A simplified method for CRISPR-Cas9 engineering of *Bacillus subtilis*

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Abstract

The clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 system from *Streptococcus pyogenes* has been widely deployed as a tool for bacterial strain construction. Conventional CRISPR-Cas9 editing strategies require design and molecular cloning of an appropriate guide RNA (gRNA) to target genome cleavage and a repair template for introduction of the desired site-specific genome modification. Here, we present a streamlined method that leverages the existing collection of nearly 4000 *Bacillus subtilis* strains (the BKE collection) with individual genes replaced by an integrated erythromycin (*erm*) resistance cassette. A single plasmid (pAJS23) with a gRNA targeted to *erm* allows cleavage of the genome at any non-essential gene, and at sites nearby to many essential genes. This plasmid can be engineered to include a repair template, or the repair template can be co-transformed with the plasmid as either a PCR product or genomic DNA. We demonstrate the utility of this system for generating gene replacements, site-specific mutations, modification of intergenic regions, and introduction of gene-reporter fusions. In sum, this strategy bypasses the need for gRNA design and allows the facile transfer of mutations and genetic constructions with no requirement for intermediate cloning steps.

Importance

Bacillus subtilis is a well-characterized Gram-positive model organism and a popular platform for biotechnology. Although many different CRISPR-based genome editing strategies have been developed for *B. subtilis*, they generally involve the design and cloning of a specific gRNA and repair template for each application. By targeting the *erm* resistance cassette with an anti-*erm* gRNA, genome-editing can be directed to any of nearly 4000 gene disruptants within the existing BKE collection of strains. Repair templates can be engineered as PCR products, or specific alleles and constructions can be transformed as chromosomal DNA, thereby bypassing the need for plasmid construction. The described method is rapid, free of off-target effects, and facilitates a wide-range of genome manipulations.

"Healing is a matter of time, but it is sometimes also a matter of opportunity." – *Hippocrates*

Introduction

Bacillus subtilis is a Gram-positive model bacterium and serves as an important platform for industry, including the production of commodity chemicals, metabolites, and proteins (1-3). A wide variety of approaches are available for genome modification and editing, which are further aided by the natural competence of many laboratory strains.

One of the earliest methods for introducing directed mutations was transformation with plasmids that lack an active origin in *B. subtilis*. Selection for a plasmid-borne antibiotic resistance gene results in recovery of plasmid integrants, which allows for gene disruptions or the generation of reporter fusions. This approach, as implemented in the pMUTIN series of plasmids (4), was widely employed in early functional genomics studies of *B. subtilis*. However, plasmids integrated by single-crossover can have polar effects, and the integrational event is reversible when selection is removed, leading to genetic instability.

Further refinements were soon introduced to allow introduction of mutations stable in the absence of ongoing selection. For example, with long-flanking homology PCR (LFH-PCR), double-crossover integration occurs at regions upstream (UP) and downstream (DO) of the desired site of genome modification (5). This can be used to introduce null or altered function mutations, epitope- or fluorescent protein tags, or reporter fusions at a specific locus as long as the desired strain can be isolated by selection or identified by screening. If there is no selection, as is often the case, an

80 antibiotic resistance marker can be included in the region between the UP and DO
81 regions of homology for selection. Mutations introduced in this manner are generally
82 stable in the absence of ongoing selection, but engineering strains with multiple
83 mutations can be limited by the available resistance cassettes. This technique was
84 therefore further refined using Cre/Lox-based recombination to remove the antibiotic
85 resistance cassette (6), and by using MazF as a counter-selectable marker (7, 8).
86 The development of the BKE/BKK collection of strains has greatly simplified the
87 generation of *B. subtilis* strains carrying null mutations (9). These collections, with
88 nearly 4000 strains each, contain erythromycin-resistant (BKE) or kanamycin-resistant
89 (BKK) single-gene replacements, and are available from the Bacillus Genetic Stock
90 Center (BGSC, USA) and the National BioResource Project (NBRP, Japan). Typically,
91 null mutations are introduced into the genetic background of choice by transformation of
92 genomic DNA from the chosen BKE or BKK strain with selection for the antibiotic
93 cassette. In a subsequent step, the strain can be by transformed with pDR244, a
94 temperature-sensitive plasmid expressing the Cre recombinase to excise the antibiotic
95 cassette leaving behind an in-frame gene deletion with a 150 bp (50 amino acid) bar-
96 coded scar (9). This process can be repeated to generate strains containing multiple
97 null mutations.
98 The CRISPR-Cas9 system, originally from *Streptococcus pyogenes*, has been
99 widely adapted for bacterial genome editing including several variants for *B. subtilis* (10-
100 13). CRISPR-Cas9 genome editing facilitates genetic manipulations, including the
101 introduction of point mutations, large deletions, epitope- or fluorescent protein tags,
102 regulatory site mutations, and expression of non-native genes. In addition, targeting of

