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1 **Bacterial NHEJ: A never ending story**

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16 ***Mycobacterium***

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19

20 **Abstract**

21 Double-strand breaks (DSBs) are the most detrimental DNA damage encountered by bacterial
22 cells. DSBs can be repaired by homologous recombination thanks to the availability of an intact
23 DNA template or by Non-Homologous End Joining (NHEJ) when no intact template is available.
24 Bacterial NHEJ is performed by sets of proteins of growing complexity from *Bacillus subtilis* and
25 *Mycobacterium tuberculosis* to *Streptomyces* and *Sinorhizobium meliloti*. Here we discuss the
26 contribution of these models to the understanding of the bacterial NHEJ repair mechanism as
27 well as the involvement of NHEJ partners in other DNA repair pathways. The importance of NHEJ
28 and of its complexity is discussed in the perspective of regulation through the biological cycle of
29 the bacteria and in response to environmental stimuli. Finally, we consider the role of NHEJ in
30 genome evolution, notably in horizontal gene transfer.

31 I- Bacterial NHEJ: A two-component DNA repair mechanism?

32 Until *in silico* identification of putative NHEJ actors in bacterial genomes, it was thought that
33 Homologous Recombination (HR) was the major double-strand break (DSB) repair mechanism in
34 prokaryotes. However, HR needs an intact template to ensure repair of the break, a condition
35 encountered by bacteria only during their dividing state and raising the question of the repair of
36 DSB in other phases of their life cycle. The identification of a bacterial NHEJ answered, at least in
37 part, this question since, in eukaryotes, NHEJ was known to ligate DNA ends together without
38 any homologous template. This mechanism has been well documented in eukaryotes and
39 involves a dozen of actors in human, including the Ku70/Ku80 heterodimer, and the ligase IV
40 (Chang *et al.*, 2017).

41 The first hint for the existence of a bacterial NHEJ was provided by *in silico* approaches leading
42 to the identification of Ku70/80 homologs and ATP-dependent DNA-ligase homologs (Doherty *et*
43 *al.*, 2001; Aravind and Koonin, 2001). These Ku and DNA-ligase encoding genes are often
44 colocalized, or in an operonic structure. The DNA-ligase genes encode a putative ligase domain
45 (LigDom or LIG), fused or not with a polymerase domain (PolDom, POL or Prim-Pol) and/or a
46 nuclease domain (NucDom or PE for Phosphoesterase). Whatever the domain combination,
47 these multifunctional ligases were collectively named LigD, in reference to the main NHEJ ligase
48 in Mycobacteria (Gong *et al.*, 2005).

49 The first experimental validation of a bacterial NHEJ came from the study of Weller *et al.* showing
50 that *in vitro* the *Mycobacterium tuberculosis* LigD protein (LigD_{Mtub}) is indeed an ATP-dependent
51 DNA ligase that is stimulated by its cognate Ku_{Mtub} partner, possibly via an interaction between
52 Ku_{Mtub} bound to DNA and LigD_{Mtub} (Weller *et al.*, 2002). In parallel, authors described the

53 deleterious effect of the deletion of the *Bacillus subtilis* genes coding for LigD_{Bsub} and Ku_{Bsub}
54 (*ykoU-ykoV* operon) on the survival of cells in stationary phase after ionizing radiation (IR). IR is
55 a cause of multiple DNA lesions, including DSBs that are particularly hazardous. These two
56 proteins from *B. subtilis* have been later shown to also perform NHEJ repair *in vitro* (de Vega,
57 2013). The Ku and/or LigD-dependent ability of *M. tuberculosis* and *Mycobacterium smegmatis*
58 to recircularize linear plasmid DNA after transformation, and the IR sensitivity of *M. smegmatis*
59 *ku* and *ligD* mutants in stationary phase, have given further evidence *in vivo* that several bacterial
60 species are NHEJ proficient (Stephanou *et al.*, 2007).

61 LigD_{Mtub} is not a common ligase. Its PolDom provides LigD_{Mtub} with DNA-dependent DNA
62 polymerase, DNA-dependent RNA polymerase and primase and non-templated nucleotide
63 addition activities (Della *et al.*, 2004). Moreover, the Poldom interacts *in vitro* with Ku_{Mtub}-DNA
64 complex suggesting that it could be responsible for the recruitment of LigD_{Mtub} on Ku-bound DSB
65 *in vivo* (Pitcher *et al.*, 2005). LigD_{Mtub} also shows a 3'-5' exonuclease activity most probably carried
66 by its NucDom (Della *et al.*, 2004). LigD from *Pseudomonas aeruginosa* (LigD_{Paer}) and
67 *Agrobacterium tumefaciens* (LigD2) display similar activities accordingly to their identical domain
68 composition, though different domain organization, compared to LigD_{Mtub} (Zhu and Shuman,
69 2005a; Zhu and Shuman, 2005b; Zhu *et al.*, 2005; Zhu and Shuman, 2007). Thus, LigD displays all
70 activities allowing to process DNA ends, if necessary, and ligate them without the need for other
71 proteins, except Ku. Accordingly, expression of Ku and LigD from *M. tuberculosis* i) partially
72 restores NHEJ in yeast *ku70* or *dnl4* mutant strains (Della *et al.*, 2004) and ii) renders *Escherichia*
73 *coli* NHEJ-proficient (Malyarchuk *et al.*, 2007).

74 Altogether, these studies have led to the following model for the bacterial NHEJ: Ku homodimer
75 binds to DNA ends generated by a break and recruits LigD (one multidomain polypeptide, such

76 as LigD_{Mtub}, or a holoenzyme consisting of several subunits similar to the LigDom, PolDom and/or
77 NucDom of LigD_{Mtub}) probably via a direct physical interaction on the DNA between Ku and the
78 PolDom of the LigD holoenzyme. LigD is able to process DNA ends, if broken ends are non-directly
79 ligatable, thanks to its Poldom and/or Nucdom, before ligation by LigDom (Pitcher *et al.*, 2007a).

80 Most of the past studies on bacterial NHEJ have already been extensively reviewed (Bowater and
81 Doherty, 2006; Pitcher *et al.*, 2007a; Shuman and Glickman, 2007; Gu and Lieber, 2008; Brissett
82 and Doherty, 2009), with the exception of the most recent ones on structural data of different
83 LigD domains and mechanistic details of DNA end processing by NHEJ actors before ligation (Nair
84 *et al.*, 2010; Zhu and Shuman, 2010; Brissett *et al.*, 2011;; Brissett *et al.*, 2013). The archaea
85 *Methanocella paludicola* contain genes coding for homologs of PolDom, NucDom, LigDom and
86 Ku. Their characterization have also deepened our knowledge on the processing of DNA ends
87 during NHEJ (Bartlett *et al.*, 2013; Brissett *et al.*, 2013; Bartlett *et al.*, 2016). In the present report,
88 we wished to focus on recent data obtained with different bacterial models highlighting the
89 complexity of the prokaryotic NHEJ pathway in terms of involved actors, their interactions, their
90 regulation, their links with other DNA repair pathways and finally their impact on bacterial
91 evolution.

