* does not cause an extra fitness cost when compared to the Δ*recU*, Δ*recG* or Δ*ruvAB*
191 cells. The viability of the Δ*radA* cells was similar to or slightly reduced (<1.5-fold) relative to *rec*$^+$
192 cells, but the viability of the Δ*rarA* Δ*radA* double mutant strain was reduced ~10-fold compared to
193 the *rec*$^+$ strain (Fig. 1A), suggesting that absence of the RarA and so far less well characterized
194 RadA/Sms functions poses a considerable threat for cell viability.

195 Previous studies demonstrated that in the absence of any external DNA damage, the Δ*recO* and
196 *recF*15 mutations (impaired in RecA nucleation and filament formation) only slightly affect (<1.4-
197 fold) the number of CFUs at mid-exponential phase (Fig. 1B), but the Δ*recA* mutation lead to a
198 strong reduction (~10-fold) (17, 34). Interestingly, the absence of RarA caused a ~15-, ~60- and
199 ~145-fold reduction in the number of CFUs at mid-exponential phase in the Δ*rarA recF*15, Δ*rarA*
200 Δ*recO* or Δ*rarA* Δ*recA* backgrounds, respectively, compared to the Δ*rarA* single mutant strain (Fig.
201 1B). Thus, there is a strong synthetic defect of combining the Δ*rarA* deletion with loss-of function
202 in RecA accessory proteins or most severely with loss of RecA itself. Similarly, the *E. coli* Δ*rarA*
203 Δ*recA* cells have low viability when compared to Δ*recA* cells (9), revealing a strong parallel in this
204 aspect.

205 Moving on with our analyses, we chose the double mutant strains with the lowest viability
206 (Δ*recO* Δ*rarA* and Δ*recA* Δ*rarA*). To investigate whether this reduced viability in Δ*recO* Δ*rarA* and
207 Δ*recA* Δ*rarA* correlates with membrane-compromised cells, two different fluorophores were used
208 (SYTO 9 and PI). Exponentially grown cells (OD_{560} = 0.4) were stained with SYTO 9 (in green)
209 and PI (in red). The proportion of exponentially growing *rec*$^+$ and Δ*rarA* cells stained with PI
210 (membrane compromised/dead) was low (~1% and ~1.8% of total cells, respectively). The
211 proportion of Δ*recO* and Δ*recA* cells stained with PI was 9.8% and 5.6% of total cells, respectively
212 (Fig. 1C). The absence of RarA increased the proportion of PI stained cells by only ~1.2 fold in
213 Δ*recA* cells, but the number increased by ~4-fold in the Δ*recO* background (Fig. 1C). Thus, the

214 strong decrease in CFUs in Δ*recA* Δ*rarA* cells (~145-fold) does not correlate with the number of
215 membrane compromised cells (7.1% of total cells), but it partially does in Δ*recO* Δ*rarA* cells (~60-
216 fold reduction in CFUs versus 39.2% PI staining cells) (Fig. 1C). These results show that Δ*recO*
217 Δ*rarA* and Δ*recA* Δ*rarA* double mutant strains show a gross cell proliferation defect (Fig. 1B) and
218 that RecO is crucial to alleviate the membrane compromise defect (Fig. 1C).

219 **Experimental approach for repair-by-recombination studies**

220 To gain further insight into the involvement of RarA in repair-by-recombination the double (triple
221 in case of Δ*rarA* Δ*addAB* or Δ*rarA* Δ*ruvAB*) mutant strains were exposed to DNA damaging agents,
222 for 15 min, at concentrations that are bacteriostatic to *rec*$^+$ cells growing in nutrient broth (NB)
223 medium. MMS and H_2O_2 were chosen, because both induce modifications in DNA bases, but in the
224 presence of Fe(II), H_2O_2 treatment additionally generates DNA nicks (35). MMS- or H_2O_2-damaged
225 bases are mainly repaired by direct DNA damage reversal, such as the guanine oxidation
226 prevention/repair system, base excision repair or mismatch repair (35-37). Unrepaired MMS- or
227 H_2O_2-lesions primarily halt elongation by the replicative DNA polymerase, and thereby stall
228 replication fork progression. Stalled forks can be repaired by different repair-by-recombination or
229 postreplication repair pathways (36-38). The H_2O_2 generated nicks collapsed replication forks, and
230 these intermediates can be repaired by different repair-by-recombination pathways (1, 27, 39). Our
231 previous work showed that RarA single mutants are very sensitive to H_2O_2-, but resistant to MMS-
232 induced lesions (17), showing that RarA deals differently with the effect of the two drugs.

233 We classified the different outcomes into "moderately sensitive" when the viability was reduced
234 less than 10^2-fold, into "sensitive" when it was reduced less than 10^3-fold, into "very sensitive"
235 when viability was reduced from more than 10^3-fold and up to 10^5-fold, and when the viability was

236 reduced more than 10^5-fold the mutant strain was considered "extremely sensitive" to the damaging
237 agent.

