

1 **Requirement for specific bacterial genome maintenance pathways in repair**
2 **of C8-linked pyrrolobenzodiazepine (PBD) bi-aryl monomer-mediated DNA**
3 **damage**

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20 **Keywords**

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22 antibiotics, DNA lesion, DNA damage

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27 **Abstract**

28 Pyrrolobenzodiazepines (PBDs) are naturally occurring DNA binding compounds that possess
29 anti-tumor and anti-bacterial activity. Chemical modifications of PBDs can result in improved
30 DNA binding, sequence specificity and enhanced efficacy. More recently, synthetic PBD
31 monomers have shown promise as payloads for antibody drug conjugates and antibacterial
32 agents. The precise mechanism of action of these PBD monomers and their role in causing
33 DNA damage remains to be elucidated. Here we characterized the damage-inducing potential
34 of two C8-linked PBD bi-aryl monomers in *Caulobacter crescentus* and investigated the
35 strategies employed by cells to repair the same. We show that these compounds cause DNA
36 damage and efficiently kill bacteria, in a manner comparable to the extensively used DNA
37 cross-linking agent mitomycin-C (MMC). However, in stark contrast to MMC which employs a
38 mutagenic lesion tolerance pathway, we implicate essential functions for error-free
39 mechanisms in repairing PBD monomer-mediated damage. We find that survival is severely
40 compromised in cells lacking nucleotide excision repair and to a lesser extent, in cells with
41 impaired recombination-based repair. Loss of nucleotide excision repair leads to significant
42 increase in double-strand breaks, underscoring the critical role of this pathway in mediating
43 repair of PBD-induced DNA lesions. Together, our study provides comprehensive insights into
44 how mono-alkylating DNA-targeting therapeutic compounds like PBD monomers challenge
45 cell growth, and identifies the specific mechanisms employed by the cell to counter the same.

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48 Introduction

49 Persistent DNA damage can be problematic to cells across domains of life, from unicellular
50 bacteria to multicellular eukaryotes. It can have deleterious effects on basic cellular processes
51 as well as organismal functions, and subsequently lead to cell death (Surova & Zhivotovsky,
52 2013). However, when designed and used appropriately, DNA damage can also work as tools
53 to eliminate hazardous microorganisms or malignant tissues (de Almeida et al., 2021).

54 DNA damage can be mediated by endogenous or exogenous agents, leading to broad
55 spectrum of DNA modifications (Chatterjee & Walker, 2017). For example,
56 methylmethanesulfonate (MMS) leads to methylation on a single base (G, A or C) causing
57 mono-alkylated adducts such as 7-MeG, 1-MeA and 3-MeC (Beranek, 1990). UV exposure
58 induces covalent linkages between adjacent pyrimidines, creating intra-strand crosslinks
59 including cyclobutane pyrimidine dimers (CPDs) and pyrimidine-(6-4)-pyrimidone
60 photoproducts (Chatterjee & Walker, 2017). On the contrary, mitomycin-C (MMC) causes
61 inter-strand crosslinks in addition to mono-alkylated adducts and intra-strand crosslinks
62 (Bargonetti et al., 2010; Tomasz, 1995).

63 Pyrrolobenzodiazepines (PBDs) are a group of DNA damaging agents that bind to the minor
64 groove of DNA and alkylate DNA in a sequence-specific manner (Gerratana, 2012; Mantaj et
65 al., 2017). PBDs typically contain an aromatic A-ring, a diazepine B-ring and a pyrrolidone C-
66 ring, a structure that fits into the minor groove of DNA. Once secured within the minor groove,
67 an electrophilic imine moiety in the B-ring establishes a covalent link with the C2-NH₂ group
68 of guanine base on the DNA, preferably within Pu-G-Pu sequences. Several PBDs including
69 anthramycin, tomaymycin and sibiromycin, naturally produced by actinomycetes are mono-
70 alkylators of DNA and exhibit strong anti-microbial and anti-tumor properties (Gerratana,
71 2012; Hurley, 1977; Leimgruber et al., 1965)

72 Diversity in PBDs can be typically brought about by the variations in the A- and C-rings (Mantaj
73 et al., 2017). For instance, the A ring can be functionalized with electron-donating sidechains
74 at the C7- and C8-positions resulting in increased alkylating potential and DNA binding
75 capability of PBDs (Mantaj et al., 2017; Thurston et al., 1999). Research in the last three
76 decades has led to development of several synthetic PBDs by engineering modifications to

77 the basic PBD structure resulting in enhanced sequence specificity, stability and functionality
78 (Bose et al., 1992; Gregson et al., 2001; Mantaj et al., 2017; Rahman et al., 2012).

79 Of these, synthetic PBD dimers formed by linking two PBD monomers via their aromatic A-
80 ring has the potential to form cross-links on DNA, similar to cross-links formed by mitomycin
81 C (MMC) and have been extensively studied for their chemotherapeutic value (Kung
82 Sutherland et al., 2013; Puzanov et al., 2011; Rahman et al., 2011). Few of these dimers are
83 being evaluated as payloads for antibody drug conjugates (ADCs) in clinical trials to specifically
84 target and kill tumor cells (Kung Sutherland et al., 2013; Morgensztern et al., 2019). However,
85 there have been a number of failures in the clinical development of PBD-dimer containing
86 ADCs due to their toxicity (Jackson et al., 2018). This has resulted in significant interest in PBD-
87 pseudodimer containing only one N10-C11 imine group, PBD-monomers and structurally
88 related pyridinobenzodiazepines (PDD) monomers (Hoffmann et al., 2020; Kovtun et al.,
89 2018), which are considered less toxic as they can only mono-alkylate DNA. C8-linked PBD bi-
90 aryl monomers are a group of synthetic PBD monomers where a heteroaromatic group like
91 pyrrole or imidazole is directly linked to a phenyl group using C-C coupling or fused with a
92 phenyl ring and these bi-aryl units are attached to the C8 position of the PBD A-ring. These
93 PBD monomers exhibit a strong preference for GC-rich DNA, leading to formation of mono-
94 alkylated adducts on guanine. One such monomer, KMR-28-39 has shown low nanomolar to
95 picomolar *in vitro* cytotoxicity against a panel of cancer cell lines and *in vivo* anti-tumor
96 activity to breast cancer and pancreatic cancer xenografts in mouse models (Rahman et al.,
97 2013). Interestingly, many of these PBD monomers also exhibited anti-bacterial activity
98 against a range of Gram-positive bacteria, including methicillin-resistant *Staphylococcus*
99 (Rahman et al., 2012). Another set of PBDs containing a terminal heteroaliphatic ring has
100 similarly shown excellent activity against a panel of multidrug resistant Gram-negative
101 bacteria (Picconi et al., 2020), without noticeable toxicity against eukaryotic cells. The
102 therapeutic potential exhibited by PBD bi-aryl monomers (Andriollo et al., 2018; Brucoli et al.,
103 2016; Picconi et al., 2020; Rahman et al., 2012; Rosado et al., 2011) is encouraging as they
104 appear to possess significant anticancer and antibacterial activity and their eukaryotic toxicity
105 can be tuned by altering the C8-side chain to make them selective against either eukaryotic
106 or prokaryotic cells.

107 While induction of cross-links and DSBs by PBD dimers (Arnould et al., 2006; Jenkins et al.,
108 1994) and their repair via endonuclease ERCC1 and homologous recombination (Hartley et
109 al., 2010; Xing et al., 2019; Zhong et al., 2019) are well characterized, there has been little
110 research in elucidating the mechanism of action of PBD monomers beyond their ability to
111 inhibit transcription factors (Corcoran et al., 2019; Kotecha et al., 2008; Rahman et al., 2013).
112 The contribution of DNA-damage mediated effect on their overall cytotoxicity or antibacterial
113 activity needs to be studied to properly evaluate their potential as chemotherapeutic agents.
114 Furthermore, as efforts progress towards developing DNA-interactive PBD monomers as
115 payloads for antibody drug conjugate, it is also important to elucidate the mechanism(s) that
116 lead to repair of such PBD lesions, and identify the outcome of their repair on both survival
117 and mutagenesis.

118 The main objective of this work was to identify the pathways(s) involved in the repair or
119 tolerance of lesions induced by PBD monomers and assess the possible involvement of error-
120 prone repair or tolerance mechanisms (such as translesion synthesis) that can impact
121 damage-induced mutagenesis, contributing to development of resistance. We used the non-
122 pathogenic Gram-negative bacteria *Caulobacter crescentus* as our model system. Unlike *E.*
123 *coli*, this GC-rich organism shares several key genome maintenance features, including error-
124 prone lesion tolerance mechanisms, with pathogenic bacteria such as *Pseudomonas*
125 *aeruginosa* and *Mycobacterium tuberculosis* (Alves et al., 2017; Boshoff et al., 2003; Galhardo,
126 2005; Jatsenko et al., 2017; Warner et al., 2010). Using two prototypes (described in Fig. 1A
127 and below), we found that C8-linked PBD bi-aryl monomers induced DNA damage and
128 efficiently killed *Caulobacter crescentus*. Repair of these lesions was predominantly mediated
129 by nucleotide excision repair; lack of repair led to generation of double-strand breaks and
130 severely compromised survival. In contrast to MMC we found that mutagenic translesion
131 synthesis was not essential for PBD monomer-mediated damage tolerance or repair. Taken
132 together, our study uncovers, for the first time, the mechanisms involved in repair of DNA-
133 monoalkylations induced by PBD monomers and their overall impact on genome integrity and
134 survival of bacterial cells.

135 **Results**

136 **Chemically synthesized C8-linked PBD bi-aryl monomers KMR-28-33 and KMR-28-35 cause**
137 **DNA damage in *Caulobacter crescentus***

138 To assess the DNA damaging potential of PBD monomers, we chose two C8-linked PBD bi-aryl
139 monomers (KMR-28-33 and KMR-28-35) (Fig. 1A) as prototypes. These compounds, which
140 have propensity to bind to DNA (preferentially to GC-rich tracts (Rahman et al., 2013)),
141 showed strong cytotoxic (Rahman et al., 2013) and antibacterial activity towards Gram-
142 positive bacteria in the earlier studies (Rahman et al., 2012). As a comparison, we evaluated
143 the repair of lesions caused by a conventional and well-characterized DNA cross-linking agent
144 (mitomycin-C, MMC).