103 inactive nucleases to specific promoter regions allows for interference with gene
104 expression (CRISPRi) and thereby gene repression (10, 14, 15). As often applied, the
105 Cas9 endonuclease is targeted to a specific site by an ~20 nt guide RNA (gRNA), and
106 homologous recombination with a cloned repair template allows for chromosome repair
107 and cell survival. The gRNA and repair template can be introduced together on a single-
108 plasmid system as implemented in plasmid pJOE8999 (11). While this plasmid provides
109 a powerful tool for a wide-variety of genome manipulations, each construct requires
110 design and cloning of both an appropriate gRNA and the desired repair template
111 containing the intended mutation together with sufficient flanking DNA for homologous
112 recombination. These multiple cloning steps present a bottleneck for the rapid
113 deployment of CRISPR-Cas9 genome engineering. This has motivated efforts to
114 increase the rapidity with which genomes can be edited by using strains with Cas9
115 expressed from an inducible chromosomal locus (10), plasmids optimized for rapid
116 fragment exchange (16), and introduction of repair templates by co-transformation with
117 PCR products (10).

118 Here, we have sought to streamline genome editing by leveraging the extant
119 BKE collection of nearly 4000 *B. subtilis* strains with individual genes replaced by an
120 integrated erythromycin (*erm*) resistance cassette (9). We have designed a plasmid
121 (pAJS23) containing a gRNA targeted to the erythromycin (*erm*) cassette. pAJS23 can
122 be modified to incorporate repair template DNA, or the repair template can be provided
123 by co-transformation with either a PCR product or chromosomal DNA. This approach
124 allows one-step genome editing with no need for gRNA design or plasmid cloning steps.

125 This single plasmid system will allow for greater accessibility and efficiency in genome
126 editing of *B. subtilis*.
127
128 **Results**
129
130 **Plasmid pAJS23 encodes an *erm*-directed gRNA.** Plasmid pJOE8999 has been
131 widely used for CRISPR-Cas9 mediated engineering in *B. subtilis* (11). Rather than
132 designing a unique gRNA for each application, we sought to leverage the large BKE
133 collection of single-gene *erm* replacements by design of an anti-*erm* gRNA. The
134 designed gRNA is specific to the *erm* gene, and the closest homology in the *B. subtilis*
135 168 genome has 4 mismatches, which thereby prevents off-target effects. The resultant
136 plasmid, pAJS23, allows selective cleavage of the chromosome at any site with an
137 integrated *erm* gene (Fig. 1). Subsequent to chromosome cleavage, homologous
138 recombination with a cognate repair template restores chromosome integrity to allow
139 survival. Here, we present several examples of genome-editing using DNA repair
140 templates either cloned into pAJS23, or co-transformed as either a PCR product or
141 genomic DNA.
142
143 **Genome-editing using pAJS23 with a cloned guide RNA.** As shown in previous
144 studies, repair DNA templates for CRISPR-Cas based *B. subtilis* genome-editing can be
145 provided by cloning into a plasmid (11), but can also be provided by transformation of
146 PCR products into cells, albeit with reduced efficiency (10). We first explored the utility
147 of pAJS23 for genome editing by cloning appropriate repair templates into pAJS23. For