238 **RarA is not required for end-resection but affects the outcome of repair events in end-**
239 **resection mutants**

240 In *B. subtilis,* there are two alternative DNA end resection pathways: the AddAB complex, and RecJ
241 single-stranded exonuclease in concert with a RecQ-like DNA helicase (RecQ or RecS) (40). Both
242 machineries contribute to the processing of 5´-termini at both ends of the break, generating a 3´-
243 tailed duplex intermediate that is the substrate for RecA nucleation and filament growth, and the
244 latter also resects single strand gaps (27, 41). The lack of AddAB and RecJ renders cells extremely
245 sensitive to DNA-damaging agents, with a sensitivity similar to that of Δ*recA* cells (40), showing
246 that HR is no longer operative in their absence. In our experiments, Δ*addAB* mutations rendered
247 cells very sensitive and the Δ*recS*, Δ*recQ* and Δ*recJ* mutations cells sensitive to H_2O_2 or MMS
248 exposure (Fig. 2A and 3A) (40), suggesting a certain hierarchical order in the processing of the
249 broken molecules by the AddAB or RecJ-RecQ(RecS) complexes.

250 The acute lethal H_2O_2 dose that reduced Δ*rarA* cells survival by 99% (LD_{99}) was ~0.38 mM
251 (Table 2). A Δ*rarA* mutation rendered cells very sensitive to acute exposure to H_2O_2, with an LD_{99}
252 >16-fold lower than for the *rec*$^+$ control (Fig. 2, Table 2) (17). Curiously, the survival rate of Δ*addAB*
253 Δ*rarA* cells was increased ~12-fold when compared to the parental Δ*rarA* or Δ*addAB* strains (Fig.
254 2A, Table 2), suggesting that in the absence of both RarA and AddAB the recombinational
255 intermediates are channelled towards another repair pathway(s). The DNA repair defect of *rarA*
256 mutant cells was also partially suppressed when the mutation was combined with *recQ* or *recS*,
257 resulting in an LD_{99} to H_2O_2 that was ~5-fold higher than that of Δ*rarA* cells (Fig. 2A, Table 2).

258 Thus, Δ*addAB*, Δ*recQ* or Δ*recS* mutations suppressed the DNA repair defect of the Δ*rarA* mutation
259 upon exposure to H_2O_2.
260 The connection between *rarA* and *recJ* mutations was somewhat different than expected with
261 regard to the above mentioned mutations. The survival rate of Δ*recJ* Δ*rarA* was reduced ~9-fold
262 compared to Δ*recJ*, and the LD_{99} was comparable to that of the Δ*rarA* control (Fig. 2A, Table 2).
263 At a higher H_2O_2 dose a different outcome was observed. At 2 mM H_2O_2 the survival rate increased
264 ~4-fold, and at 4 mM of H_2O_2 the survival of the Δ*recJ* Δ*rarA* mutant strain increased ~17-fold
265 compared to the Δ*rarA* control (Fig. 2A), suggesting that the absence of *recJ* partially suppressed
266 the DNA repair defect of Δ*rarA* cells. The differences observed between the *recJ* and the other
267 functions involved in end-pprocessing in combination with Δ*rarA* could be due to the different
268 activities. RecJ is involved in base excision repair, methyl-directed mismatch repair and repair-by-
269 recombination (27, 39, 42), whereas no role other than repair-by-recombination has been described
270 for AddAB, RecQ or RecS (26, 27). In none of the cases of double mutant cells, we observed an
271 epistatic effect, nor strong synergistic effects. Therefore, we have to assume that RarA is not
272 required for end resection.
273 To further evaluate the contribution of RarA to end resection, exponentially growing cells were
274 acutely exposed to increasing MMS concentrations for 15 min (Fig. 3). The acute LD_{99} dose for
275 MMS for rec^+ cells (10 mM) was lower than that for Δ*rarA* cell (>50 mM) (Table 2), confirming
276 that in the absence of RarA, cells remain recombination proficient, and apparently more capable of
277 repairing MMS-induced DNA damage than *wt* cells (17). AddAB cells were very sensitive to MMS,
278 but the additonal mutation in *rarA* rescued this phenotype: the LD_{99} to MMS was increased by ~55-
279 fold in Δ*addAB* Δ*rarA* cells relative to the Δ*addAB* mutant strain (Fig. 3A, Table 2). The survival
280 rate in Δ*recS* Δ*rarA*, Δ*recJ* Δ*rarA* or Δ*recQ* Δ*rarA* was enhanced ~2-fold when compared to the
281 single Δ*recS*, Δ*recJ* or Δ*recQ* strains (Fig. 3A, Table 2). These findings suggest that inactivation of

282 *rarA* makes canonical DSB repair deleterious for cell survival, because the absence of functions
283 involved in long-range 5′→3′ end resection (e.g., AddAB, RecJ, RecQ, RecS) partially suppressed
284 the DNA repair defect of Δ*rarA* cells in response to H_2O_2 or MMS (Fig. 2A and 3A). This is in
285 agreement with a previous report showing that WRNIP1 is directly involved in preventing
286 uncontrolled MRE11-mediated degradation of stalled replication forks (13). The observed genetic
287 interactions are in line with the observation that exponentially growing Δ*addAB*, Δ*recS*, Δ*recQ* or
288 Δ*recJ* cells show strongly reduced RarA mobility (16), *i.e*. the activity of RarA with respect to its
289 binding to DNA is considerably altered in end-resection mutants.