145 Indeed, molecular modelling of KMR-28-33 and KMR-28-35 binding to DNA also lent strong
146 support to the possibility that these PBD monomers could result in DNA lesions, without
147 distorting the DNA helix itself, as they snugly fit within the DNA minor groove (Fig. 1B, S2A.
148 S2B). Our *in vitro* FRET-based DNA melting assays corroborated the binding of these PBD
149 monomers to DNA (Fig. S2C). Given these observations, we investigate whether these PBD
150 monomers cause DNA damage and if so, what mechanisms are employed by cells to repair
151 the same. For this, we use the GC-rich model system *Caulobacter crescentus*, where we
152 deleted genes involved in repair of specific types of DNA damage (Fig. S2E).

153 We first assessed cell survival upon treatment with KMR-28-33 and KMR-28-35 (synthesis
154 described in Fig. S1A-B) in a wild type background. As a reference, we also exposed cells to
155 well-characterized DNA damaging agents, that are known to induce specific types of damage
156 (Fig. 2A and S2D). In particular, we compared the effects of KMR-28-33 and KMR-28-35 to
157 mitomycin-C (MMC). Both KMR-28-33 and KMR-28-35 can only form DNA monoadducts while
158 MMC can form intra-strand and inter-strand crosslinks in addition to monoadducts (Rahman
159 et al., 2012; Warren et al., 1998).

160 We observed that both C8-linked PBD bi-aryl monomers caused cell death in a dose-
161 dependent manner (Fig. 2A). We next asked whether these PBD monomers resulted in DNA
162 damage. For this, we measured SOS response induction in wild type *Caulobacter* after
163 treatment with the KMR-28-33 and KMR-28-35. We specifically quantified the expression of
164 a fluorescence marker (*YFP*) induced under an SOS promoter (P_{sidA}) integrated on the
165 *Caulobacter* chromosome at the *xyl* locus (Badrinarayanan et al., 2015; Joseph et al., 2021).
166 We found that exposure of cells to both PBD monomers resulted in significant accumulation
167 of SOS-induced YFP (comparable to that observed in case of MMC, at concentrations that

168 similarly affected cell growth in all three damages), suggesting that KMR-28-33 and KMR-28-
169 35 caused DNA damage (Fig. 2B).

170 **Requirement for RecA, but not the SOS response, in C8-linked PBD bi-aryl monomer-treated** 171 **cells**

172 Based on the above observations we wondered whether specific pathways under the SOS
173 response were required to repair KMR-28-33 and KMR-28-35-mediated damage. For
174 example, in the case of MMC, the error-prone translesion synthesis polymerase, DnaE2, has
175 been found to be essential (Boshoff et al., 2003; Galhardo, 2005; Joseph et al., 2021). In
176 support of the possibility that these PBD monomers indeed induce DNA damage, we found
177 that cells lacking *recA* were compromised for survival upon treatment with both PBD-
178 monomers (Fig. 3A). The difference in sensitivity for $\Delta recA$ cells across KMR-28-33, KMR-38-
179 35 and MMC suggested that there may be distinct repair mechanisms at play. We thus
180 uncoupled key DNA damage-specific functions of RecA (recombination and SOS induction), to
181 assess the contribution of the two towards survival. For this, we generated a strain where the
182 SOS repressor, *lexA*, is deleted. To circumvent the problem of cell length elongation in this
183 background, previous studies have additionally deleted the SOS-induced division inhibitor,
184 *sidA* (Modell et al., 2011). In this constitutive 'SOS-ON' background, deletion of *recA* would
185 predominantly eliminate its function in recombination. Thus, this triple deletion of
186 *lexAsidArecA* can be used as a genetic read-out to test requirement of SOS vs recombination
187 functions of RecA upon DNA damage treatment.

188 In case of MMC-damage, SOS-ON cells performed better than those deleted for *recA* (Fig. 3A),
189 suggesting that a pathway regulated under the SOS response contributed significantly to cell
190 survival under MMC damage. Indeed, this phenotype can be attributed to the expression of
191 the TLS pathway (including the error-prone polymerase DnaE2) in SOS-ON cells, but not in
192 cells deleted for *recA*. Previous studies have implicated an important role for this mechanism
193 in tolerance of MMC-induced lesions, independent of *recA* (Galhardo, 2005; Joseph et al.,
194 2021) (Fig. 3B). In contrast to MMC, we found that $\Delta recA$ or $\Delta lexAsidArecA$ cells were similarly
195 compromised in growth when treated with the C8-linked PBD bi-aryl monomers, suggesting
196 that SOS function was not required for combatting KMR-28-33 and KMR-28-35-mediated
197 damage (Fig. 3A). In line with this observation, we also found that TLS polymerase DnaE2 was
198 not essential to tolerate C8-linked PBD bi-aryl monomer-mediated damage (Fig. 3B).

199 Given the sensitivity of $\Delta recA$ cells (independent of SOS) to treatment with the KMR
200 compounds, we asked whether recombination-mediated repair contributed to cell survival
201 under PBD monomer-mediated damage. For this we deleted genes involved in specific
202 recombination-based repair: a. *recF*, *recO* and *recR* that function in single-strand gap (SSG)
203 repair and b. *addAB* and *recN* that function in double-strand break (DSB) repair (Rocha et al.,
204 2005; Spies & Kowalczykowski, 2014). It is important to note that although we categorize the
205 genes in this manner, there is also evidence to suggest that they may have overlapping
206 functions (Pages, 2003).

207 In case of MMC damage, we found that cells deleted for *recF*, *recO* or *recR* were similarly
208 sensitive to damage, and comparable with a *recA* deletion (Fig. 4A-B). Given the $\Delta recA$ -like
209 sensitivity in these backgrounds, it is tempting to speculate that these proteins may play a
210 role in loading RecA at ssDNA gaps to enable SOS induction, apart from contribution to
211 recombination-based repair. In line with this possibility, *addAB* and *recN* deleted cells were
212 less compromised in growth when compared to the $\Delta recA$ cells (Fig. 4A). In contrast to MMC
213 damage, cells treated with KMR-28-33 or KMR-28-35 were similarly compromised in growth
214 in the absence of SSG or DSB repair (Fig. 4A-B). Importantly, the sensitivity observed in case
215 of these deletions mirrored that of *recA*, suggesting that both recombination pathways may
216 contribute to repairing PBD bi-aryl monomer-induced damage.

217 **Nucleotide excision repair (NER) is essential for survival under KMR-28-33 and KMR-28-35-** 218 **induced DNA damage**

219 Although we implicated a role for recombination in repair of C8-linked PBD bi-aryl monomer-
220 mediated damage, these PBD monomers are thought to predominantly form DNA mono-
221 alkylations and are not known to directly induce double-strand breaks. To estimate the DSB-
222 inducing potential of KMR-28-33 and KMR-28-35, we adapted the Gam-GFP reporter system
223 previously described in *E. coli* (Shee et al., 2013) to mark DSB ends *in vivo* in *Caulobacter*.
224 Using this system, we estimated the percentage of cells with Gam localization in the presence
225 and absence of the lesion-inducing damaging agents (Fig. S3A). In the absence of any damage,
226 <1% cells had detectable foci. As anticipated, in the presence of the KMR compounds, this
227 number increased only nominally to ~5% after 2 h of treatment with the compounds. This was
228 similar to observations made for MMC-induced damage as well (Fig. S3A). Interestingly loss
229 of *recN*, required for recombination repair, only resulted in a modest increase in DSBs under

230 KMR compounds or MMC (Fig. S3A). Together, this suggested to us that recombination may
231 only be a minor repair pathway, with some other mechanism(s) likely enabling lesion repair
232 or tolerance.

233 We thus wondered which lesion repair or tolerance pathways were required to repair damage
234 induced by the C8-linked PBD bi-aryl monomers. As shown earlier, unlike MMC, we had ruled
235 out a role for TLS polymerase DnaE2 (Fig. 3A-3B). Indeed, the lack of SOS response essentiality
236 also eliminated a role for the other TLS polymerase, DinB (Galhardo, 2005; Joseph &
237 Badrinarayanan, 2020), in this case (Fig. 3A). We next assessed survival in cells compromised
238 for alkylation repair (*alkB*) (Colombi & Gomes, 1997) or mismatch repair (*mutL*) (Chai et al.,
239 2021) and found that these pathways also did not contribute to repair of KMR-induced
240 damage (Fig. S3B).

241 We thus turned to Nucleotide Excision Repair (NER). NER is an important mechanism of DNA
242 lesion repair, that predominantly acts on helix-distorting lesions (Jia et al., 2009; Liu et al.,
243 2011). Although damage induced by these PBD monomers is thought to not cause significant
244 distortion to the DNA helix, we found that cells lacking *uvrA* (the lesion scanning component
245 of the NER pathway) were severely compromised in survival under KMR-28-33 or KMR-28-35
246 damage (Fig. 5A). This was in contrast to MMC, where UvrA is required but not as essential,
247 as seen in case of the C8-linked PBD bi-aryl monomers (Fig. 5A). Indeed, differential sensitivity
248 of $\Delta uv r A$ strains to UV, MMS and norfloxacin damage further underscored the specificity of
249 this repair pathway (Fig. S4A).