92 **II- Multiple components NHEJ pathways**

93 The above model of minimal NHEJ, with one Ku and one LigD holoenzyme performing bacterial
94 NHEJ, does not take into account the existence of other proteins involved in this mechanism.
95 These proteins can be orthologues of Ku or of domains of the LigD holoenzyme, and/or proteins
96 interacting with them. Figure 1 illustrates diverse levels of NHEJ complexity in four bacterial
97 models.

98 **NHEJ complexity: multiplicity of NHEJ-like genes**

99 A recent and large scale search for *ku*-like genes suggests the existence of a potential NHEJ repair
100 system in about 25% of the prokaryotes for which a genome was available in public databases
101 (McGovern *et al.*, 2016), with a scattered distribution. Among the bacterial species predicted to
102 possess a NHEJ repair system, some actually encode several putative Ku homologs, mainly
103 actinobacteria of the streptomycetes family, and proteobacteria of the α subdivision (McGovern
104 *et al.*, 2016). For instance, more than 70% of *Streptomyces* species encode more than one *ku*
105 gene (up to 4 in *Streptomyces coelicolor*) (Hoff *et al.*, 2016), and more than half of α -
106 proteobacteria possessing a *ku* gene actually have two or more (up to 6 in *Rhizobium*
107 *leguminosarum bv trifolii*). Some of the *ku* genes are located on plasmids, suggesting their
108 putative acquisition via horizontal gene transfer. Importance of multiple *ku* genes in some NHEJ
109 proficient bacteria is discussed further below.

110 Putative NHEJ ligases are also often present in multiple copies. However, because of the more
111 variable organization of LigD, it is more difficult in this case to predict whether the genes encode
112 *bona fide* NHEJ ligases. This difficulty is reinforced by the fact that several ATP-dependent DNA
113 ligases can be used to perform NHEJ in the same bacterium. For instance, *M. smegmatis* encodes
114 four ATP-dependent DNA ligases (LigB_{Msm}, LigC1_{Msm}, LigC2_{Msm} and LigD_{Msm}) (Gong *et al.*, 2005).
115 Among them, LigD_{Msm} is the main NHEJ ligase and contains the PolDom, LigDom and NucDom
116 domains, as its homolog LigD_{Mtub}. However, LigC1_{Msm} (a LigDom-only enzyme) can act with Ku in
117 an alternative NHEJ in *M. smegmatis* (Gong *et al.*, 2005; Akey *et al.*, 2006; Aniukwu *et al.*, 2008;
118 Bhattarai *et al.*, 2014). This LigC1_{Msm}-dependent alternative NHEJ requires the PolDom of
119 LigD_{Msm}, the LigDom and NucDom being dispensable for this mechanism (Bhattarai *et al.*, 2014).
120 This result, supplemented by the fact that the PolDom of LigD_{Msm} plays a structural role

121 independent of its polymerase activity in the efficiency of recircularization of linearized plasmid
122 *in vivo* (Aniukwu *et al.*, 2008), highlights that the PolDom could serve as an interaction platform
123 for an ATP-dependent ligase protein containing only a LigDom. Other LigC proteins, LigC1 and
124 LigC2 from *A. tumefaciens* are endowed with ligase activities that are stimulated by Ku (Zhu and
125 Shuman, 2007), suggesting that alternative NHEJ pathways are conserved across bacterial
126 species. It has to be noticed that *M. smegmatis* contains two *polD* genes expressing two PolDom-
127 only polymerases that can suppress partially the sensitivity to IR of a mutant encoding a
128 polymerase deficient LigD, suggesting that, as observed with LigC1, several PolDom proteins can
129 be used in the NHEJ pathway in Mycobacteria (Bhattarai *et al.*, 2014).

130 In *Streptomyces*, PolDom, LigDom and NucDom only exist as stand-alone domains and not as
131 multifunctional proteins, making even more complex the identification of actual NHEJ partners.
132 The analysis of 38 *Streptomyces* genomes revealed a conserved gene set, composed of two loci,
133 *kuA* and its neighbor *polK* and *ligC* (encoding a putative ligase displaying an higher level of
134 homology with LigC1 from *M. smegmatis* compared to LigD_{M_{sme}}) and its neighbor *polR* (*polK* and
135 *polR* being two PolDom encoding genes). Surprisingly, *ligD*, whose product is the closest homolog
136 of LigD_{M_{tub}}, is not ubiquitous in these genomes (present in 75% of the analyzed species),
137 suggesting that the core *ligC* gene provides the main NHEJ ligase function. The variable set of
138 *Streptomyces* NHEJ-like genes also includes *kuB* and *kuC*, the latter often being plasmid borne
139 and often laying in the close vicinity of *ligD*. Although they are not conserved, these genes take
140 part in DNA repair since their mutation in *Streptomyces ambofaciens* confers spore sensitivity to
141 IR and increases the sensitivity of the core gene set mutants (Hoff *et al.*, 2016). In *S. ambofaciens*,
142 the involvement of KuA in a NHEJ system has been demonstrated together with the existence of

143 a secondary illegitimate DSB repair system that could imply these variable genes (Hoff *et al.*,
144 2018).

145 The *Streptomyces* organization also questions the role of the two conserved PolDom encoding
146 genes (*polK* and *polR*). In this regard, *Streptomyces* is not an exception since *M. smegmatis* (see
147 above and below, PolD1, PolD2) and numerous eukaryotes, especially vertebrates, also possess
148 several polymerases involved in NHEJ (Ramsden and Asagoshi, 2012). A first hypothesis to explain
149 this observation is based on the difference in expression profile: indeed, *S. ambofaciens polR*
150 seems constitutively expressed whereas *polK* transcription was not detected in the tested
151 conditions (Hoff *et al.*, 2016), but might be induced in response to some genotoxic stress.
152 Alternatively or concomitantly, these PolDoms could display different template requirements, as
153 for the four polymerases of the X family found in vertebrates (Pol β , Pol λ , Pol μ and TdT, for review
154 see Ramsden and Asagoshi, 2012). Therefore, each PolDom could act specifically on different
155 types of damaged DNA ends. In favor of this hypothesis, in *M. smegmatis*, a mutation of the
156 PolDom of LigD abolishes the nucleotide addition but not the fill-in activity suggesting that at
157 least two polymerase activities (probably driven by PolD1 and/or PolD2, Bhattarai *et al.*, 2014)
158 would be required for full DSB repair achievement (Aniukwu *et al.*, 2008). Finally, due to its
159 similarity with the mycobacterial locus encoding *ligC1* and *prim-polC* involved in a BER system
160 (Płociński *et al.*, 2017), we surmise that the *Streptomyces ligC-polR* locus could also be involved
161 in this DNA repair pathway in addition to its role in NHEJ.