290 **Branch migration or HJ processing of recombination intermediates activities do not**
291 **require RarA, but their loss partially suppress *rarA* phenotypes**

292 A branch migration translocase binds to HJs, formed as HR intermediates (double-HJ), or when
293 replication forks stall and reverse (HJ-like structure), and promotes HJ migration (43-45). When its
294 cognate site becomes available, the RecU resolvase cleaves the double-HJ, in concert with the
295 RuvAB translocase, to preferentially generate non-crossover products, and rarely crossover products
296 (postsynaptic step) (27, 42, 44-46). It is unknown whether RecU can cleave the reversed forks
297 generated by RecG in *B. subtilis*. In any event, RecU has two activities: to mediate HJ cleavage in
298 concert with a branch migration translocase (47), and to modulate RecA nucleoprotein filament
299 formation (48, 49).

300 In our assays, the Δ*recG*, Δ*ruvAB* and Δ*recU* mutations rendered cells very sensitive and the
301 Δ*radA* mutation sensitive to H_2O_2 or MMS exposure (Fig. 2B and 3B) (21, 33, 50, 51). The survival
302 rate to H_2O_2 of Δ*radA* Δ*rarA* or Δ*ruvAB* Δ*rarA* mutant cells was increased compared to the less
303 sensitive single mutant strain, with an LD_{99} to H_2O_2 ~12-fold or ~3-fold higher than the Δ*rarA* strain,
304 respectively (Fig. 2B, Table 2). The LD_{99} to H_2O_2 of the Δ*recG* Δ*rarA* or Δ*recU* Δ*rarA* mutant

305 strains was similar to the more sensitive single mutant strain (Fig. 2B, Table 2). However, at a H_2O_2
306 dose as high as 2 mM, the survival rate of Δ*recG* Δ*rarA* or Δ*recU* Δ*rarA* mutant strains increased
307 ~16-fold and ~25-fold relative to the Δ*rarA* strain (Fig. 2A), suggesting that Δ*recG* or Δ*recU*
308 partially suppressed the DNA repair defect at high H_2O_2 concentrations. When cells were acutely
309 exposed to increasing MMS concentrations (Fig. 3B), the sensitivity of Δ*recU* Δ*rarA*, Δ*recG* Δ*rarA*
310 and Δ*radA* Δ*rarA* cells to MMS was lower than that of the single mutants, with LD_{99} to MMS of
311 ~2-, ~2- and ~12-fold higher than the Δ*radA*, Δ*recG* and Δ*recU* mutant strains, but the LD_{99} of the
312 Δ*ruvAB* Δ*rarA* cells was similar to that of the Δ*ruvAB* strains (Fig. 3B). At MMS doses as high as
313 20 mM, the survival rate of Δ*ruvAB* Δ*rarA* mutant strain increased ~3-fold compared to the Δ*ruvAB*
314 control (Fig. 2A), suggesting that Δ*rarA* partially suppressed the DNA repair defect of Δ*ruvAB* cells
315 at moderate MMS concentrations.

316 Taken together, it can be stated that i) the absence of RuvAB, RecG, RadA/Sms or RecU
317 partially suppressed the acute sensitivity to high H_2O_2 concentrations of Δ*rarA* cells (Fig. 2B); ii)
318 the absence of RarA partially suppressed the repair defect seen in the absence of the branch
319 migration translocase (RadA/Sms) or of the HJ resolvase (RecU) upon exposure to MMS, but not
320 of RuvAB or RecG (Fig. 3B). This is consistent with the observation that in the absence of HJ-
321 processing enzymes, the static RarA population decreases in *ruvAB*, *recG* and *radA* cells, meaning
322 that RarA is less often bound to DNA, but increased in *recU* cells (16), i.e. RarA becomes more
323 engaged with DNA in cells lacking RecU.

324 RarA is epistatic to RecO and RecF in response to DNA damage

325 *In vitro*, *B. subtilis* RecA cannot nucleate on the SsbA-ssDNA complexes, and AddAB cannot
326 activate RecA to catalyze DNA strand exchange (52). The two-component mediator SsbA and RecO
327 (in conjunction with RecR), together with positive (RecF) and negative modulators (RecX, RecU),

328 load RecA on a ssDNA gap or a 3′-tailed duplex ssDNA, facilitate RecA filament growth and
329 activate RecA to catalyze DNA strand exchange *in vitro*, with SsbA, RecO, RecR, RecF and RecX
330 collectively acting *in vivo* (23, 53-55).

331 As previously shown, *recF*15 and Δ*recO* mutations rendered cells very sensitive to H_2O_2 or MMS
332 exposure (Fig. 2C and 3C) (22, 56). The double Δ*recO* Δ*rarA* or *recF15* Δ*rarA* mutant strains were
333 equally sensitive to H_2O_2 or to MMS as the more sensitive single mutant strain, suggesting epistasis
334 (Fig. 2C, 3C and Table 2). This is consistent with the observation that *rarA* is epistatic to *recA* in
335 response to H_2O_2- or MMS-induced DNA damage (17). Moreover, the ratio of DNA bound to freely
336 moving RarA is altered in Δ*recO* or *recF*15 cells upon exposure to DNA damaging agents (16),
337 showing that the genetic interaction is reflected in the presumed activity of RarA. As described for
338 *B. subtilis rarA* (Fig. 2C, 3C), eukaryotic WRNIP1 functions in the same pathway as the Rad51
339 mediator BRCA2 (13).