250 NER primarily functions via two ways: transcription-coupled repair (TCR) and global genomic
251 repair (GGR). In case of TCR, Mfd plays a central role in recruiting Uvr components to the site
252 of lesion for excision, followed by gap filling (C. Selby & Sancar, 1993; Strick & Portman, 2019).
253 On the other hand, in case of GGR, UvrA is thought to scan and recognize lesions across the
254 genome, and subsequently initiate repair (Kisker et al., 2013). We thus deleted the *mfd*
255 homolog in *Caulobacter* and found that cells lacking the ability to engage in TCR were not as
256 severely compromised in survival, when compared to $\Delta uv r A$ deleted cells (Fig. S4B). These
257 data suggest that the GGR arm of NER is primarily responsible for repair of DNA lesions
258 induced by KMR-28-33 and KMR-28-35. Indeed, alternate pathways for repair coupled to
259 transcription, independent of Mfd, have also been proposed (C. P. Selby, 2017). We have not

260 investigated the role of such mechanisms, which are currently not well-characterized or
261 identified in *Caulobacter*.

262 Importantly, the absence of NER resulted in severe genome instability in cells treated with
263 KMR-28-33 or KMR-28-35. As shown above, only 5-6% wild type cells treated with KMR
264 compounds or with MMC had Gam localizations, indicating DSBs. In contrast, the lack of *uvrA*
265 resulted in a significant increase in cells with Gam localizations in case of the PBD monomers,
266 with 56% cells having DSBs upon KMR-28-33 treatment and 40% cells with DSBs on KMR-28-
267 35 treatment (Fig. 5B). This was not found to be the case for MMC-treated cells, where *uvrA*
268 deletion only led to modest increase in percentage cells with DSBs (6% in wild type to 10% in
269 NER-compromised cells) (Fig. 5B). Such marked genome instability was observed only when
270 NER action was compromised, as deletion of *recN* did not result in increase in localization of
271 Gam-GFP (Fig. S3A).

272 Together these results highlight: a. the essentiality of NER in repairing C8-linked PBD bi-aryl
273 monomer-mediated damage and b. the distinct mechanisms of DNA damage repair in case of
274 the PBD monomers, when compared to an extensively studied DNA cross-linking agent, MMC
275 (Fig. S5).

276 **Discussion**

277 Earlier studies have affirmed the specificity of action of pathways for repair of DNA damage.
278 For example, MrfA and MrfB in *Bacillus subtilis*, and MmcA and MmcB in *Caulobacter*
279 *crescentus* are essential for repair of only MMC-induced lesions (Burby & Simmons, 2019;
280 Lopes-Kulishev et al., 2015). Similarly, NER has been implicated as the primary repair pathway
281 in case of nitrofurazone damage in *E. coli* (Ona et al., 2009). In many instances, the
282 requirement for different repair components is likely driven the structural variations in lesions
283 induced by specific damaging agents (Cole et al., 2018; Ona et al., 2009; Williams et al., 2013).
284 Such difference in function can be observed even within a pathway, when the type of lesion
285 differs. Both prokaryotic and eukaryotic TLS polymerases exhibit substrate specificity which
286 defines their lesion bypass properties including efficiency of bypass and fidelity (Inomata et
287 al., 2021; Ippoliti et al., 2012; Prakash et al., 2005; Waters et al., 2009). In case of *Caulobacter*,
288 requirement for TLS polymerase DnaE2 is relatively higher for repair of MMC lesions than UV
289 lesions (Galhardo, 2005; Joseph et al., 2021).

290 Harnessing this specificity in function of repair pathways, in this study, we determined the
291 damage-inducing potential of two C8-linked PBD bi-aryl monomers and delineated the
292 strategies employed by bacterial cells to repair the same. Our results indicate that base
293 modifications (in the form of mono-alkylated adducts) caused by the KMR compounds are
294 predominantly repaired by NER and do not employ error-prone TLS mechanisms. Indeed, it is
295 tempting to attribute the difference between MMC and the KMR compounds to repair/
296 tolerance of monoalkylations vs inter and intra-strand DNA crosslinks. While the C8-linked
297 PBD bi-aryl monomers can only form mono-adducts, MMC treatment can result in mono-
298 adducts as well as both inter and intra-strand DNA crosslinks. Thus, the relative contribution
299 of various repair components could differ between lesions that are structurally and chemically
300 distinct, but mechanistically similar.

301 In addition to NER, we find contribution of recombination-mediated repair to cell survival in
302 case of the KMR compounds. The exact sequence of event(s) that lead to conversion of a
303 mono-adduct into a DSB remains elusive. It is speculated that cellular processes like
304 transcription, replication and even incomplete repair can lead to generation of single
305 stranded gaps as well as double stranded breaks (Aguilera & Gaillard, 2014; Mehta & Haber,
306 2014). The observation that the NER mutant is far more compromised in survival and
307 generates much higher proportion of DSBs than a recombination mutant supports the idea
308 that recombination may act secondary to lesion repair via NER. Indeed, we did consider
309 making a strain impaired in both NER and recombination to test this possibility. However, the
310 very high sensitivity of the *ΔuvrA* strain to KMR compounds precludes our ability to do so with
311 reliability. Future work aimed at quantitative estimation of the levels and types of DNA
312 damage induced *in vivo* in all three cases (KMR compounds and MMC) will enable us to further
313 discern the hierarchy of requirement and action of repair pathways.

314 In sum, our work highlights the importance of studying the mechanism of action of potential
315 DNA-interactive therapeutics like PBD monomers in depth, to understand how they may
316 affect cell growth and what strategies may be employed by the cell to respond to the same.
317 For example, when considering a DNA damaging agent for therapeutic purposes, it is
318 important to understand the fidelity of repair mechanisms that could be employed by the
319 cells. Mutagenic repair can be a major source of stress-induced mutagenesis and subsequent
320 development of resistance (Fitzgerald et al., 2017; Ippoliti et al., 2012; Joseph &

321 Badrinarayanan, 2020). Our findings suggest that C8-linked PBD bi-aryl monomer-induced
322 lesions are likely non-mutagenic, and are predominantly repaired by nucleotide excision
323 repair, thus negating an important driver for development of chemoresistance and
324 antimicrobial resistance. This contrasts with PBD dimers which are known to cause
325 mutagenesis and non-selective toxicity, and makes the case for using PBD monomers as ADC
326 payloads to overcome recent clinical failures observed with PBD dimers (Jackson et al., 2018).
327 Identifying the dependency on NER (specifically Uvr components, which are restricted to
328 bacteria) further opens up possibilities for considering inhibitors for Uvr components to use
329 in combination with the PBD monomers. Indeed, chemical inhibitors for specific repair
330 pathways have been identified previously, including for *E. coli* RecBCD, *H. pylori* AddAB and
331 *M. smegmatis* Uvr proteins (Amundsen et al., 2012; Mazloum et al., 2011). Combining a DNA
332 damaging drug with a small molecule inhibitor capable of dampening damage repair in the
333 pathogen can potentiate the efficacy of the drug and reduce pleotropic cytotoxicity (Lim et
334 al., 2019).

335

336

337 **Materials and methods**

338 **Synthesis of KMR-28-33 and KMR-28-35**

339 The PBD component of the hybrids was synthesized from vanillin as previously described in
340 the literature (Rahman et al., 2013) and is summarized in Fig. S1A. A four-carbon linker was
341 used to connect the PBD component with the non-covalently interactive subunits, as chains
342 of this length had proven optimal in previous hybrid SAR studies (Wells et al., 2006). The linker
343 was located at the C8 position of the molecule to allow an isohelical fit of the non-covalent
344 component of the hybrid along the minor groove upon covalent PBD binding. After the
345 synthesis of the PBD core, non-covalently interactive side chains were constructed using
346 combinations of benzofused (benzothiophene, KMR-28-33), five membered heterocyclic
347 structures (N-methyl pyrrole/N-methyl imidazole) and MPB (4-(1-methyl-1H-pyrrol-3-
348 yl)benzenamine, KMR-28-35) moieties. The MPB subunit was synthesized using Suzuki-
349 Miyuara conditions previously described (Rahman et al., 2013) (Fig. S1B). These moieties
350 were linked via Steglich amide bond formation at positions which maintained the overall fit of
351 the hybrid for the DNA minor groove (C2/C5 for benzofused, C1/C4 for heterocyclic
352 components), and finally the N10/C11 imine component of the molecule was activated using
353 tetrakis palladium and pyrrolidine.

354 **Bacterial strains and growth conditions**

355 Bacterial strains, plasmids and primers used in the study are listed in Table S1-S3.
356 Chromosomal deletions and integrations were performed using either a two-step
357 recombination method with a *sacB* counter-selection marker (Skerker et al., 2005) or using
358 integrating vectors from Thanbichler et al. (Thanbichler et al., 2007). Transductions were
359 carried out with Φ CR30 (Ely, 1991). *Caulobacter crescentus* cultures were routinely grown at
360 30°C in PYE (0.2% peptone, 0.1% yeast extract and 0.06% MgSO₄). For strains expressing *Gam*-
361 *GFP* under P_{xyI}, 0.3% xylose was added 3h prior to imaging.

362 **Survival assay**

363 *Caulobacter* cultures were grown in PYE to O.D₆₀₀ of 0.3. Serial dilutions in 10-fold increments
364 were made and 6 μ l of each dilution (10⁻¹ to 10⁻⁸) were spotted on PYE agar containing
365 appropriate concentrations of different DNA damaging agents. For UV damage, serial
366 dilutions of the culture were spotted on PYE agar plates and exposed to specific energy

367 settings in a UV Stratalinker 1800 (STRATAGENE). Growth was assessed from the number of
368 spots on the plates after two days of incubation at 30°C. Percentage survival for each strain
369 was calculated by normalizing growth of that specific strain on different doses of DNA damage
370 to that on media without DNA damage.