162 Another bacterium displaying a complex organization is *Sinorhizobium meliloti*, an α -
163 proteobacterium which encodes four Ku and four LigD orthologues (Galibert *et al.*, 2001); here,
164 LigD proteins are defined as multidomain ATP-dependent ligases, with LigD3 and LigD1 lacking a
165 Nuc and a Pol domain, respectively. The Ku proteins of α -proteobacteria can be classified in

166 several families based on sequence similarity, but a “representative” Ku protein present in all α -
167 proteobacteria could not be identified. Single and multiple *S. meliloti* *ku* mutations conferred IR
168 sensitivity to stationary phase bacteria, suggesting a role of these genes in DNA repair (Kobayashi
169 *et al.*, 2008; Dupuy *et al.*, 2017). Involvement of the various *ku* and *ligD* genes in NHEJ was
170 assessed more precisely using a linear plasmid recircularization assay, which showed the
171 existence of at least two distinct NHEJ pathways (Fig. 1) (Dupuy *et al.*, 2018). The first major
172 pathway is dependent on *ku2* and *ligD2*, two adjacent genes apparently organized in operon,
173 although they can be transcribed from independent promoters. A second, less efficient pathway,
174 dependent on *ku3*, *ku4* and *ligD4* was also detected in stress conditions (see further below). Both
175 *ku3* and *ku4* are organized in operon, whereas *ligD4* is located elsewhere in the genome, but all
176 three genes are co-regulated (see further below) and *ku3*, *ku4* and *ligD4* orthologues are often
177 found in close proximity, mostly in operon, in α -proteobacterial genomes. Interestingly, genetic
178 analyses showed that both *ku3* and *ku4* are needed for activity of this second pathway. This
179 observation, together with the quasi-systematic co-occurrence and co-regulation of *ku3*- and
180 *ku4*-like genes in NHEJ equipped α -proteobacteria, led to the suggestion that Ku3 and Ku4 might
181 function as heterodimers, like eukaryotic Ku proteins. Although LigD4 was systematically found
182 associated to the Ku3-Ku4-dependent pathway, LigD2, and to a much lesser extent LigD3, are
183 also able to function, probably aspecifically, with Ku3 and Ku4 on certain types of DNA ends and
184 in certain genetic contexts. Both *ku1* and *ligD1* are weakly expressed, and consistently, no NHEJ
185 activity could be associated with these genes in the tested conditions. PolD1 (also called LigD1p,
186 Kobayashi *et al.*, 2008), encoded by the gene located just downstream *ligD1*, is likely the vestigial
187 PolDom of a larger ancestral LigD1 protein. Deletions of *ligD1* analyzed by Dupuy *et al.* (2018)
188 encompassed *polD1*, showing that this gene is not involved in NHEJ in the tested conditions.

189 *S. meliloti* also encodes three *ligC* and one *ligB* genes, whose role in NHEJ or another DNA repair
190 system has not been tested so far (Fig. 1).

191 Inactivation of NHEJ in *M. smegmatis* and *S. meliloti* unmasked a background level of Ku- and
192 LigD-independent end-joining activity, called alternative End-Joining (A-EJ), observed during
193 recircularization of linear transforming plasmids (Aniukwu *et al.*, 2008; Dupuy *et al.*, 2018).
194 Depending on the bacterial species and the DNA end type, the observed efficiencies and fidelity
195 of repair greatly varied. It is unclear whether A-EJ occurs by the same mechanism in all cases, but
196 as it was observed in *M. smegmatis* *ligBCD* mutants, it is believed to depend on the replicative
197 ligase LigA. Interestingly, LigA-dependent, LigB-independent A-EJ has been described in the NHEJ-
198 deficient *E. coli* (Chayot *et al.*, 2010). When not resulting in faithful ligation of compatible DNA
199 ends (which occurred in some cases with 100% efficiency; Aniukwu *et al.*, 2008; Dupuy *et al.*,
200 2018), A-EJ repair events mostly involved deletions of variable lengths, with frequent occurrence
201 of microhomologies at the junctions (Aniukwu *et al.*, 2008), very similar to the observed products
202 of A-EJ in *E. coli* (Chayot *et al.*, 2010). These observations highlight the importance of DNA end
203 resection for homology search, which in *E. coli* is in part due to RecBCD. The resection enzymes
204 involved in the A-EJ of NHEJ-proficient bacteria have not been characterized yet.

205 **NHEJ complexity: are there other actors involved?**

206 Proteins other than Ku and LigD domains have been suggested to be involved in NHEJ, either via
207 their physical and/or functional interaction with Ku and/or LigD or via the impact of their
208 inactivation on DSB repair. This is the case for the helicase UvrD1 from Mycobacteria, which
209 interacts with Ku in a yeast two-hybrid assay and which forms ternary complex with Ku and
210 dsDNA (Sinha *et al.*, 2007). The fact that the helicase activity of UvrD1 is stimulated in presence
211 of Ku_{Msm} and that the deletion of *uvrD1* in *M. smegmatis* sensitizes this bacterium to IR makes

212 sense with a role of UvrD1 in DSB repair via NHEJ. However, its role in this process remains to be
213 elucidated.

214 Another protein interacting with the NHEJ actors in Mycobacteria is Sir2. This deacetylase
215 interacts physically with both Ku and LigD and its inactivation decreased the NHEJ efficiency *in*
216 *vivo* (Li *et al.*, 2011). Its putative role in the NHEJ process is discussed below (see “Regulation of
217 NHEJ”).