340 Δ*rarA* partially suppresses the DNA repair defect of Δ*recD2* or Δ*recX* cells to 341 treament with H_2O_2

342 The negative modulator RecX has been shown to disassemble RecA nucleoprotein filaments (23,
343 55), and preliminary data from our laboratory has suggested a similar role for RecD2, whose funstion
344 in HR is poorly understood (32, 57). Investigating the genetic connection between RarA and RecX
345 or RecD2, we found Δ*recX* and Δ*recD2* mutants to be sensitive to acute H_2O_2 or MMS exposure
346 (Fig. 2D and 3D), as described earlier (23, 32). The LD_{99} to H_2O_2 of the Δ*recD2* Δ*rarA* or Δ*recX*
347 Δ*rarA* double mutant strain was not significantly different that the Δ*rarA* strain (Fig. 2D, Table 2).
348 However, at a H_2O_2 dose as high as 2 mM, the survival rate of Δ*recX* Δ*rarA* or Δ*recD2* Δ*rarA* mutant
349 strain was increased ~4-fold or ~100-fold, respectively, compared to the Δ*rarA* control (Fig. 2A),
350 suggesting that Δ*recX* and Δ*recD2* partially suppress the DNA repair defect in the Δ*rarA* context at

351 high H_2O_2 concentrations. With respect to MMS treatment, the Δ*recD*2 mutation partially
352 suppressed the DNA repair defect of Δ*recD*2 Δ*rarA* cells (Fig. 3D, Table 2), whereas the Δ*recX*
353 Δ*rarA* strain was slightly more sensitive to MMS than the single Δ*recX* mutant strain (Fig. 3D, Table
354 2). Thus, while the *rarA* deletion has a suppressor phenotype to high H_2O_2 concentrations with
355 regards to *recX* and *recD2* deletions, Δ*recX* Δ*rarA* cells show higher sensitivity to MMS treatment
356 than the Δ*recX* control (Fig. 3D). Interestingly, RarA dynamics decreased in the Δ*recX* strain (RarA
357 was more stronlgy bound to DNA than in *wt* cells), and the opposite behaviour was observed in the
358 Δ*recO* or *recF*15 backgrounds (16). Thus, there is a strong connection between RecX and RarA in
359 a genetic and cell biological aspect.

360 The threshold for maximal RecA levels after DNA damage is increased in Δ*rarA* cells

361 The previous results suggest that RarA has two roles: it may protect DNA from deleterious action
362 of recombination proteins, and additionally it may work as a RecA accesory protein. *In vitro, B.*
363 *subtilis* RecA·ATP cannot nucleate onto SsbA coated ssDNA, and cannot catalyze DNA strand
364 exchange between circular ssDNA and linear duplex in the absence of accessory factors (52, 58,
365 59). Thus, RecA activity is regulated by accessory proteins (28). Accessory factors can be divided
366 into two general groups: mediators that act before and the modulators that act during homology
367 search and the DNA strand exchange reaction (presynaptic step) (27, 28). Mediators and modulators
368 can be further divided into two classes, acting positively or negatively on RecA nucleation and/or
369 filament growth (60). The mediators and modulators are partially conserved between *B. subtilis* and
370 the genetically distant *E. coli*. For example, DinI, which antagonizes the role of RecX, and RdgC,
371 which inhibits RecA-dependent LexA autocleavage, are missing in *B. subtilis* cells. Also different
372 from *E. coli*, none of the *B. subtilis* mediators and modulators are part of the SOS response (27, 61).

373 Damages in the DNA template block DNA replication in a concentration dependent manner, 374 leading to extended ssDNA regions coated by SsbA. *B. subtilis* RecA·ATP acts as a sensor of 375 excessive ssDNA, and with the help of mediators, it assembles onto the SsbA-coated ssDNA to 376 generate RecA* (a right-handed RecA·ATP nucleoprotein filament) that conducts all the catalytic 377 steps of HR (23, 53, 54)), with the help of the RecF, RecX and RecU modulators (23, 53, 54). Then, 378 different dynamic RecA* filaments chaperone the LexA transcriptional repressor, and facilitate its 379 auto-cleavage (62), thereby de-repressing ~33 genes (*recA* among them) (61), and activating the 380 SOS response (63). A more general RecA-dependent DNA damage response is triggered following 381 MMC-induced replication arrest, with ~140 genes showing altered expression, including LexA-382 dependent (*e.g*., *ruvA* gene) and LexA-independent (*e.g*., *recN* gene) genes (64, 65).