371 **Fluorescence microscopy and image analysis**

372 Saturated overnight cultures were back diluted in fresh PYE and allowed to grow at least for
373 two generations (approx. 3h) until OD₆₀₀ was 0.1. Images were taken without damage
374 treatment (no damage control) and after treatment with specified doses of DNA damage. 1
375 ml aliquots of cultures were taken at specified time points, pelleted and resuspended in 100
376 µl of growth medium. 2 µl of cell suspension was spotted on 1% agarose pads (prepared in
377 water). Imaging was performed on a wide-field epifluorescence microscope (Eclipse Ti-2E,
378 Nikon) equipped with a 60X oil immersion objective (plan apochromat objective with NA 1.41)
379 and pE4000 light source (CoolLED). Images were acquired with Hamamatsu Orca Flash 4.0
380 camera using NIS-elements software (version 5.1). For quantifying YFP induction under *P_{sidA}*
381 promoter, cells were segmented using Oufiti (Paintdakhi et al., 2016) in MatLab, and
382 fluorescence intensities normalized to cell lengths were extracted. Percentage cells with DSBs
383 were quantified by counting cells with Gam-GFP foci using the Cell Counter plugin in ImageJ.
384 Graphs were plotted in GraphPad Prism 7.

385 **Molecular Modelling**

386 The 3D structures of desired B-form DNA sequences from the NA1000 (*Caulobacter*
387 *crescentus*) genome sequence (15 bp from *dnaE* ORF: 5'-ATCGGCAAGCTGGCC-3', LexA box
388 within the promoter of *recA*: 5'-GTTCGCAAGATGTTC-3' and *CCNA_RR0074 sRNA*: 5'-
389 CCCCTCGCCCTCCT-3',) were generated using PyMOL 2.5 structure Builder. For small
390 molecule ligands used in this study, 3D structures were generated using Chem3D 20.0
391 program. The DNA structures were processed (energy minimization and addition of polar
392 hydrogens) using MGLTools v1.5.7 (<https://autodock.scripps.edu/>). The grid box was
393 configured for each DNA macromolecule to cover the whole length of the structure so that
394 the ligand was able to find best possible binding sites along with the DNA structures including
395 both the major and minor grooves. The small molecular ligands were also processed with the
396 same tools. Finally, the molecular docking was performed using opensource AutoDock Vina

397 v.1.2.0 (<https://vina.scripps.edu/>) (Trott & Olson, 2009). The default flexible docking
398 parameters were kept for docking. The post processing of the output files was curated using
399 PyMOL 2.5 and the molecular interactions were visualized using BIOVIA Discovery Studio
400 Visualizer.

401 **FRET-based DNA melting:**

402 All FRET duplexes and hairpins were purchased as pairs of complimentary or self-
403 complimentary single-stranded oligonucleotides in lyophilised form from Eurogentec Ltd. The
404 oligonucleotides were fluoro-tagged at the 5' position with TAM and 3' position with TAMRA.
405 Sequences used were as follows; AT-rich sequence (seq-1): 5'-FAM-TAT-ATA-TAG-ATA-TTT-
406 TTT-TAT-CTA-TAT-ATA-TAMRA-3'; GC-rich sequence (seq-2): 5'-FAM-TAT-AGG-GAC-AGC-CCT-
407 ATA-3', 3'-TAMRA-ATA-TCC-CTG-TCG-GGA-TAT-5'. Nuclease-free water was used to prepare
408 stock solutions (20 μ M) of the oligonucleotide hairpins/duplex strands. These stock solutions
409 were diluted to concentrations of 400 nM using FRET buffer (50 mM potassium cacodylate,
410 pH 7.4). The solutions were then heated to 85 °C/80 °C for five/ten minutes (hairpin/duplex
411 solutions, respectively) using a heating block (Grant-Bio). The solutions were allowed to cool
412 to room temperature overnight and cooled to -20 °C to complete the annealing process.
413 Annealed stock solutions were diluted to concentrations of 100 and 10 nM using FRET buffer
414 to prepare working solutions. PBD monomers, GWL-78 (Wells et al., 2006) and mitomycin C
415 to be incubated with the DNA duplexes were dissolved in DMSO to form 5 mM solutions.
416 Working solutions of PBD monomers and mitomycin C (5 μ M and 1 μ M) were prepared using
417 FRET buffer. The working solutions of the compounds and DNA hairpins/duplexes were mixed
418 (1:1 ratio, 25 μ L of each solution) in the wells of a 96 well plate (Bio-Rad). The wells were
419 covered and placed in a DNA Engine Opticon system for melting. The samples were heated
420 over a range of 30-100 °C, with fluorescence readings (incident radiation 450-495 nm,
421 detection 515-545 nm) taken at intervals of 0.5 °C. Experimental data was imported into
422 Origin (OriginLab Corp.), where the curves were smoothed and normalised. Using a script, the
423 point of inflection of the first derivative of the melting point for each sample on the plate was
424 calculated. The difference between the melting temperature of each sample and that of the
425 blank (i.e., the ΔT_m) was used for comparative purposes. Mean is shown from three
426 independent repeats.

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429 **References**

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705 **Author contributions**

706 AJ led the project, generated tools and reagents, carried out *in vivo* experiments in
707 *Caulobacter* and conducted data analysis. SD contributed tools and reagents, and carried out
708 *in vivo* experiments. KN and MMH carried out experiments pertaining to the KMR compounds
709 synthesis and *in vitro* characterization. RL and TL contributed tools and reagents. KMR and AB
710 conceived and supervised the project. AJ, KMR and AB procured funding and wrote the
711 manuscript, with feedback from all authors.

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717 **Declaration of interests**

718 None declared.

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720 **Main Figure legends:**

721 **Figure 1: C8-linked PBD bi-aryl monomers KMR-28-33 and KMR-28-35.** (A): Structures of
722 KMR-28-33 and KMR-28-35; pyrrolo(2,1-c)(1,4)benzodiazepine (PBD) unit is highlighted by
723 the red square, Py - 1-methylpyrrol-3-amine, BztMC - methyl 5-aminobenzothiophene-2-
724 carboxylate, MPB - 4-(1-methyl-1H-pyrrol-3-yl)benzenamine. (B) Molecular docking of KMR-
725 28-33 and KMR-28-35 with 15 bp DNA sequence taken from the ORF of *dnaE* gene (5'-
726 ATCGGCAAGCTGGCC-3'; GC content – 66%) suggests snug fit of both KMR-28-33 and KMR-28-
727 35 within the DNA minor groove.

728 **Figure 2: KMR-28-33 and KMR-28-35 treatment causes cell death and induces the SOS**
729 **response.** (A) Representative images of wild type *Caulobacter crescentus* growth on
730 increasing concentrations of DNA damaging agents KMR-28-33, KMR-28-35 and MMC. Grey
731 triangle at the bottom of each image panel depicts increasing dilution of the bacterial culture
732 from left to right. Minimum of two independent experiments were performed for each dose.
733 (B) [left] SOS induction is measured by assessing the expression of YFP from an SOS-inducible
734 promoter (*P_{sidA}-yfp*). Representative images of cells expressing the reporter with and without
735 treatment with KMR-28-33 (0.5 µg/ml), KMR-28-35 (1 µg/ml) or MMC (0.5 µg/ml). Scale bar
736 – 4 µm. [right] Total fluorescence intensity normalized to cell area plotted for indicated
737 duration of damage treatment. Each dot represents a single cell. Mean and SD are shown in
738 black (n ≥ 215).

739 **Figure 3: Requirement for RecA, but not the SOS response, in C8-linked PBD bi-aryl**
740 **monomer-treated cells.** (A) Survival of wild type, $\Delta recA$ and $\Delta recA\Delta lexA\Delta sidA$ strains under
741 increasing doses of KMR-28-33, KMR-28-35 and MMC. Minimum of three independent
742 experiments were performed for each strain. Mean and SEM from all repeats for each strain
743 is plotted. (B) Survival of wild type and $\Delta dnaE2$ strains under increasing doses of KMR-28-33,
744 KMR-28-35 and MMC. Minimum of three independent experiments were performed for each
745 strain. Mean and SEM from all repeats for each strain is plotted (wild type data from Figure
746 3A for comparison).

747 **Figure 4: Recombination-mediated repair contributes to survival under DNA damage caused**
748 **by KMR-28-33 and KMR-28-35.** (A) Survival of wild type, $\Delta recA$, $\Delta addAB$ and $\Delta recN$ strains
749 under increasing doses of KMR-28-33, KMR-28-35 and MMC. Minimum of three independent

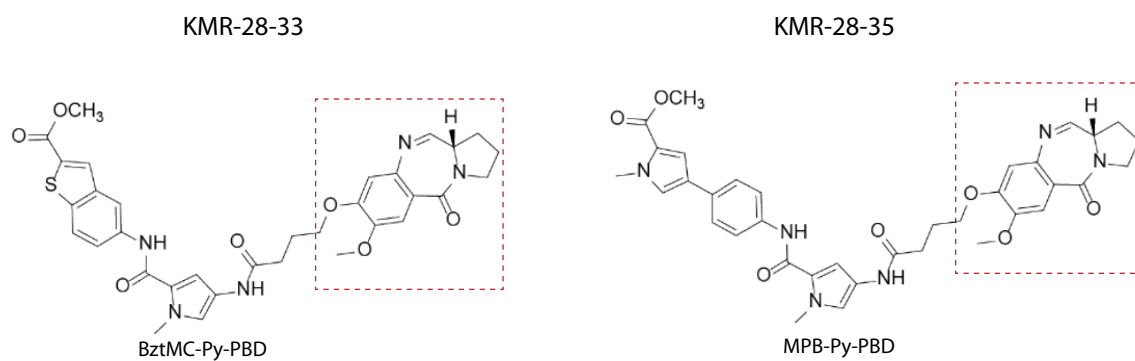
750 experiments were performed for each strain. Mean and SEM from all repeats for each strain
751 is plotted (wild type and $\Delta recA$ data from Figure 3A for comparison). (B) Survival of wild type,
752 $\Delta recF$, $\Delta recO$ and $\Delta recR$ strains under increasing doses of KMR-28-33, KMR-28-35 and MMC.
753 Minimum of three independent experiments were performed for each strain. Mean and SEM
754 from all repeats for each strain is plotted (wild type data from Figure 3A for comparison).