218 **NHEJ complexity: the multiple DNA binding abilities of Ku**

219 Bacterial Ku is composed of a N-terminal Ku core domain homologous to the central domain of
220 the Ku70 and Ku80 eukaryotic homologs (Aravind and Koonin, 2001; Kushwaha and Grove,
221 2013a; McGovern *et al.*, 2016; Pitcher *et al.*, 2007a). Ku70/80 heterodimer has been shown to
222 bind DSB and to form a ring (involving the Ku core domains of the two subunits) able to encircle
223 a dsDNA molecule (Walker *et al.*, 2001). This particular structure of the eukaryotic Ku allows its
224 threading inward the DNA molecule when additional Ku proteins enter by the ends (de Vries *et*
225 *al.*, 1989; Paillard and Strauss, 1991; Downs and Jackson, 2004). This threading ability is
226 conserved at least for Ku from *B. subtilis*, *M. tuberculosis* and most probably *M. smegmatis*
227 (Weller *et al.*, 2002; Kushwaha and Grove, 2013a; McGovern *et al.*, 2016). The Ku core domain of
228 bacterial Ku is associated to a variable C-terminal (C-ter) tail which contains the ubiquitous
229 minimal C-ter domain and, in most cases, an extended C-ter tail (McGovern *et al.*, 2016). The
230 minimal C-ter is required for the interaction of Ku_{Bsub} with LigD_{Bsub}, is involved in the stimulation
231 of the ligase activity of LigD_{Bsub} and LigD_{Paer} and is contained in a segment of Ku_{Mtub} interacting
232 with LigD_{Mtub} in a yeast two-hybrid assay (Gong *et al.*, 2005; Zhu and Shuman, 2010; McGovern
233 *et al.*, 2016). These observations led to the proposal that the minimal C-ter is a part of the LigD-

234 interacting domain in bacterial Ku. The extended C-ter is predominantly a domain rich in basic
235 residues (lysine and arginine) that is involved, but not required, in the Ku-dependent stimulation
236 of the ligase activity of LigD in *P. aeruginosa* and *B. subtilis* (Zhu and Shuman, 2010; McGovern *et*
237 *al.*, 2016). This domain also confers to Ku the ability to bind DNA without free ends (Kushwaha
238 and Grove, 2013b; McGovern *et al.*, 2016) and limits the threading of Ku_{Bsub} inward the DNA
239 molecule. Its function could be to reduce putative negative effects of this threading inside the
240 chromosome on other DNA repair processes. The regulation of the threading of Ku may also be
241 important for the recruitment of LigD near, or at the DSB site, ensuring an efficient NHEJ repair
242 since LigD alone is a poorly efficient ligase without its Ku partner *in vitro* (Weller *et al.*, 2002; Della
243 *et al.*, 2004; Zhu and Shuman, 2010; de Ory *et al.*, 2014; McGovern *et al.*, 2016). The function of
244 Ku in the NHEJ could be more proactive than previously anticipated by allowing the bridging of
245 DNA ends, thanks to its extended C-ter (McGovern *et al.*, 2016), an ability shared by the human
246 Ku (Ramsden and Gellert, 1998). The different functions of this extended C-ter domain raise the
247 question about the efficiency of the NHEJ in bacteria encoding a Ku without this domain and/or
248 about the role of Ku and its extended C-ter in another DNA repair mechanism (for instance the
249 BER process, see below). In *Streptomyces*, one of the accessory Ku (KuB), present in only 8 of 38
250 species (Hoff *et al.*, 2016), possesses an extra C-ter domain weakly related to the RhoN domain
251 (Pfam 07498) which role in Ku protein remains to be elucidated.

252 Finally, a very recent study demonstrated that the eukaryotic Ku orthologue encoded by the
253 bacteriophage Mu (d'Adda di Fagagna *et al.*, 2003) is able to promote NHEJ repair of plasmidic
254 and chromosomal DSBs, most probably by interacting with and stimulating the *E. coli* replicative
255 ligase LigA (Bhattacharyya *et al.*, 2018). Surprisingly, MuGam also stimulates *in vitro* the LigB
256 ligase of *E. coli*, although MuGam and LigB do not seem to interact on DNA. These results raise

257 the possibility that a bacterial Ku protein might also increase the ligase activity of a non-NHEJ
258 ligase. In this line, Ku_{Msmeg} (Kushwaha and Grove, 2013a), but not Ku_{Mtub} (Weller *et al.*, 2002), has
259 been shown to stimulate the T4 DNA ligase *in vitro*. To our knowledge, no study assessed the
260 possibility that a bacterial Ku redirects the activity of a bacterial replicative ligase.

261 **III- Regulation of NHEJ**

262 In eukaryotes, NHEJ repairs DSBs throughout the whole cell cycle and is predominant in G1 phase
263 while HR relies on the presence of an intact sister chromatid and therefore functions in S/G2
264 phase (Ira *et al.*, 2004; Aylon *et al.*, 2004; Clerici *et al.*, 2008). Similarly, during quiescent states
265 such as sporulation (*Bacillus*, *Streptomyces*) or late-stationary phase (*Mycobacterium*,
266 *Sinorhizobium*) in bacteria, HR would not be operational because stationary phase cells and
267 spores are assumed to be single genome compartments. Note however that spores (Setlow and
268 Setlow, 1996; Moeller *et al.*, 2007; Hoff *et al.*, 2016) or stationary phase bacteria (Pitcher *et al.*,
269 2007b; Dupuy *et al.*, 2017) of HR-deficient *recA* mutants display IR sensitivity. This is most likely
270 because of the involvement of *recA* in the repair of other DNA damages, in particular via its role
271 in the SOS response, since i) spores of *B. subtilis addAB* and *recJ* mutants, deficient in the
272 resection step of HR (no other genes coding for end resection nucleases are known to be involved
273 in HR in *B. subtilis*), displayed IR resistance similar to the one of wild type spores (Vlašić *et al.*,
274 2014), ii) the effects of *recA* and NHEJ mutations on IR sensitivity of *B. subtilis* and *S. ambofaciens*
275 spores or *M. smegmatis* and *S. meliloti* stationary phase bacteria were found to be additive
276 (Stephanou *et al.*, 2007; Pitcher *et al.*, 2007b; Moeller *et al.*, 2012; Hoff *et al.*, 2016; Dupuy *et al.*,
277 2017). Thus, DSB repair in quiescent cells would essentially rely on NHEJ using available free non-
278 homologous DNA ends. Furthermore, DSB repair is essential during these quiescent states
279 because cells are more exposed to harsh environmental conditions such as desiccation, that can

280 damage the DNA. How the balance between NHEJ and HR is regulated is far from understood but
281 crucial regarding the consequences of the repair.