383 Exponentially growing cells were estimated to contain ~4,800 RecA monomers/CFU as judged 384 by Western blot (Fig. 4A) and by integrated mass spectrometry and 2-D gel-based proteomics 385 analyses (66). This is good agreement with the literature (23, 65). In rec^{+} cells, RecA reached its 386 maximal level of expression at ~0.6 μM MMC, and its maximal induction caused a ~5-fold increase 387 to 26,000 ± 1,000 RecA/CFU (Fig. 4A), similar to what was shown before (26,000 ± 1,000) in the 388 wt as well as in the Δ*lexA* background (23, 65), suggesting that this MMC concentration provides 389 the DNA damage threshold necessary to fully de-repress RecA expression. Under similar 390 experimental conditions, *recA* promoter utilization increased 6- to 10-fold (67). For comparison, 391 undamaged *E. coli* cells have 7,000 - 15,000 RecA monomers/cell and these levels increase to 392 ~100,000 RecA/cell upon DNA damage (68). When MMC was replaced H_2O_2, similar RecA 393 expression levels were observed, but here the correlation between RecA accumulation and H_2O_2 394 concentrations were less pronounced (65).

395 Two different outcomes can be envisioned upon addition of increasing MMC concentrations in 396 the absence of a RecA mediator or modulator. First, in the absence of a mediator or a positive

397 modulator, a negative RecA modulator will promote a net RecA filament disassembly, with
398 subsequent reduction in the probability of LexA repressor autocleavage. Thus, a higher MMC dose
399 should be required to reach maximal RecA expression levels. Secondly, in the absence of negative
400 modulators, the positive mediators and/or modulators will facilitate RecA filament assembly, so that
401 the probabilities of RecA filament increase as well as the interaction with LexA. Thus, a lower dose
402 of DNA damage should be sufficient for RecA to stimulate LexA auto-cleavage, so maximal RecA
403 levels are obtained at lower MMC doses in the absence of negative regulators. For example, in the
404 absence of the positive modulator RecF, an MMC dose higher than the one needed in the *rec*$^{+}$ control
405 was required to maximal RecA expression levels, but in the absence of negative modulator RecX, a
406 lower MMC dose was sufficient (Fig. 4A) (23, 55).

407 We then tested whether RarA contributes to RecA nucleoprotein filament formation and
408 compared its RecA levels with that in the absence of RecO (positive mediator) or RecF (positive
409 modulator). In uninduced Δ*rarA*, Δ*recF*15 or Δ*recO* cells, RecA levels were maintained at a similar
410 basal level estimated to be 4,600 ± 1,200 RecA monomers/CFU during mid-log phase of cell growth
411 (Fig. 4A). The absence of RarA reduced maximal RecA levelsn (from ~26,000 to 16,000 ± 900
412 RecA/CFU) that were reached at ~0.75 µM MMC, and did not barely change at 1.5 µM MMC (Fig.
413 4A). Similarly, a higher MMC dose is necessary to facilitate maximal RecA expression in cells
414 impaired in the RecF modulator, but no increase is observed in cells lacking RecO (Fig. 4A) (23).
415 Because RarA and RecO both interacts with SsbA rather than with RecA (18), it is unlike that RarA
416 binds to the RecA filament and competes with LexA binding, preventing its autocleavage. Thus, we
417 can exclude this alternative explanation for a higher MMC dose required for maximal RecA
418 expression levels, suggesting that RarA is a true mediator or modulator of RecA, and that it faciliates
419 and/or stabilises RecA filaments onto ssDNA.

420 **RarA is required for efficient RecA filament formation *in vivo***

421 To analyse whether RarA participates in RecA nucleation or facilitates RecA filament growth, we
422 used a functional RecA-mVenus (mVenus is a variant of fluorescent protein YFP), for the
423 visualization of RecA filaments (termed "threads") in live cells. The C-terminal fusion was
424 integrated at the original gene locus, such that the fusion is the sole source of RecA expresed in
425 cells, under the control of the original promoter. The RecA-mVenus fusion is repair proficient, as
426 the RecA-mVenus strain was as viable as wild type cells after induction of DNA damage, in contrast
427 to the highly sensitive *recA* deletion strain. RecA-mVenus changed from a localization pattern
428 throughout the cells ("diffuse") or at discrete spots to form striking filemantous structures upon
429 induction of DNA damage (Fig. 5). These filamantous structures have been described before (69)
430 and were termed "threads", because it is still unclear if these structures correspond to RecA-ssDNA
431 observed *in vitro*. Although evidence for this notion has been described (70), we will maintain the
432 term "threads" to describe the structures observed by epifluorescence microscopy. Formation of
433 RecA threads was maximal 40 min after induction of DNA damage, and thereafter, threads
434 dissipated in favour of the diffuse or spot-lioke localization seen in the absence of DNA damage
435 (Fig. 5). Strikingly, even at 40 min after addition of H_2O_2, Δ*rarA* mutant cells only showed the RecA
436 patch- or spot-like structures that occasionally had short filamentous extensions (Fig. 6A). The
437 failure to form discrete RecA threads can be most conveniently seen in the demographs (Fig. 6B),
438 which do not reflect different levels of RecA-mVenus, but visualize the presence or absence of
439 sharply contrasted fluorescent structures, *i.e.* RecA threads. In order to follow the dynamics of
440 formation of RecA threads, we scored the number of cells containing diffusely localized RecA,
441 RecA spots or RecA threads, during exponential growth (no damage) or in 10 min intervals
442 following damage induction. Fig. 6C shows that while less than 10% of exponentially growing cells