755 **Figure 5: Nucleotide excision repair (NER) is essential for survival under KMR-28-33 and**
756 **KMR-28-35-induced DNA damage.** (A) Survival of wild type, $\Delta recA$ and $\Delta uvrA$ strains under
757 increasing doses of KMR-28-33, KMR-28-35 and MMC. Minimum of three independent
758 experiments were performed for each strain. Mean and SEM from all repeats for each strain
759 was plotted (wild type and $\Delta recA$ data from Figure 3A for comparison). (B) [left]
760 Representative images for cells with Gam-GFP foci upon treatment with KMR-28_33, KMR-
761 28-35 and MMC in wild type and $\Delta uvrA$ strains (wild type images from Figure S3A for
762 comparison). Scale bar – 4 μ m. [right] Percentage cells with Gam-GFP foci upon treatment
763 with KMR-28-33, KMR-28-35 and MMC in wild type and $\Delta uvrA$ strains (wild type data from
764 Figure S3A for comparison). Mean and SD for data from three independent experiments is
765 plotted ($n \geq 330$ cells).

766

Figure 1

A.



B.

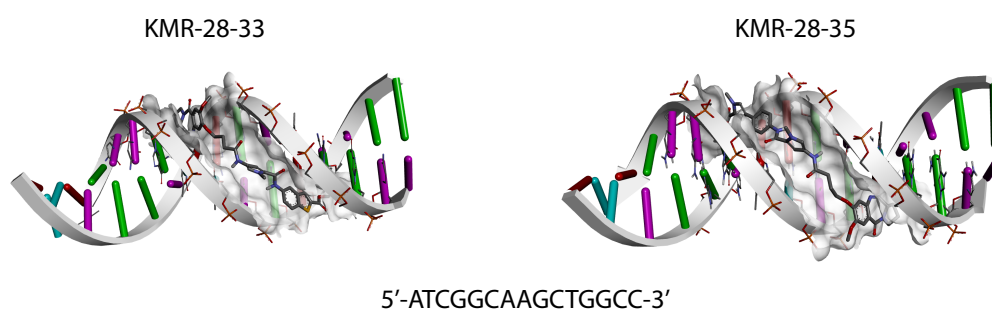
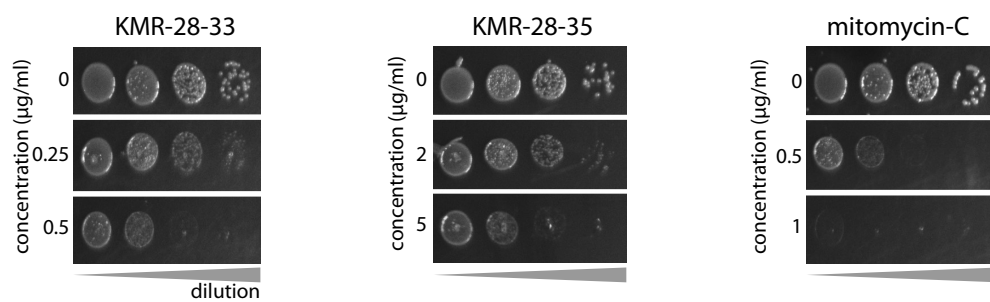


Figure 2

A.



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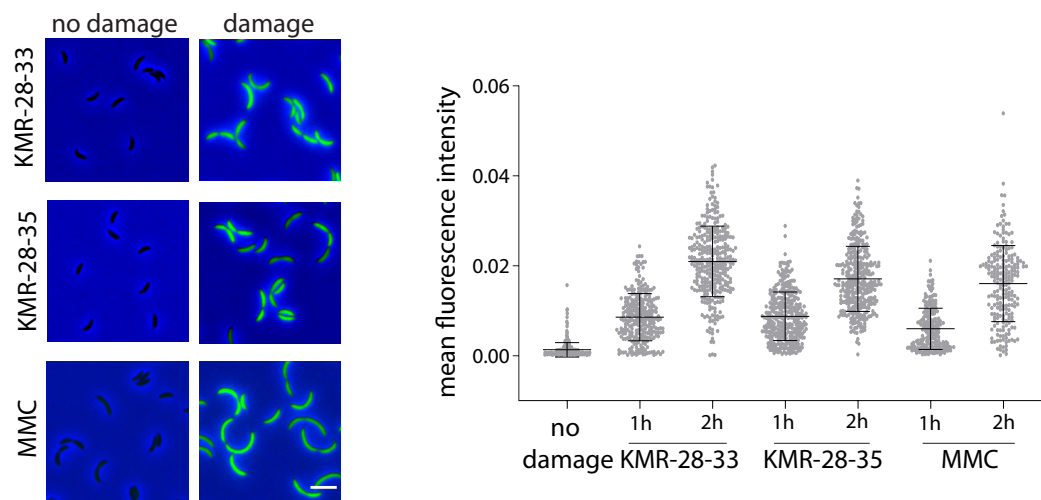


Figure 4

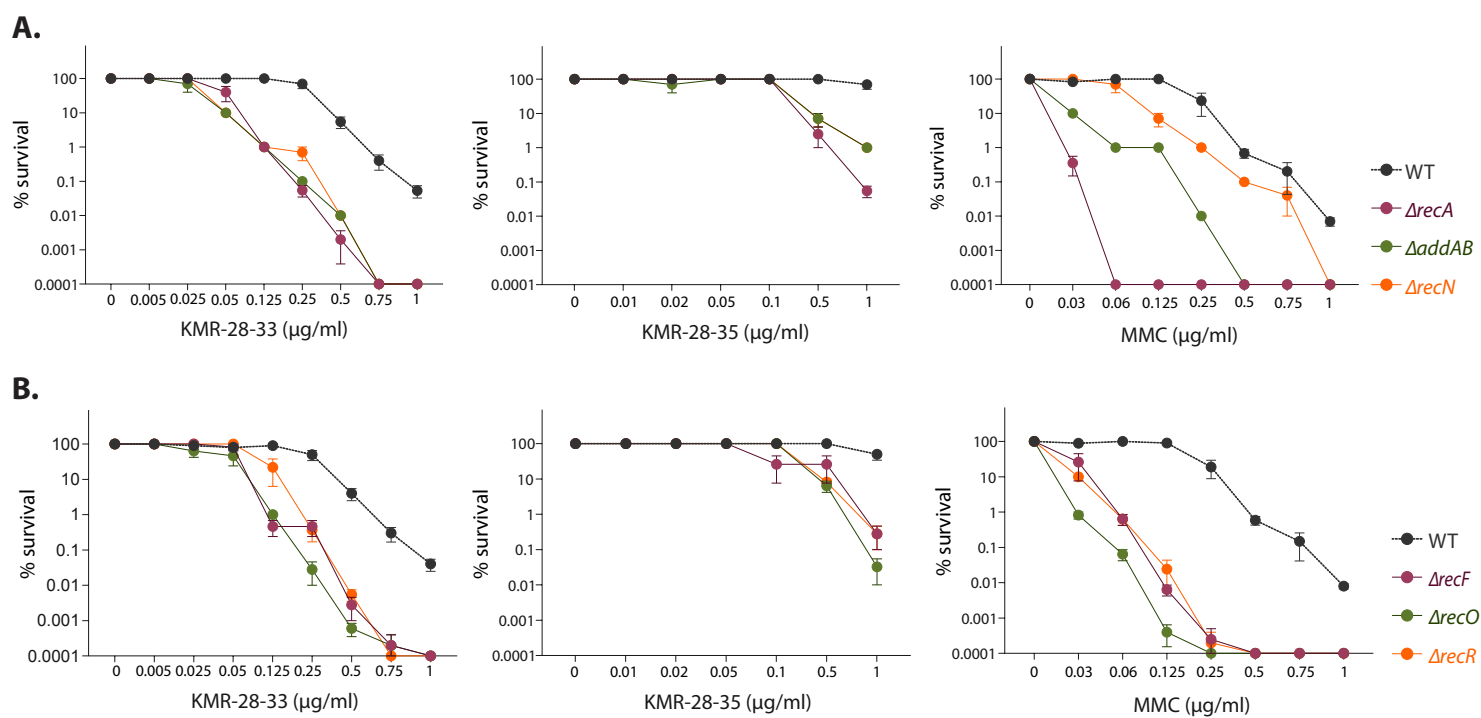
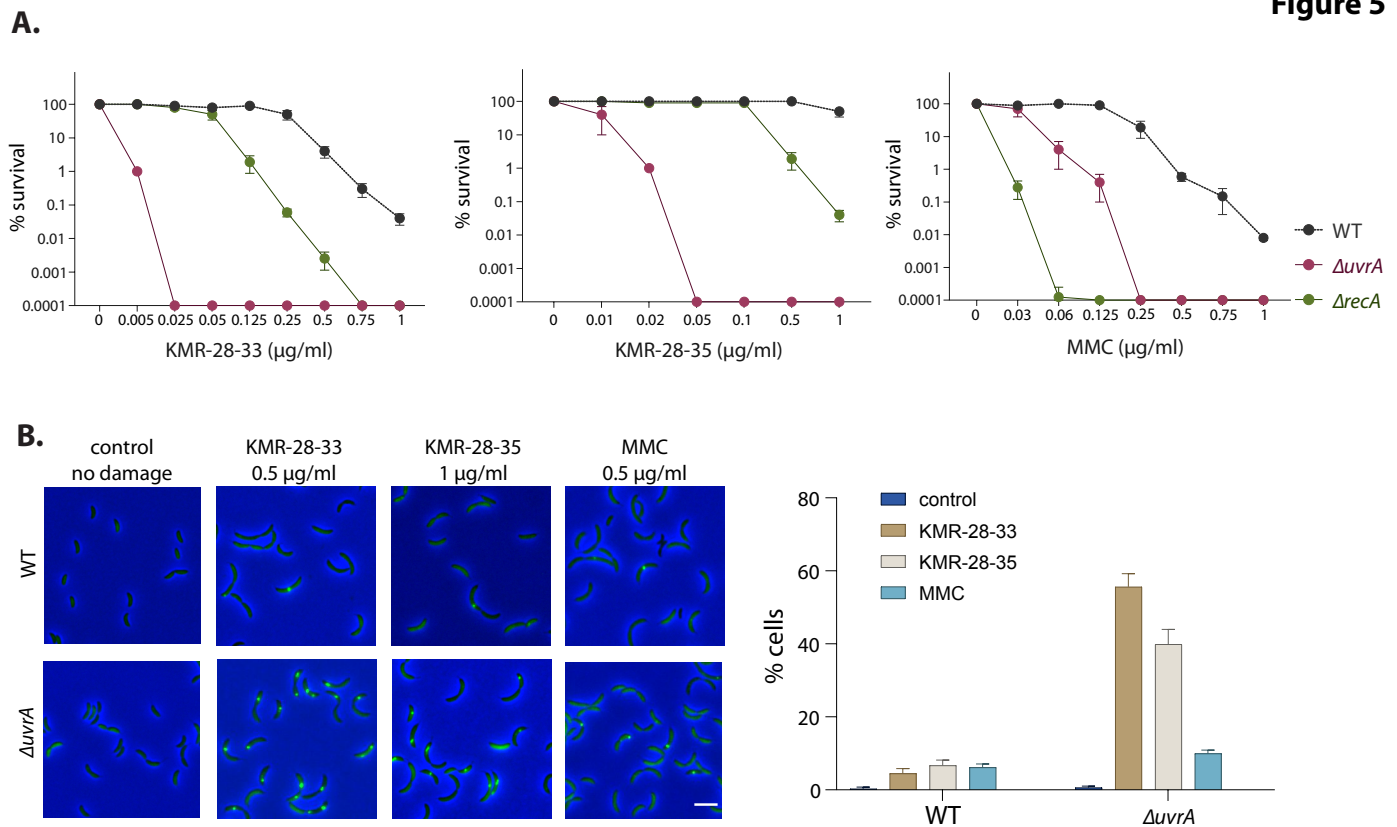