282 The best characterized bacterial NHEJ regulation comes from a study aiming at characterizing
283 gene expression in the forespore of *B. subtilis* (Wang *et al.*, 2006). The operon containing *ku_{Bsub}*
284 and *ligD_{Bsub}* is expressed inside the forespore under the control of the sigma factor SigG and the
285 transcriptional factor SpoVT. Their role in the DSB repair during spore germination (LigD is an
286 ATP-dependent ligase and ATP is present at very low levels in spores, Korza *et al.*, 2016) has been
287 demonstrated by the localization of a Ku-GFP fusion on the spore nucleoid after initiation of the
288 germination, and by the high sensitivity of NHEJ deficient spores (mutated for *ku_{Bsub}* or *ku_{Bsub}* and
289 *ligD_{Bsub}*) to dry heat, a cause of DSB inside spores (Wang *et al.*, 2006). Other sources of DNA
290 lesions, such as vacuum desiccation, heavy ions bombardment and ultraviolet radiations have a
291 higher toxic effect on NHEJ deficient compared to wild type spores of *B. subtilis* (Moeller *et al.*,
292 2007; Moeller *et al.*, 2008; Moeller *et al.*, 2014). According to the study of Wang *et al.*, expression
293 of *ku_{Bsub}* and *ligD_{Bsub}* was observed three to four hours after *B. subtilis* cells initiate their
294 sporulation program, concomitantly with the expression of SigG and SpoVT (Nicolas *et al.*, 2012).
295 However, it was found that the *ku_{Bsub} ligD_{Bsub}* operon was slightly up-regulated (~2 times)
296 compared to its basal level (i.e. in non sporulating conditions) when bacteria encounter a cold
297 stress. In this condition neither SpoVT nor SigG were expressed suggesting that the NHEJ genes
298 in *B. subtilis* could be regulated by other transcriptional factors (Nicolas *et al.*, 2012). The NHEJ
299 proficiency of these cold stressed bacteria remains to be assessed. Besides the overexpression of
300 the *ku_{Bsub}* and *ligD_{Bsub}* genes during the sporulation, lack of impact of inactivation of the HR-end
301 resection genes on the X-ray radiation sensitivity of *B. subtilis* spores led to the proposal for a
302 mechanism that specifically inhibits the action of AddAB and RecJ nucleases inside germinating

303 spores, rendering the NHEJ pathway the only efficient DSB repair mechanism in this condition
304 (Vlašić *et al.*, 2014; Raguse *et al.*, 2017). Moreover, no trace of *ku* and *ligD* expression was
305 observed at the transcriptional (Nicolas *et al.*, 2012) and translational (Sinai *et al.*, 2015) levels
306 during the revival of spores. Ku (Ku-GFP fusion) expressed during sporulation is detectable
307 associated to the nucleoid during the first 30 min of the spore revival (Wang *et al.*, 2006), i.e.
308 before initiation of DNA replication and expression of DNA repair genes such as *recA* (Sinai *et al.*,
309 2015). Then, *in B. subtilis*, NHEJ and HR do not seem to compete for the repair of two dsDNA
310 ends.

311 NHEJ is not a hallmark of bacterial sporulation since *ku* and *ligD* genes are also present in non-
312 spore forming bacteria and all spore-forming bacteria do not encode these proteins (Aravind and
313 Koonin, 2001; Bowater and Doherty, 2006; McGovern *et al.*, 2016). In Mycobacteria and in *S.*
314 *meliloti*, *ku* and *ligD* inactivation leads to a sensitization against IR of mutant bacteria grown in
315 stationary phase and have no or little effect in logarithmic phase, even if *recA* is inactivated
316 (Stephanou *et al.*, 2007; Pitcher *et al.*, 2007b; Kobayashi *et al.*, 2008; Dupuy *et al.*, 2017). On the
317 other hand, *ku* and/or *ligD* inactivation strongly reduces the capacity of logarithmically grown
318 Mycobacteria or *S. meliloti* to repair enzymatically linearized plasmid DNA upon transformation
319 (Gong *et al.*, 2004; Gong *et al.*, 2005; Aniukwu *et al.*, 2008; Dupuy *et al.*, 2018) as well as
320 enzymatically induced DSB on the chromosome (Stephanou *et al.*, 2007; Gupta *et al.*, 2011;
321 Dupuy *et al.*, 2018). Moreover, *ligD* is required in exponentially growing *M. smegmatis* bacteria
322 for infection by the mycobacteriophages Omega and Corndog, where it is thought to promote
323 circularization at the *cos* site of the phage genomes, thanks to the Ku expressed by the phages
324 (Pitcher *et al.*, 2006). Altogether, these observations show that although not involved in the
325 repair of DSB induced by IR in logarithmic phase, Ku- and LigD-dependent NHEJ is well active in

326 this growth stage in several bacterial species. This is consistent with the detection of similar
327 amounts of Ku and similar activity of its promoter in logarithmic and early stationary phases in
328 *M. smegmatis* (Bhattarai *et al.*, 2014; Zhou *et al.*, 2015), even if an increased activity of the Ku
329 promoter was observed in late stationary phase cells (Bhattarai *et al.*, 2014). This is also
330 consistent with the expression of a *S. meliloti ku2-lacZ* transcriptional fusion in both log and
331 stationary phase, and the visualization of Ku2-YFP foci after X-ray treatment of cells in both
332 phases (Kobayashi *et al.*, 2008). Together, these observations suggest that the action of NHEJ for
333 repair of IR induced DSBs, which have most probably similar molecular configurations in
334 logarithmic and stationary phases, is negatively regulated in logarithmic phase. The mechanism
335 of this inhibition does not seem dependent on RecA since the deletion of *recA* in *M. smegmatis*
336 does not increase the NHEJ (Gupta *et al.*, 2011), however it could involve the competition with
337 presynaptic actors of the HR.

338 In *S. meliloti*, NHEJ activity as measured using a linear plasmid repair assay, displays a significant
339 stimulation when bacteria enter stationary phase, or are exposed to a thermic stress (Dupuy *et al.*,
340 2018). This stimulation mainly results from the upregulation of *ku* gene expression. The *ku3*
341 *ku4* operon, as well as the *ligD4* gene, appeared to be under transcriptional control of the general
342 stress response EcfG sigma factor RpoE2. Examination of *ku* promoter regions in α -
343 Proteobacteria revealed the presence of putative EcfG binding sites upstream at least one *ku*
344 gene in about 90 % of the species, suggesting that stress control of *ku* and *ligD* transcription is
345 widespread in this bacterial class. Accordingly, stress induction of *ku* and/or *ligD* gene expression
346 was described in *Mesorhizobium loti* and *Bradyrhizobium japonicum* (Cytryn *et al.*, 2007;
347 Alexandre *et al.*, 2014). How *ku2* is up-regulated in stress conditions is currently unknown, but it
348 appears not to be part of the general stress response, the heat shock response, the SOS response,

349 the stringent response nor to be regulated by quorum sensing (Kobayashi *et al.*, 2008; Dupuy *et*
350 *al.*, 2018). Whether stress control of NHEJ gene expression is conserved beyond α -Proteobacteria
351 remains to be tested experimentally. In *Streptomyces*, major NHEJ genes are expressed in late-
352 growth phase (Hoff *et al.*, 2016) and gene products accumulate in spores. However, no induction
353 of the NHEJ-like genes could be detected in *S. ambofaciens* in response to DNA damaging agents
354 (i.e., Mitomycin C, Hoff *et al.*, 2016).