148 each application, the repair template is generated containing the desired cargo (gene of
149 interest with or without modifications) flanked by UP and DO regions of homology for
150 integration into the chromosome. For cloning of the repair template into pAJS23 the
151 outermost primers used for UP and DO region amplification contain SfiI recognition sites
152 corresponding to the two SfiI sites in the parent plasmid pJOE8999 (11). Note that SfiI
153 recognizes the sequence GGCCNNNN/NGGCC and leaves a 3 nt 3'-overhang. The two
154 SfiI sites in pJOE8999 leave different overhangs (5'ACG3' and 5'TAT3'), and the
155 primers used to amplify the repair template should include SfiI sites that generate 3'-
156 overhangs (5'CGT3' and 5'ATA3') that are compatible with the vector overhangs.
157 For genome engineering, it is recommended to first transform the relevant gene
158 disruptant from the BKE collection into the desired parent strain prior to genome-editing.
159 This helps to ensure that all strains are isogenic except for the modified region. Next,
160 the pAJS23 derivative carrying the desired repair template is transformed into the strain
161 containing an *erm* cassette in the region between the UP and DO regions of homology.
162 Cleavage of the chromosome within the *erm* cassette is a potentially lethal event, and
163 surviving cells are enriched for those where chromosome integrity is restored by
164 recombination at the UP and DO homologous regions.
165
166 **Gene replacement at locus.** As a first application, we sought to replace an
167 endogenous *B. subtilis* gene with an ortholog from another organism. We targeted
168 *cpgA*, the third gene in the *prpC-prkC-cpgA-rpe-yloS* operon. The *cpgA* gene encodes a
169 ribosome assembly GTPase (required for growth at lower temperature) that additionally
170 functions as a metabolite proofreading phosphatase important under glycolytic

171 conditions (17). We sought to determine whether these functions are conserved among
172 related organisms using gene replacement to substitute, at locus, the *cpgA* coding
173 sequence with that of the *Staphylococcus aureus* ortholog *rsgA*.
174 For this application, we first generated LFH-PCR products containing three DNA
175 fragments corresponding to (i) an upstream region of homology from *prkC* (UP), (ii) the
176 *Staphylococcus aureus rsgA* gene (*cpgA* ortholog), and (iii) a downstream region of
177 homology from *rpe* (DO). The resulting 2.4 kb LFH-PCR fragment was cloned into
178 pAJS23 between the adjacent SfiI restriction sites to generate pAJS24, which was
179 transformed into BKE15780 (*cpgA*::*erm trpC2*). Transformants were selected on LB
180 plates at 30 ˚C with kanamycin (to select for the temperature-sensitive plasmid) and
181 mannose (to induce gRNA expression) for 48 h. The resultant Kan^R clones contain the
182 plasmid and express the anti-*erm* gRNA, so there is a strong selection for
183 recombinational repair. We then patched the transformants onto LB plates without
184 antibiotics at 42 ˚C to facilitate plasmid curing. After three passages at 42 ˚C, the
185 majority (89%) of the test clones were MLS sensitive (MLS^S), indicative of loss of the
186 *erm* resistance cassette (Table 1). In each case tested (10/10), the resultant *erm*
187 sensitive colony contained the desired gene replacement, with *cpgA* replaced by the *S.*
188 *aureus rsgA* gene (Table 1). These *cpgA::rsgA-Sau* transformants were capable of
189 rescuing growth defects of *cpgA* null mutants as visualized through serial dilution at
190 permissive (37 ˚C) and non-permissive (30 ˚C) temperature for ribosome assembly
191 defects (Fig 2A).
192

193 **Introduction of an epitoge-tag.** As a second application, we sought to incorporate a
194 FLAG epitope tag by modification of the *B. subtilis sodA* gene encoding superoxide
195 dismutase. Since *sodA* deletion strains are defective in competence (18), we chose to
196 use the adjacent *yqgC*::*erm* BKE strain as recipient. We generated plasmid pAJS25 to
197 include a 3.9 kb repair template containing the desired *sodA*-FLAG gene flanked by
198 upstream (*yqgB*-*yqgC*) and downstream (*yqgE*) genes (Fig. 2B). In this case,
199 recombinational repair could occur downstream of *sodA*, leading to successful
200 integration of the *sodA*-FLAG gene, or within the *sodA* gene. After curing of the plasmid,
201 most of the recovered clones (66%, Table 1) were MLS^S, consistent with loss of the *erm*
202 cassette. All 10 of the MLS^S transformants characterized encoded the *sodA*-FLAG
203 protein, which was monitored as a function of cell growth by immunoblotting with anti-
204 FLAG rabbit primary antibody and HRP conjugated-anti rabbit mouse secondary
205 antibodies (Fig. 2B).
206
207 **Generation of a GFP-translational fusion.** As a third application, we modified the
208 *yqgC* gene to encode a translational green fluorescent protein (GFP) fusion protein.
209 Plasmid pAJS26 was engineered to contain a 4.3 kb repair template encoding *yqgC*-*gfp*
210 together with flanking upstream and downstream DNA. The *yqgC::erm* strain was the
211 recipient for genome editing. We again recovered MLS^S colonies, and all those tested
212 had the desired gene fusion as verified by DNA sequencing (Table 1). The resulting
213 strain expressed GFP, as visualized during late stationary growth with fluorescence
214 microscopy (Fig 2C).
215