Figure 5



Supplementary Information

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Requirement for specific bacterial genome maintenance pathways in repair of C8-linked pyrrolobenzodiazepine (PBD) bi-aryl monomer-mediated DNA damage

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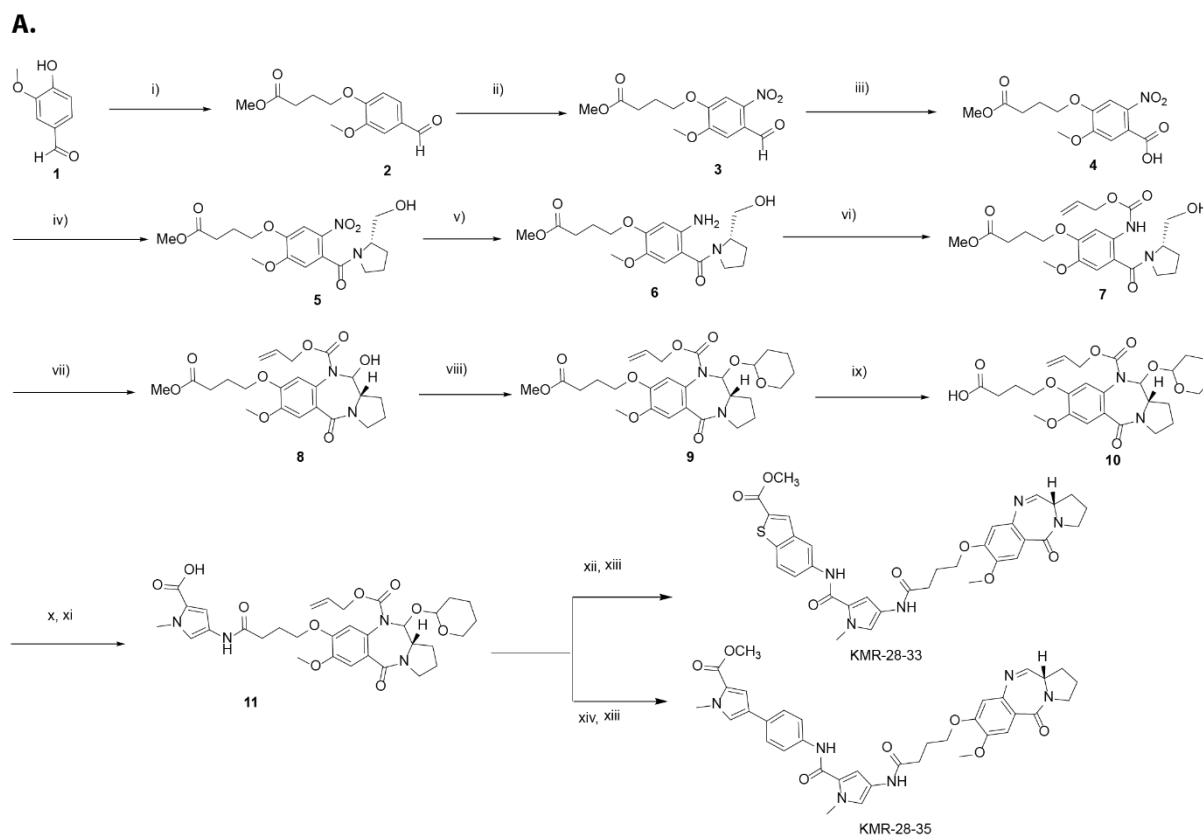
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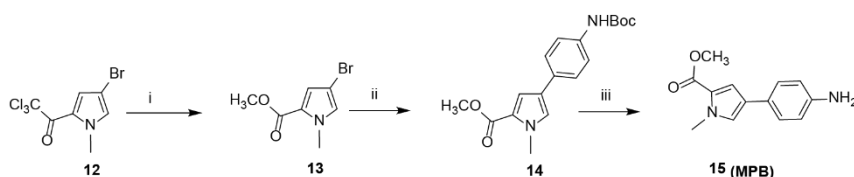
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22 Supplementary Figures:

Figure S1



B.



Reagents and Conditions

i) Na-Methoxide, MeOH (anhydrous), H₂SO₄, reflux; ii) 4-(tert-butoxycarbonylamino)phenylboronic acid, (PPh₃)₄Pd, K₂CO₃, Ethanol:Toluene:Water 9:3:1, MW iv) 4M HCl in Dioxane, RT.

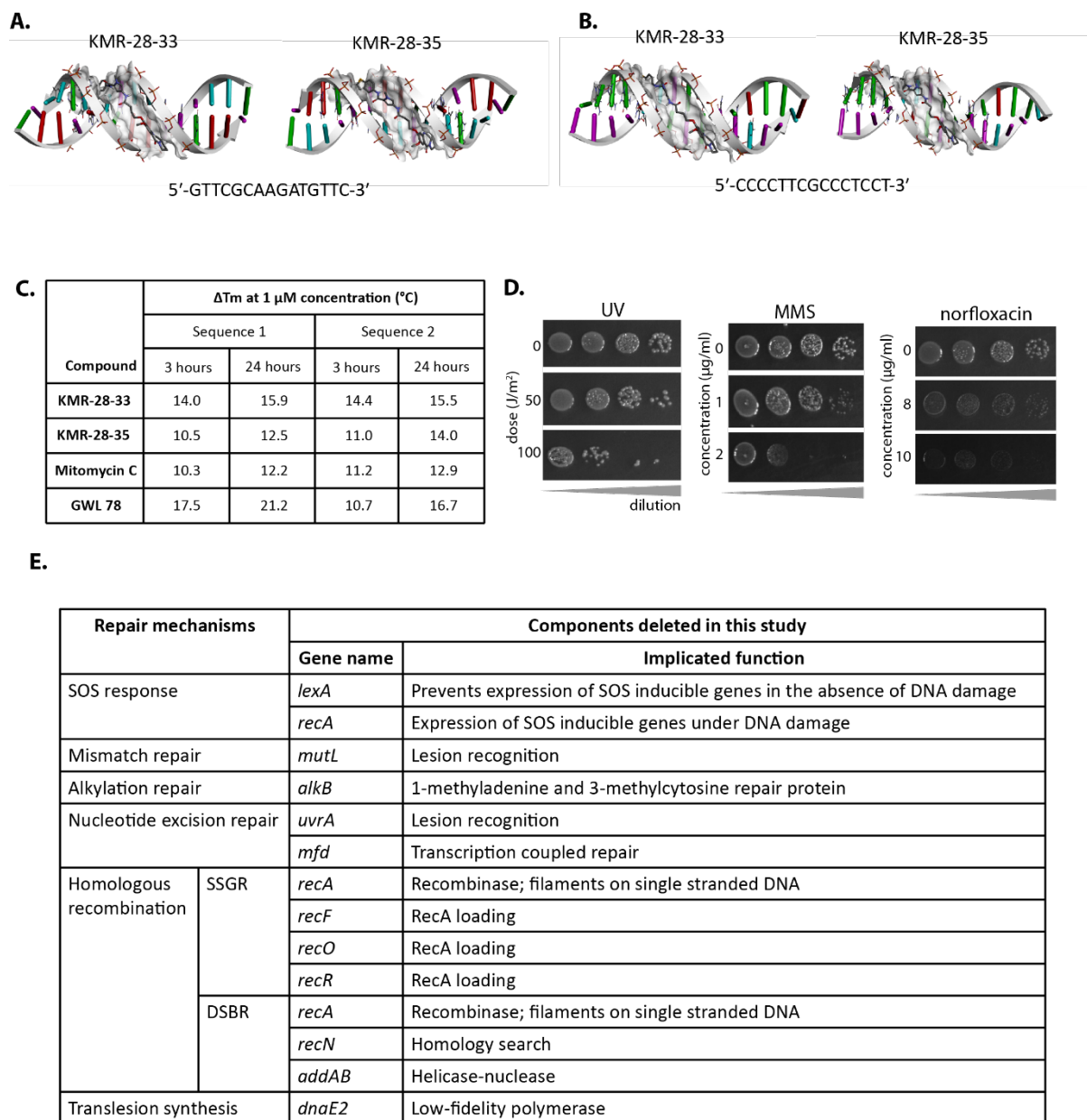
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24 **Figure S1:** (A) General synthetic scheme for the synthesis of PBD core (10) and the conjugation
 25 of C8-side chain to the PBD core to obtain KMR-28-33 and KMR-28-35. (B) Synthesis of MPB
 26 building block 15 which is present in KMR-28-35.