355 Besides transcriptional regulation, post-translational modification of NHEJ proteins could also
356 regulate NHEJ activity in bacteria. Indeed the deacetylase Sir2 from *M. smegmatis* is required for
357 an efficient NHEJ repair *in vivo* (Li *et al.*, 2011). Overexpression of *sir2* leads to a decreased
358 amount of Ku in bacteria, without altering the quantity of the corresponding mRNA. In line with
359 this interaction, Ku_{Msm} is acetylated at least at the residue K29, which is conserved among
360 bacterial Ku, and this modification is mainly observed in stationary phase (Zhou *et al.*, 2015). Even
361 if the mechanism by which Sir2 regulates NHEJ proteins remains to be clarified, it seems likely
362 that acetylation could regulate the activity of NHEJ (Zhou *et al.*, 2015).

363 **IV Cross talk with other DNA repair mechanisms**

364 As mentioned in the section III, DSB repair in quiescent cells would essentially rely on NHEJ, but
365 NHEJ and HR repair mechanisms may both co-exist, at least in some NHEJ proficient bacteria
366 during replicating phases. Moreover, in *Streptomyces*, recent studies (Hoff *et al.*, 2018) on the
367 consequences of DSB repair in the terminal region of the linear chromosome revealed events of
368 HR repair involving homologous sequences at distinct loci suggesting that HR can occur even in
369 the absence of a replicated copy of the chromosome (quiescent phases). This raises the question
370 of how the choice between the two pathways is performed and if there is a competition between

371 both mechanisms. In eukaryotes, it is known that Ku proteins rapidly sequester DSB free ends
372 and shield them from unscheduled exonucleases, preventing HR to proceed. The removal of Ku
373 involves multiple pathways including ubiquitination, neddylation, phosphorylation and the
374 nuclease activity of the MRE11-dependent complex MRX (yeast) /MRN (mammals) which plays a
375 pivotal role in NHEJ/HR balance (for review see Shibata, 2017). Similarly, in *Mycobacterium*, *in*
376 *vitro* analyses revealed that Ku_{Mtub} protects DSBs from resection by AdnAB helicase-nuclease
377 (Sinha *et al.*, 2009) and Ku_{Mtub} but not LigD_{Mtub} inactivation is compensated by an elevated HR *in*
378 *vivo* (Gupta *et al.*, 2011). Likewise, in *S. avermitilis*, the deletion of *kuSave1* and *kuSave2* seemed
379 associated with an increased efficiency of HR (Zhang *et al.*, 2012). Recent electronic beam
380 irradiation assays in *S. ambofaciens* (Hoff *et al.*, 2016, Leblond *et al.*, unpublished) also suggest
381 that NHEJ inactivation (*kuSamb* triple deletion or *ligCSamb* and *ligDSamb* double deletion) is
382 compensated for by an increased HR repair. However, unlike in *Mycobacterium* where loss of HR
383 is not compensated by increased NHEJ, a reciprocal effect is observed in *S. ambofaciens* since a
384 *recA* mutant is 100 times more affected by NHEJ inactivation than the wild-type strain.

385 Ku and LigD proteins may help coupling NHEJ to other DNA repair pathways. IR-induced cellular
386 damages on DNA include oxidized bases, abasic sites and single-strand breaks (SSB). However,
387 due to the presumed “clustered damage” induced by IR (Ward, 1994), several DNA lesions can
388 be present in close proximity and, if they are not repaired efficiently, can lead to DSB (Ward,
389 1994; Blaisdell and Wallace, 2001; Cox and Battista, 2005). Then, DSBs induced by IR are
390 potentially surrounded by multiple other DNA lesions. Part of these lesions are taken in charge
391 by the base excision repair (BER) pathway, the nicking of the phosphodiester bond 3’ to an
392 apurinic/apyrimidinic (AP) site by an AP-lyase being one possible step of this pathway (for review
393 see Almeida and Sobol, 2007). Human Ku, besides its function as a scaffolder of the NHEJ

394 machinery, is also an AP-lyase suggested to be involved in the repair of AP site near DSBs (Roberts
395 *et al.*, 2010). This activity is conserved in Ku from *B. subtilis* and *P. aeruginosa* (de Ory *et al.*,
396 2014). As Ku_{Bsub} is able to thread inward DNA molecules (McGovern *et al.*, 2016), via loading on
397 their broken ends, this could facilitate the repair of abasic sites surrounding a DSB. The interplay
398 between NHEJ and BER in bacteria is reinforced by the discovery of an AP-lyase activity driven by
399 the other NHEJ factor: LigD (de Ory *et al.*, 2016). The involvement of LigC1_{Msm} in BER during
400 stationary phase of *M. smegmatis* (Płociński *et al.*, 2017) shows that the link between NHEJ and
401 BER can even be extended to alternative NHEJ proteins. This multiplicity of NHEJ actors involved
402 in BER is not specific to bacteria, human DNA polymerase λ is one example (see Bebenek *et al.*,
403 2014).

404 Remarkably, Ku_{Bsub} is also able to linearize a supercoiled plasmid containing AP-sites (de Ory *et*
405 *al.*, 2014). This counter-intuitive effect for a protein intended to repair DSB is supposed to be
406 dependent on its AP-lyase activity and its ability to bind end-free dsDNA molecules, DSB arising
407 by the nicking of phosphodiester bonds of proximal AP-sites on opposite strands of a dsDNA
408 molecules containing numerous such lesions (de Ory *et al.*, 2014). Then it is tempting to propose
409 that NHEJ could have evolved from a Ku-dependent BER pathway, primarily independent on the
410 presence of DSB, that has acquired the ability to join Ku-catalyzed broken DNA ends, via the
411 recruitment of LigD, in conditions where the bacterial genome contains numerous AP-sites. This
412 hypothesis and the fact that most of the bacterial Ku proteins contain an extended C-ter tail that
413 is required for their interaction on end-free DNA (Kushwaha and Grove, 2013b; McGovern *et al.*,
414 2016) but for which the deletion has only a mild effect *in vitro* on the NHEJ efficiency (Zhu and
415 Shuman, 2010; McGovern *et al.*, 2016) suggest that the extended C-ter of Ku could play an
416 important role in a Ku-dependent BER acting on AP-sites embedded in duplex regions of DNA.