443 contained visible RecA threads or spots (no damage), ~65% of cells contained RecA threads and
444 ~15% RecA spots as early as 20 min after addition of H_2O_2, which declined thereafter back towards
445 the pattern seen in untreated cells. In stark contrast, only a maximum of ~15% of Δ*rarA* cells
446 contained RecA threads, but ~60% RecA spots only. Assuming that the accumulation of RecA into
447 spots represents RecA loading events onto ssDNA, and the formation of threads extended filament
448 formation, we can propose that RarA plays an important role in the formation of RecA threads by
449 promoting the extension of filaments, stabilizing the RecA nucleprptein filament or by
450 downregulating the activity of negative modulators. Thus, RarA plays a dual role during HR, in
451 addition to its activity in replication re-initiation (15), it also strongly affects the formation of RecA
452 threads, which have been shown to be the active form of RecA during HR (69).

453 **RarA counteracts the action of RecU and RecX modulators**

454 Previously, it has been shown that inactivation of *recX* reversed the effect of the *recF*15 mutation
455 with regard to the level of RecA, with RecA levels comparable to *rec*$^+$ cells (23). We favour the
456 view that RarA acts as an antagonizer of RecX and/or of RecU. In the absence of MMC, RecA levels
457 were estimated to be 4,600 ± 1,200 RecA monomers/CFU in Δ*recU* cells (Fig. 4A). As expected for
458 a negative modulator, a significant net RecA accumulation was observed upon exposure to low
459 MMC concentrations in Δ*recU* cells. As low as 0.07 μM MMC already increased RecA levels, and
460 the maximal level of RecA accumulation was reached at ~0.3 μM MMC (26,000 ± 1,100
461 RecA/CFU) (Fig. 4A). Similar results were observed in the absence of the negative modulator RecX
462 (Fig. 4A) (23, 55). To test whether RarA may antagonize the action of RecX or RecU, the expression
463 levels of RecA were measured in Δ*recX* Δ*rarA* or Δ*recU* Δ*rarA* cells. The basal level of RecA in
464 the Δ*recU* Δ*rarA* and Δ*recX* Δ*rarA* strains was slightly lower than in the *rec*$^+$ cells (~4,100 RecA

465 monomers/CFU) (Fig. 4B). In the presence of increasing MMC, RecA expression in Δ*recU* Δ*rarA*
466 or Δ*recX* Δ*rarA* cells was similar to *rec*$^{+}$ cells up to 0.15 μM MMC, but no further increase was
467 observed at higher MMC concentrations (Fig. 4B). These results show that the absence of RarA
468 partially counteracted the effect of the absence of RecU or RecX, and it reduced the maximal rate
469 of RecA accumulation in Δ*recU* Δ*rarA* (10,000 ± 1,200 RecA /CFU) or Δ*recX* Δ*rarA* (8,400 ± 900
470 RecA /CFU) cells (Fig. 4A-B), suggesting that the absence of RarA counteracts the inactivation of
471 *recU* or *recX*. This is consistent with the observation that RarA focus formation and its dymanic
472 interaction with RecO and RecF differs from those with RecX; foci formation observed in the latter
473 was decreased while in the other two mutant strains it was enhanced compared to *wt* cells, and
474 additionally, it occurred earlier with regard to damage induction. This may be related to the
475 formation of RecA-ssDNA nucleoprotein filaments, which is facilitated by RecF (16).

476 **RarA acts as a positive contributor to RecA filament formation**

477 To test whether RarA works as a positive modulator of RecA, its expression levels were measured
478 upon exposing *recF*15 Δ*rarA* cells to increasing MMC concentrations (Fig. 4B). The RecA basal
479 level of *recF15* Δ*rarA* cells was slightly lower than in the *rec*$^{+}$ cells (~4,100 ± 900 RecA
480 monomers/CFU) (Fig. 4B). In the double mutant background increasing concentration of MMC
481 failed to stimulate RecA expression (~3,900 RecA/CFU) above the RecA basal levels (Fig. 4B),
482 suggesting that RecF and RarA might work as a alternative positive modulators. In the absence of
483 both RarA and RecF modulators, RecA can nucleate onto SsbA-coated ssDNA by the action of
484 RecO, but these short filaments are likely destabilized by RecX and/or RecU.

485 The estimation of the RecA basal level in the Δ*recO* Δ*rarA* strain generated uncertainties (~3200
486 ± 1900 RecA/estimated cell) due to the low viability of the Δ*recO* Δ*rarA* strain (see Fig. 1B). The
487 strain was not further analyzed.

488 **Conclusions**

489 Genetic analyses reveal that RarA acts in the context of arrested replication forks in conjunction
490 with a network of proteins that affect the activity of the RecA recombinase. Our work indicate that
491 RarA prevents uncontrolled DNA end resection and processing of stalled replication forks, with
492 subsequent fork reversion by the action of branch migration translocases (Fig. 2A-B and 3A-B).