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Figure S2

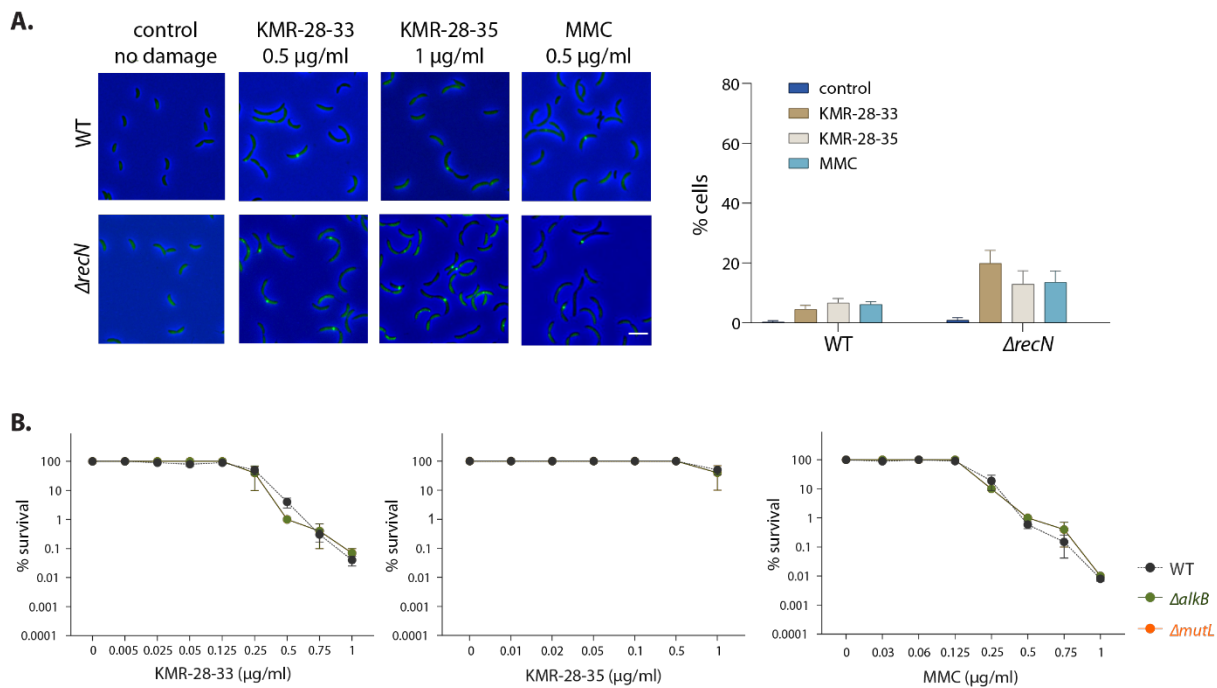


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30 **Figure S2:** (A) Molecular docking of KMR-28-33 and KMR-28-35 with 15 bp DNA sequence
31 taken from the LexA box within the promoter of *recA* gene (5'-GTTCGCAAGATGTTTC-3'; GC
32 content – 46%). (B) Molecular docking of KMR-28-33 and KMR-28-35 with 15 bp DNA
33 sequence taken from the sRNA gene *CCNA_R0074* (5'-CCCCTTCGCCCTCCT-3'; GC content –
34 73%) (C) DNA thermal stabilization data for KMR-28-33, KMR-28-35 and mitomycin C with an
35 AT-rich (seq-1) and a GC-rich (seq-2) hairpin DNA sequence. As a positive control, previously
36 characterized compound GWL-78 is used. Average from three replicates shown (see methods
37 for details of experimental setup). (D) Representative images of wild type *Caulobacter*
38 *crescentus* growth on increasing concentrations of DNA damaging agents UV, MMS and
39 norfloxacin. Grey triangle at the bottom of each image panel depicts increasing dilution of the
40 bacterial culture from left to right. Minimum of three independent experiments were

41 performed for each condition. (E) Table of all the repair components deleted in this study and
42 their ascribed functions; SSGR – single-strand gap repair, DSBR – double-strand break repair.
43

Figure S3



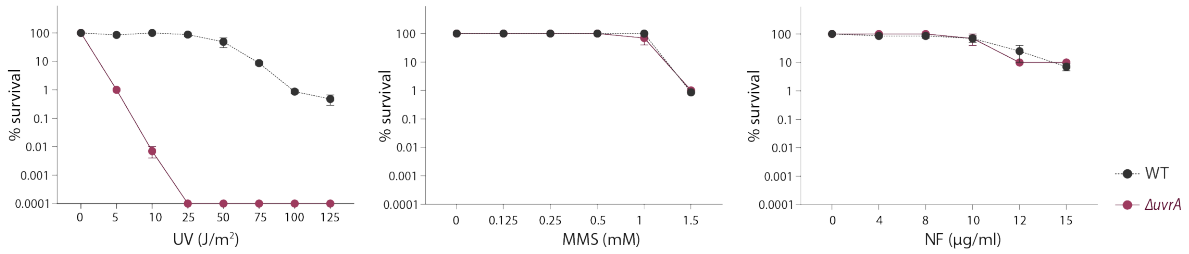
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45 **Figure S3:** (A) [left] Representative images for cells with Gam-GFP foci upon treatment with
 46 KMR-28-33, KMR-28-35 and MMC in wild type and $\Delta recN$ strains. Scale bar – 4 µm. [right]
 47 Percentage cells with Gam-GFP foci upon treatment with KMR-28-33, KMR-28-35 and MMC
 48 in wild type and $\Delta recN$ strains. Mean and SD for data from three independent experiments is
 49 plotted ($n \leq 330$ cells). (B) Survival of wild type, $\Delta mutL$ and $\Delta alkB$ strains under increasing
 50 doses of KMR-28-33, KMR-28-35 and MMC. Minimum of three independent experiments
 51 were performed for each strain. Mean and SEM from all repeats for each strain is plotted
 52 (wild type data from Figure 3A for comparison).

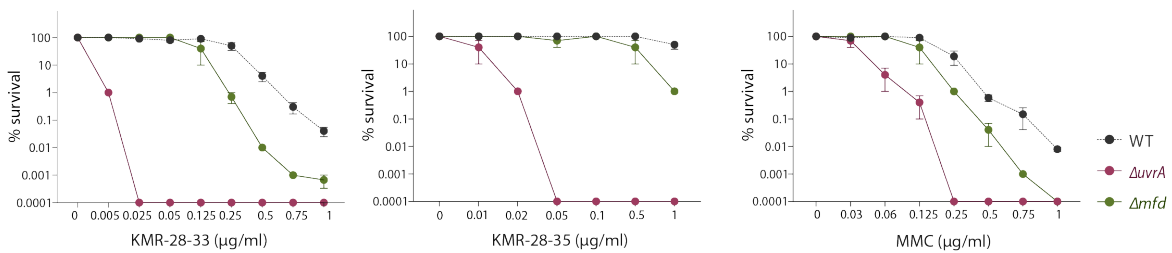
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Figure S4

A.



B.



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55 **Figure S4:** (A) Survival of wild type and $\Delta uvrA$ strains under increasing doses of UV, MMS and
56 norfloxacin (NF) damage. Minimum of three independent experiments were performed for
57 each strain. Mean and SEM from all repeats for each strain is plotted. (B) Survival of wild type,
58 $\Delta uvrA$ and Δmfd strains under increasing doses of KMR-28-33, KMR-28-35 and MMC.
59 Minimum of three independent experiments were performed for each strain. Mean and SEM
60 from all repeats for each strain is plotted (wild type and $\Delta uvrA$ data from Figure 3A for
61 comparison).

62

Figure S5

DNA damage	Lesion type	Repaired by
KMR-28-33	Mono-adducts	Nucleotide excision repair Recombination repair
KMR-28-35	Mono-adducts	Nucleotide excision repair Recombination repair
Mitomycin C (MMC)	Mono-adducts intra-strand crosslinks inter-strand crosslinks	Nucleotide excision repair Translesion synthesis Recombination repair

63

64 **Figure S5:** Table summarizing findings from this study on DNA repair mechanisms essential
65 for repair/tolerance of lesions induced by KMR-28-33, KMR-28-35 and MMC.