417 A recent study has functionally linked the NHEJ of *P. aeruginosa* to the MutL protein, a
418 component of the mismatch repair (MMR) pathway. Searching for the molecular mechanism at
419 the basis of a large genomic deletion in the *P. aeruginosa* chromosome allowing to prevent
420 infection by a bacteriophage, Shen *et al.* (2018) have shown that MutL_{Paer} is required for getting
421 the deletion. MutL_{Paer} is an endonuclease promoting SSB and, directly or indirectly, DSB (as a
422 consequence of multiple SSB in dsDNA). Surprisingly, the MutL_{Paer}-dependent genomic deletion
423 observed after the bacteriophage infection is not dependent on MutS_{Paer}, the partner of MutL_{Paer}
424 in MMR, suggesting a new function of MutL in this bacterium. Whatever the mechanism, the
425 reduced frequency of the corresponding deletion in a NHEJ deficient mutant led to the proposal
426 that DSB generated by MutL_{Paer} are repaired by the NHEJ pathway in this bacterium (Shen *et al.*,
427 2018). On the other hand, the majority of archaeal species and many actinobacteria lack MutHLS
428 system but display genes putatively involved in a novel mismatch repair system, called
429 NucS/EndoMS, which has been shown in *Corynebacterium glutamicum* to cleave both strands at
430 double-strand DNA mismatched substrate during the repair process (Castañeda-García *et al.*,
431 2017; Ishino *et al.*, 2018). Whether these induced DSBs are thereafter processed by NHEJ or HR
432 is not yet known, but one can suggest that chromosomes of bacteria possessing a NucS/EndoMS
433 system are more prone to DSBs and consequently to mutations and rearrangements, especially
434 if they also feature a NHEJ machinery, like many actinomycetes.

435 Another mechanism that induces DSBs is the type II CRISPR-Cas (Clustered Regularly Interspaced
436 Short Palindromic Repeats and associated proteins) system which is conferring adaptive
437 immunity against phages and exogenous mobile genetic elements (for review see Marraffini,
438 2015) and which has been engineered to provide an efficient genome editing method (Jinek *et*
439 *al.*, 2012). Since the CRISPR-Cas system generates DSBs, it could display antagonistic interactions

440 with DSB repair systems. Bernheim and co-authors (2017) revealed that NHEJ and type II-A
441 CRISPR-Cas systems do not co-occur in all but one of more than 5000 sequenced bacterial
442 genomes. On the other hand, this strong avoidance of co-occurrence was not observed with type
443 II-C system, which is consistent with the fact that, unlike type II-A system, type II-C system lacks
444 Csn2, a multimeric toroidal binding protein that binds double stranded DNA ends and has similar
445 properties to Ku protein (Arslan *et al.*, 2013). The binding of Csn2 at DNA ends could block access
446 to Ku and inhibit efficient repair by NHEJ. In contrast, *B. subtilis* NHEJ system does not impair
447 *Streptococcus pyogenes* or *Streptococcus thermophilus* type II-A CRISPR-Cas9 system (Bernheim
448 *et al.*, 2017).

449 **V- NHEJ and genome evolution**

450 NHEJ is inherently unfaithful, as DNA ends of DSB are often remodeled before ligation, thus
451 generating short insertions or deletions at the repair junction (although repair fidelity seems to
452 vary depending on the tested organism, the type of DNA ends being repaired or the enzymes
453 involved). Interestingly, NHEJ is generally more active in stationary phase or during sporulation,
454 and in α -proteobacteria, is activated under stress conditions, in a process dependent on the
455 general stress response sigma factor EcfG. This is reminiscent of the so-called stress-induced
456 mutagenesis of enterobacteria, where the highly faithful HR-dependent DSB repair becomes
457 mutagenic in a process dependent on the general stress response sigma factor RpoS (Shee *et al.*,
458 2011). NHEJ was also recently suggested to participate in stationary phase mutagenesis in
459 *Pseudomonas putida* (Paris *et al.*, 2015). NHEJ could then significantly contribute to DSB-
460 dependent stress-induced mutagenesis in bacteria and could thus participate in their adaptation
461 to adverse environmental conditions.

462 In addition to the short, non-templated nucleotide insertions usually observed at NHEJ repair
463 junctions, insertion of longer DNA sequences (several tens of nucleotides) of either plasmid or
464 chromosomal origin, was described to have occurred in rare instances at plasmid or chromosome
465 DSB repair junctions in *M. smegmatis* and *S. ambofaciens* (Akey *et al.*, 2006; Aniukwu *et al.*, 2008;
466 Hoff *et al.*, 2018). Although the mechanism of such DNA insertions was not clearly established, it
467 may have resulted from the incidental capture of indigenous DNA fragments at the break by the
468 NHEJ machinery. In eukaryotes, DNA inserted into the genome by NHEJ during evolution include
469 foreign DNA fragments such as mitochondrial DNA, transposable elements, and viral DNA (Moore
470 and Haber, 1996; Ricchetti *et al.*, 1999; Lin and Waldman, 2001a; Lin and Waldman, 2001b; Nakai
471 *et al.*, 2003; Hazkani-Covo and Covo, 2008). In yeast, capture of extranuclear DNA at repaired
472 DSBs was shown to be dependent on DNA end resection and NHEJ complexes (Decottignies,
473 2005). In the same way, the low level of genome rearrangements in the yeast *Lachancea kluyveri*
474 since its separation from its ancestor with *S. cerevisiae* was assigned to the loss of NHEJ genes in
475 this phylum (Gordon *et al.*, 2011). Interestingly, Dupuy *et al.* demonstrated that linear DNA of
476 heterologous origin, introduced in *S. meliloti* by transformation, could be effectively captured in
477 a NHEJ-dependent way at an artificially generated DSB in the chromosome (Dupuy *et al.*, 2018).
478 In *Streptomyces*, since the variable NHEJ genes encode actors of the response to DNA damage
479 (Hoff *et al.*, 2016) that presumably participate to the NHEJ and other DNA repair pathways, it is
480 likely that different combinations of NHEJ genes result in variable recombination profiles, and
481 consequently in variable abilities to integrate DNA from horizontal gene transfer. Like for *E. coli*
482 mutator strains (Giraud *et al.*, 2001), such recombination proficient lineages could provide a
483 flexible adaption property to the population in the ecological context by allowing rapid and
484 intense diversification of the accessory gene set. This speculation is substantiated by the strong
485 variability of the NHEJ-gene set revealed within a *Streptomyces* population originating from a

486 forest soil (Leblond *et al.*, unpublished). Altogether, these data suggest that NHEJ could
487 participate in the acquisition of heterologous DNA during lateral transfer events, overcoming the
488 lack of sequence homology between distantly related species. Interestingly, in support of this
489 hypothesis, Popa *et al.* (2011) found that gene transfer events from distant donors are more
490 frequent when recipient genomes encode NHEJ proteins. In addition, Kidane *et al.* (2009) have
491 observed that the absence of Ku reduces natural plasmid transformation and phage DNA
492 transfection in *B. subtilis*.