493 Most importantly, we show that RarA positively regulates RecA filament extension, and
494 apparently counteracts the role of the negative RecA modulators. The *rarA* gene is epistatic to *recO*
495 or *recF* in response to DNA damage. There is a genetic interaction between *rarA* and *recX* and *recU*
496 because inactivation of *recU* or *recX* partially suppresses the defect of Δ*rarA* gene in response to
497 H_2O_2-induced DNA damage, but *rarA* is not epistatic to *recX* in response to MMS-induced DNA
498 damage (Fig 2B and D and 3B and D). These data are consistent with single molecule tracking
499 suggesting that one of the RarA functions is related to RecA and its accessory proteins (16). It has
500 been proposed that dynamic interactions of RarA with RecO and RecF differ from those with RecX
501 and RecU (16). When DNA is damaged, the RecA threads persist for a longer time in the Δ*recX*
502 cells (23), but there is a reduced number of RecA threads persistent in the Δ*rarA* cells (Fig. 5C). We
503 propose that RarA contributes to RecA filament extension in concert with the positive RecF
504 modulator, and both might counteract the role of the negative modulators RecX and RecU that
505 promote RecA filament disassembly, in order to protect stalled forks and prevent their degradation.
506 Our data are consitent with the observation that downregulation of FBH1, which is responsible for
507 the removal of RAD51 from chromatin, can compensate for loss of WRNIP1 activity, reinforcing

508 the hypothesis of a possible function of WRNIP1 in stabilizing RAD51 upon a direct protein-protein
509 interaction (13). Like eukaryotic WRNIP1 whose absence leads to extensive degradation of nascent
510 DNA strands (13), inactivation of *rarA* renders cells very sensitive to to H_2O_2-induced lesion, but
511 deletion of DNA end resection pathways partially suppresses the DNA repair defect (Fig. 2A and
512 3A). Our data thus show that there are strong parallels between eu- and prokaryotic RarA-type
513 proteins, and increase knowledge on the function of bacterial RarA at a molecular level. It will be
514 interesting to analyse if RarA directly interacts with RecA, or *via* a RecO-SsbA-RarA interaction
515 (see Introduction).
516

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722

723 **Figures**

724 Figure 1. Growth defects of the Δ*rarA* Δ*recO* and Δ*rarA* Δ*recA* strains. (A and B) Cells were grown
725 in NB to reach exponential phase (OD_{560}=0.4) serially diluted, plated on NB agar, incubated ON
726 and counted as CFU. (C) Cells were grown in NB to reach exponential phase (OD_{560}=0.4). The cells
727 were stained with SYTO 9 (green bar) and PI (red bar) to count the number of live and dead cells
728 respectively. Percentage of SYTO 9- and PI-stained cells are indicated. 100% corresponds to the
729 sum of green and red cells. The results are the average of at least three independent experiments and
730 standard errors of the mean are indicated.

731

732 Figure 2. Acute viability assays of Δ*rarA* double mutant strains upon exposure to H_2O_2. Lack of
733 RarA in cells impaired in end resection (A), in processing of recombination intermediates (*B*), in
734 RecA accessory proteins (C-D) or in Δ*recA* context (D). Cells were grown to reach exponential
735 phase (OD_{560}=0.4), exposed to different concentrations of H_2O_2 for 15 min prior to serial dilutions.
736 Cells were counted as CFU after ON growth, and results are plotted dividing these CFUs by the
737 CFU obtained in untreated cells. The results are the average of at least three independent experiments
738 and standard errors of the mean are indicated.

739

740 Figure 3. Acute viability assays of Δ*rarA* double mutant strains upon exposed to MMS. Lack of
741 RarA in cells impaired in end resection (A), processing of recombination intermediates (*B*), in RecA
742 accessory proteins (C-D) or lack RecA (D). Cells were grown to reach exponential phase
743 (OD_{560}=0.4), exposed to different concentrations of MMS for 15 min prior to serial dilutions. Cells
744 were counted as CFU after ON growth, and results are plotted dividing these CFUs by the CFU

745 obtained in untreated cells. The results are the average of at least three independent experiments and
746 standard errors of the mean are indicated
747
748 Figure 4. RecA protein accumulation upon SOS induction in different genetic backgrounds.
749 Exponential grown *wt* (*rec*$^{+}$), Δ*recX*, Δ*recU*, *recF*15, Δ*recO* and Δ*rarA* cells (A) or *wt*, Δ*recX* Δ*rarA*,
750 Δ*recU* Δ*rarA* and *recF*15 Δ*rarA* cells (B) were exposed to the indicated concentrations of MMC for
751 30 min. Then cells were collected, lysed and equivalent protein amounts subjected to 10% SDS-
752 PAGE, followed by immunoblot transfer. The number of RecA molecules/CFU are derived from a
753 standard curve of known RecA concentrations and are the average of at least three independent
754 experiments and standard errors of the mean are indicated.
755
756 Figure. 5. Time course of RecA assembly into discrete spot and extended filamentous structures
757 called "threads" after 0.5 mM H_2O_2 addition. Subcellular localization of RecA-mV after 10 min
758 intervals after H_2O_2 treatment in *wt* cells. Scale bars 5 µm.
759
760 Figure. 6. Epifluorescence microscopy showing that RecA assembly into threads is dependent on
761 RarA. (A) Subcellular localization of RecA-mV 40 min after treatment with 0.5 mM H_2O_2, in *wt*
762 (*rec*$^{+}$) and in Δ*rarA* mutant cells. Scale bars 5 µm. (B) Demographs of *B. subtillis* cells,
763 demonstrating the localization of RecA-mV to the middle regions. Cells were aligned and ordered
764 according to size. The fluorescence profiles represent the mean fluorescence values along the medial
765 axis after background subtraction and normalization such that the maximum fluorescence of each
766 cell is equal. C) Quantitative analysis of RecA thread formation in *wt* or *rarA* mutant cells.
767