66

67 **Table S1: Strains used in present study**

Strain name	Genotype	Strain construction
CB15N		
NABC2	<i>CB15N; ΔrecA</i>	(Modell et al., 2014)
NABC29	<i>CB15N; ΔdnaE2</i>	CB15N was transformed with pNABC148 plasmid to generate deletion of <i>dnaE2</i> through two-step recombination procedure.
NABC238	<i>CB15N; ΔuvrA</i>	CB15N transformed with pNABC417 plasmid to generate deletion of <i>uvrA</i> through two-step recombination procedure.
NABC239	<i>CB15N; ΔaddAB::gent</i>	CB15N transduced with lysate of strain harboring deletion of <i>addAB</i> linked to <i>gent^R</i>
NABC265	<i>CB15N; ΔrecA::kan^R, ΔlexA::tet^R, ΔsidA</i>	<i>CB15N; ΔlexA::tet^R, ΔsidA</i> strain transduced with lysate of strain harboring <i>recA</i> deletion linked to <i>kan^R</i>
NABC268	<i>CB15N; P_{xyI}-Gam-GFP::spec^R</i>	CB15N transformed with pNABC592 plasmid
NABC439	<i>CB15N; ΔrecN</i>	(Badrinarayanan et al., 2015)
NABC499	<i>CB15N; ΔrecF</i>	CB15N transformed with pYY193 plasmid to generate deletion of <i>recF</i> (3rd-100 th codon) through two-step recombination procedure.
NABC502	<i>CB15N; ΔrecR</i>	CB15N transformed with pYY115 plasmid to generate deletion of <i>recR</i> through two-step recombination procedure.
NABC505	<i>CB15N; ΔrecO</i>	CB15N transformed with pYY119 plasmid to generate deletion of <i>recO</i> through two-step recombination procedure.
NABC544	<i>CB15N; Δmfd</i>	CB15N transformed with pNABC590 plasmid to generate deletion of <i>mfd</i> through two-step recombination procedure.
NABC579	<i>CB15N; ΔalkB</i>	CB15N transformed with pNABC591 plasmid to generate deletion of <i>alkB</i> through two-step recombination procedure.
NABC580	<i>CB15N; ΔmutL</i>	CB15N transformed with pNABC416 plasmid to generate deletion of <i>mutL</i> through two-step recombination procedure.
NABC581	<i>CB15N; P_{sidA}-YFP::kan^R</i>	CB15N transformed with pNABC420 (Chimthanawala et al., 2021)

NABC582	<i>CB15N; ΔuvrA; P_{xyI}-Gam-GFP::spec^R</i>	NABC238 transformed with pNABC592 plasmid
NABC583	<i>CB15N; ΔrecN::spec^R; P_{xyI}-Gam-GFP::spec^R</i>	NABC439 transformed with pNABC592 plasmid

69 **Table S2: Plasmids used in present study**

Plasmid name	Construct details	Antibiotic marker
pNPTS138	(Skerker et al., 2005)	Kanamycin
pXGFPC1	(Thanbichler et al., 2007)	Spectinomycin
pXYFPC2	(Thanbichler et al., 2007)	Kanamycin
pNABC148	600 bp fragments upstream and downstream of <i>dnaE2</i> genomic locus were amplified from <i>C. crescentus</i> gDNA using RR_oligo_021/RR_oligo_022 (upstream fragment) and RR_oligo_023/RR_oligo_024 (downstream fragment) primer pairs. These fragments were assembled with BamH1/Nhe1 linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pNABC416	600 bp fragments upstream and downstream of <i>mutL</i> genomic locus were amplified from <i>C. crescentus</i> gDNA using PS_oligo_049/AMJ_oligo_061 (upstream fragment) and AMJ_oligo_062/PS_oligo_054 (downstream fragment) primer pairs. These fragments were assembled with BamH1/Nhe1 linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pNABC417	600 bp fragments upstream and downstream of <i>uvrA</i> genomic locus were amplified from <i>C. crescentus</i> gDNA using PS_oligo_037/AMJ_oligo_057 (upstream fragment) and AMJ_oligo_058/PS_oligo_042 (downstream fragment) primer pairs. These fragments were assembled with BamH1/Nhe1 linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pYY193	500 bp fragments upstream and downstream of <i>recF</i> (from the 3 rd to the 100 th codon) were amplified from <i>C. crescentus</i> gDNA using UPdel00158_F/UPdel00158_R (upstream fragment) and DWNdel00158_F/DWNdel00158_R (downstream fragment) primer pairs. These fragments were assembled with EcoRI/BamHI linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pYY115	500 bp fragments upstream and downstream of <i>recR</i> were amplified from <i>C. crescentus</i> gDNA using UPdel00270_F/UPdel00270_R (upstream fragment) and DWNdel00270_F/DWNdel00270_R (downstream fragment) primer pairs. These fragments were assembled with EcoRI/BamHI linearized pNPTS138 vector using Gibson assembly.	Kanamycin

pYY119	500 bp fragments upstream and downstream of <i>recO</i> were amplified from <i>C. crescentus</i> gDNA using UPdel01635_F/UPdel01635_R (upstream fragment) and DWNdel01635_F/DWNdel01635_R (downstream fragment) primer pairs. These fragments were assembled with EcoRI/BamHI linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pNABC420	pXYFPC2 vector was amplified using AB_oligo_651 and AB_oligo_652 and P _{sidA} -YFP fragment was amplified from a replicating plasmid harbouring YFP under P _{sidA} promoter using AC_oligo_322 and AC_oligo_321. The vector and insert fragments were assembled with Gibson assembly.	Kanamycin
pNABC590	600 bp fragments upstream and downstream of <i>mfd</i> genomic locus were amplified from <i>C. crescentus</i> gDNA using SD_oligo_088/SD_oligo_089 (upstream fragment) and SD_oligo_090/SD_oligo_0091 (downstream fragment) primer pairs. These fragments were assembled with BamH1/Nhe1 linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pNABC591	600 bp fragments upstream and downstream of <i>alkB</i> genomic locus were amplified from <i>C. crescentus</i> gDNA using AMJ_oligo_041/AMJ_oligo_053 (upstream fragment) and AMJ_oligo_054/PS_oligo_044 (downstream fragment) primer pairs. These fragments were assembled with BamH1/Nhe1 linearized pNPTS138 vector using Gibson assembly.	Kanamycin
pNABC592	<i>gam_GFP</i> was amplified using SD_oligo_019 and SD_oligo_020 from an <i>E. coli</i> strain harboring <i>gam-gfp</i> (Shee <i>et al.</i> , 2013) and assembled with Nde1/Nhe1 digested pXYFPC-1 using Gibson assembly.	Spectinomycin

71 **Table S3: Oligos used in present study**

Primer name	Sequence
AB_oligo_651	CTGGACCTCTTGCCCATGACCGA
AB_oligo_652	GCTAGCTGCAGCCCGGGG
AC_oligo_321	AACTAGTGGATCCCCGGGCTGCAGCTAGCTTACTTGTACAGCTCGT CCATGCCGA
AC_oligo_322	GGTCAGGTCGGTCATGGGCAAGAGGTCCAGCACCCGCCCATCACCCA CAGATGC
AMJ_oligo_041	CAAGCTTCTCTGCAGGATATCTGTGCGGCCAATCAGGCGCTTGATCG
AMJ_oligo_044	CGGAGACGCGTCACGGCCGAAGAGCCGGCGGATCGCAACCTCC
AMJ_oligo_053	AGAGTCAGATTGATCCGGCCTACGTCAAAGCCGGGGACAACGGT
AMJ_oligo_054	TTGTCCCCGGCTTTGACGTAGGCCGATCAATCTGACTCTGCGACG
AMJ_oligo_057	GCCTGCTGAGCCGCCTTAGTTTTCCGGAACGTTGGAC
AMJ_oligo_058	GTCCAACGTTCCGGAAAATAAGGCGGCTCAGCAGGC
AMJ_oligo_061	TAGGGGGCGCTCTGGCCTCACATCAAGCGGACTTTCACGGG
AMJ_oligo_062	CCCGTGAAAGTCCGCTTGATGTGAGGCCAGAGCGCCCCCTA
PS_oligo_037	CAAGCTTCTCTGCAGGATATCTGCTTGCGGATGGCGTCACCT
PS_oligo_042	CGGAGACGCGTCACGGCCGAAGTCTACGCAGACGTGGATCTTG
PS_oligo_049	CAAGCTTCTCTGCAGGATATCTGGTCAAATGCTTCTCCAGCCG
PS_oligo_054	CGGAGACGCGTCACGGCCGAAGGAAGGAGACGAGACGATGGA
SD_oligo_019	CAGACGCTCGAGTTTTGGGGAGACGACCATATGGCTAAACCAGCAAA ACGTATCAAG
SD_oligo_020	GAAGTAGTGGATCCCCGGGCTGCAGCTAGCGCAGCCGGATCCCTTA TTTGTATAGTTC
SD_oligo_088	CAAGCTTCTCTGCAGGATATCTGTTTCGCTATCGACCACTATCT
SD_oligo_089	TGGGCCGCGGTGTCCCATTCATGACCAGGGCGTCGAAGCC
SD_oligo_090	GGCTTCGACGCCCTGGTCATGAATGGGACACGCCGGCCCA
SD_oligo_091	CGGAGACGCGTCACGGCCGAAGCAGAAGTTCAAGGACCCGGAGAAA
RR_oligo_021	CAAGCTTCTCTGCAGGATATCTGGACGCTGGCGCCGTTGATC
RR_oligo_022	ATCGCGCCCCGCTCACATGTTAGGTCCTCCCCCTCGC
RR_oligo_023	GGAGGACCTAACATGTGAGCGGGGCGCGATCCT
RR_oligo_024	CGGAGACGCGTCACGGCCGAAGGCGACATGCGGGTCAGCA
UPdel01635_F	TTCTCTGCAGGATATCTGGATCCACAATCGACGGCGAGACCTGGCTGGC
UPdel01635_R	TGACGGTCTTAGAGCTCCAGGCTCAAGCGGGGTTCCCCACGCT
DWNdel01635_F	CGCTTGAGCCTGGAGCTAAGAACCGTCACAAAGCGGCGCTATC
DWNdel01635_R	TCACGGCCGAAGCTAGCGAATTCGCCGGCGTCTGCGGGCCCCGCCCCA
UPdel00270_F	TTCTCTGCAGGATATCTGGATCCATCGAGGGGAGGGGAACTTGAAGTTG
UPdel00270_R	GGGAATTTGTCAAGCGGCCATCAGGTCCTTCGGATAGGCGGTGCG
DWNdel00270_F	AAGGACCTGATGGCCGCTTGACAAATCCCGAGCGTCTTCGGGA
DWNdel00270_R	TCACGGCCGAAGCTAGCGAATTCGGGTGGCGCTCTGATCCTCCAGCAGG
UPdel00158_F	TTCTCTGCAGGATATCTGGATCCCATCCGTCGGGCGGTCTCTCTATCG
UPdel00158_R	GGTGCACGCGCCGCCATGCGGGCGTCTTAAGCGGTGTCGG
DWNdel00158_F	AACGCCCGCATGGCGGGCGCGTTCGACCGTGGGCTCGAGGGCGAG
DWNdel00158_R	TCACGGCCGAAGCTAGCGAATTCCTCGCGGGCCCGCGGGACGCCAGGG

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