493

494 **Concluding remarks**

495 As mentioned within this review, many NHEJ proficient bacteria contain several stand-alone
496 genes displaying similarities with genes coding for NucDom, Poldom, LigDom and/or *ku* genes
497 and their multiplicity renders difficult the identification of the *bona fide* NHEJ genes. The use of
498 two-hybrid screening coupled with affinity purification of the products of these genes in their
499 host and in conditions where NHEJ is active should help to characterize actual NHEJ complexes,
500 and potentially identify new components of the NHEJ machinery, or new links with other DNA
501 repair pathways (e.g. role of AP-lyase activities in response to genotoxic stresses). Structural
502 information (i.e. cryo-electron microscopy and super-resolution microscopy analyses) associated
503 to 3D structures of stand-alone NHEJ actors (LigD, Ku) and of the complete bacterial NHEJ
504 machinery will be of crucial importance for our basic understanding of this mechanism (e.g. NHEJ
505 synapsis step, DNA extremities tethering, processing of variable DNA ends). Furthermore,
506 alternative NHEJ machineries may be involved depending on the growth phase or growth
507 conditions (see above the stress-induced NHEJ machineries of *S. meliloti*). This raises the
508 questions of the competition between distinct NHEJ machineries, and between HR and NHEJ, to

509 repair DSBs in conditions where both mechanisms coexist. It is clear, based on our knowledge of
510 the eukaryotic NHEJ, that DNA ends accessibility should be a key point that affects the HR/NHEJ
511 balance. Genetic, biochemical and cellular localization studies of proteins involved in early steps
512 of these pathways over time after DSB induction should help to elucidate preferred routes of DSB
513 repair in different life states. Further, experimental evolution could constitute a powerful
514 approach for testing the involvement of NHEJ in integration of DNA acquired by horizontal gene
515 transfer and its impact on the capacity to create and maintain genetic diversity within bacterial
516 populations in their ecosystems.

517 Finally, understanding the bacterial NHEJ may have therapeutic interest. Indeed, many human
518 pathogens possess a NHEJ repair system. This is for instance the case for the most deadly human
519 pathogen *M. tuberculosis* and the nosocomial pathogen *P. aeruginosa*. Although NHEJ is not
520 directly involved in survival or virulence in different mammal hosts (Brzostek *et al.*, 2014; Heaton
521 *et al.*, 2014), it could provide a way to survive to genotoxic conditions in certain non-dividing
522 states. Identifying these states and developing anti-NHEJ compounds based on the high-
523 resolution structure of a NHEJ machinery could help to fight against these pathogens.

524

525

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532

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Submitted Version

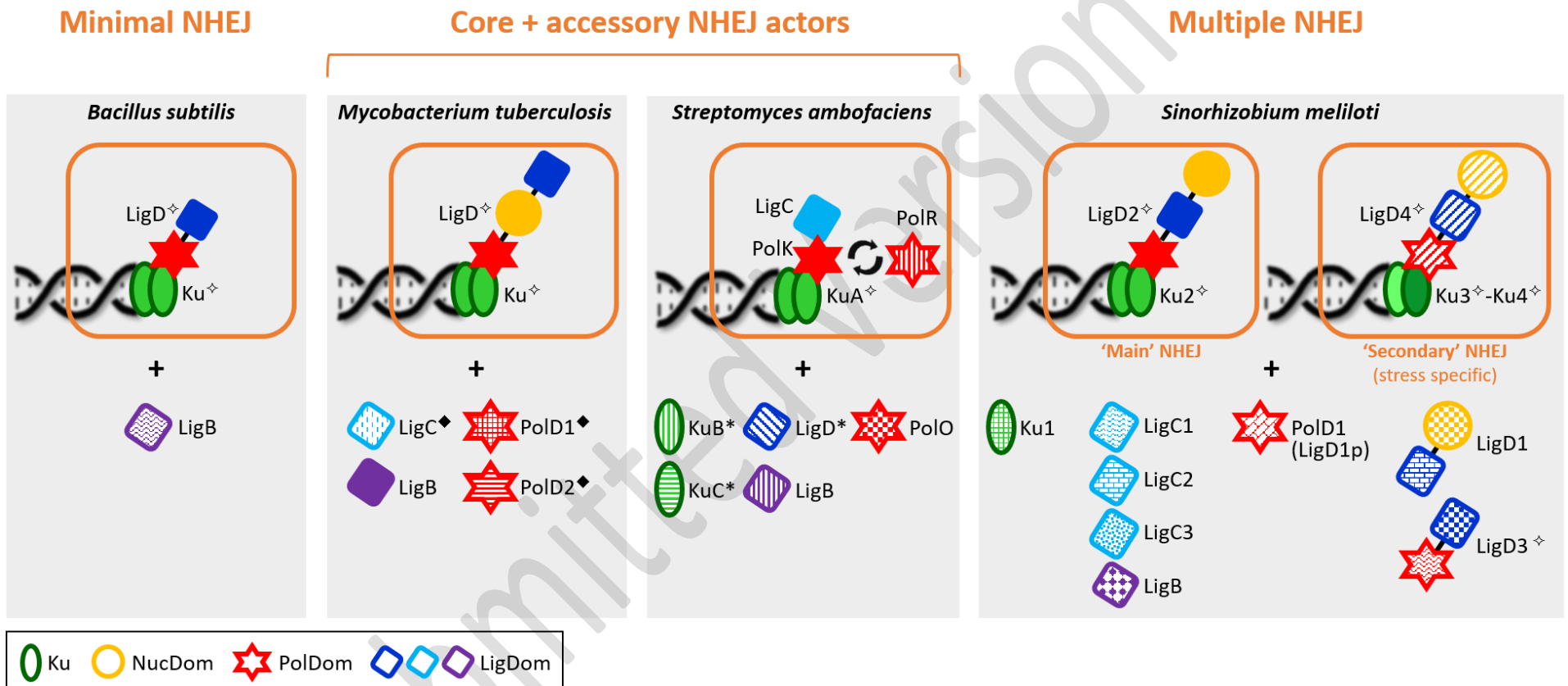


Figure 1: Repertoires of NHEJ presumed actors in model bacteria from minimal NHEJ to the more complex situations. Ku, LigDom (ATP-dependent), NucDom, PolDom identified in the genome of the bacterial species (as described in the literature) are highlighted whatever present within a single polypeptide or as standalone domains. Proteins having the same name in different organisms are not necessarily encoded by orthologous genes. [◇] indicate actors which have been experimentally involved in DSB repair. [◆] in *M. tuberculosis* denote accessory actors involved in NHEJ, based on studies of their homologs in *M. smegmatis* coding for different LigD with mutated domain (see text). In *S. ambofaciens*, accessory actors involved in response to DNA damage are highlighted by an asterisk (Hoff *et al.*, 2016, 2018). For the sake of clarity, only one dimer of Ku is shown at DNA ends and the ability of Ku to thread inward the DNA molecule has not been represented.