768 **Tables**

769 Table 1. *Bacillus subtilis* strains used

Strains	Relevant genotype[a]	Source	Strains	Relevant genotype[a]	Source
BG214	*rec*$^+$	Lab. strain	BG1067	+ Δ*rarA*	(16)
BG190	+ Δ*recA*	(71)	BG1555	+ Δ*recA* Δ*rarA*	(16)
BG439	+ Δ*recO*	(56)	BG1433	+ Δ*recO* Δ*rarA*	(16)
BG129	+ *recF15*	(22)	BG1055	+ *recF15* Δ*rarA*	(16)
BG1455	+ Δ*recD2*	(32)	BG1421	+ Δ*recD2* Δ*rarA*	(16)
BG1065	+ Δ*recX*	(23)	BG1371	+ Δ*recX* Δ*rarA*	(16)
BG1337	+ Δ*addAB*	(40)	BG1107	+ Δ*addAB* Δ*rarA*	(16)
BG675	+ Δ*recJ*	(40)	BG1059	+ Δ*recJ* Δ*rarA*	(16)
BG705	+ Δ*recQ*	(40)	BG1575	+ Δ*recQ* Δ*rarA*	(16)
BG425	+ Δ*recS*	(40)	BG1563	+ Δ*recS* Δ*rarA*	(16)
BG855	+ Δ*recU*	(72)	BG1083	+ Δ*recU* Δ*rarA*	(16)
BG1131	+ Δ*recG*	(21)	BG1103	+ Δ*recG* Δ*rarA*	(16)
BG703	+ Δ*ruvAB*	(33)	BG1351	+ Δ*ruvAB* Δ*rarA*	(16)
BG1245	+ ΔradA	(31)	BG1373	+ Δ*radA* Δ*rarA*	(16)
PG5142	+ *recA-yfp*[b]	This work	PG5143	+ *recA-yfp* Δ*rarA*	This work

770 [a]All strains are derivatives of *B. subtilis* BG214 (*trpCE metA*5 *amyE1 ytsJ*1 *rsbV*37 *xre*1 *xkd*A1

771 *att*^{SPß} *att*$^{ICE\mathit{Bs}1}$). [b]RecA-mVenus is a variant of the monomeric RecA-Yfp protein.

772

773 Table 2. LD_{99} to H_2O_2 and MMS of different *Bacillus subtilis* mutant strains

Relevant genotype	LD_{99} to H_2O_2[a] in mM	LD_{99} to MMS[a] in mM	Relevant genotype	LD_{99} to H_2O_2[a] in mM	LD_{99} to MMS[a] in mM
rec$^+$	>6.0	41.2	Δ*rarA*	0.38	>50
Δ*addAB*	0.46	0.8	Δ*addAB* Δ*rarA*	4.5	44.0
Δ*recJ*	4.3	2.2	Δ*recJ* Δ*rarA*	0.47	4.6
Δ*recQ*	2.4	2.4	Δ*recQ* Δ*rarA*	1.9	4.7
Δ*recS*	4.4	2.3	Δ*recS*,Δ*rarA*	2.0	4.8
Δ*recU*	0.45	1.7	Δ*recU* Δ*rarA*	0.47	21.3
Δ*recG*	0.44	2.2	Δ*recG* Δ*rarA*	0.53	4.8
Δ*ruvAB*	0.64	4.0	Δ*ruvAB* Δ*rarA*	1.0	5.0
Δ*radA*	2.0	17.1	Δ*radA* Δ*rarA*	4.7	36.8
Δ*recO*	0.37	0.6	Δ*recO* Δ*rarA*	0.37	0.9
recF15	0.37	0.7	*recF15* Δ*rarA*	0.37	0.8
Δ*recD2*	1.9	36.6	Δ*recD2*, Δ*rarA*	0.52	43.0
Δ*recX*	0.8	10.6	Δ*recX* Δ*rarA*	0.40	7.6

774 [a]The acute lethal dose to H_2O_2 or MMS that reduced cells survival by 99% (LD_{99}) upon 15 min exposure.

775

776 **Conflict of Interest**

777 The authors declare that the research was conducted in the absence of any commercial or financial
778 relationships that could be construed as a potential conflict of interest.

779 **Author Contributions**

780 H.R., S.A., P.L.G. and J.C.A designed the experiments. H.R., E.S., R.H-T., P.P.C., S.A., P.L.G.
781 and J.C.A. planned experiments and interpreted data; H.R., E.S., R.H-T., P.P.C. and S.A.
782 performed the experiments; H.R., E.S., R.H-T., S.A., P.L.G. and J.C.A. drafted the manuscript;
783 and S.A., P.L.G. and J.C.A. wrote the manuscript.

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