216 **Single nucleotide changes.** For a fourth application, we introduced *yceF** (an allele of
217 *yceF* with an Ile206Thr substitution) on a 2.17 kb repair template cloned into pAJS23 to
218 generate pAJS27. The *yceF* gene encodes a membrane protein from the TerC family
219 and is part of the *yceCDEFGH* operon (19). We were again able to recover the desired
220 recombinant strain with high yield (Table 1, Fig 2D).
221
222 **Deletion of an intergenic region.** As a fifth example, we deleted the intergenic region
223 within the *yqgC-sodA* operon, which codes for an uncharacterized RNA feature (S936)
224 and includes promoters for transcription of *sodA.* For this construction, the pAJS23
225 plasmid was modified to include a 2.2 kb repair template resulting in plasmid pAJS28.
226 With *yqgC*::*erm* as the recipient strain, we recovered the desired genetic construct with
227 high efficiency (Table 1), as confirmed by checking the size of PCR fragments spanning
228 the desired deletion and by DNA sequencing (Fig 2E).
229
230 **Generation of clean deletions.** As a final application, we introduced a Δ*yqgC* deletion
231 derived from the corresponding BKE strain to generate two cloned repair templates
232 differing in the extent of homology to the chromosome. Plasmid pAJS29 had a
233 combined 596 bp of homology for recombination (746 total length including 150 bp scar
234 region), whereas pAJS30 had 1594 bp (1744 bp total length). Both constructs were
235 successful, although pAJS30 gave a higher efficiency (Table 1). In general, for each of
236 these applications we had no difficulty in identifying MLS^S clones after plasmid curing,
237 although the frequency of recovery was reduced when the length of the regions of

238 homology for recombination upstream or downstream of the *erm* cassette fell below 300
239 bp (e.g. pAJS29).
240
241 **Provision of a repair template by co-transformation.** Since *B. subtilis* is naturally
242 competent, it is possible to introduce DNA repair templates as a PCR product, as shown
243 previously (10). Therefore, we decided to explore genetic constructions made by co-
244 transforming pAJS23 with a repair template provided as either a PCR product or
245 genomic DNA. Since competent cells can take up multiple DNA molecules, those that
246 acquire both the plasmid and the repair template should be enriched among those
247 colonies that pass through the standard selection procedure.
248 To test this procedure, we used the BKE *yceF::erm* strain as recipient and
249 introduced *yceF** (Ile206Thr substitution) on a repair template provided either in cis (by
250 cloning into pAJS23), as a PCR product, or as genomic DNA (Fig 2D). When the repair
251 template was provided as a PCR product spanning the *yceF** gene, the efficiency of the
252 procedure decreased to ~18% (5/28 MLS^S clones were confirmed by sequencing). We
253 were also able to obtain the desired strain with a repair template provided using
254 genomic DNA. In two separate trials using genomic DNA we observed similar
255 efficiencies (11% and 13%, Table 1). With this strategy, genome editing can be done
256 quickly since there are no required cloning steps, and in many applications the repair
257 template can be provided as genomic DNA. Even though the efficiency in these
258 examples was not as high as with a cloned repair template, it was still straightforward to
259 identify clones with the desired genetic change.
260

261 **Discussion**

262 CRISPR-Cas9 and related genome-editing systems have revolutionized biology
263 by enabling the precise editing of complex genomes (20, 21). The applications of
264 CRISPR-based tools to bacterial genetics are legion and include the introduction of
265 changes ranging from single-nucleotide mutations (11), to large-scale chromosomal
266 deletions (22), the precisely tuned modulation of gene expression using CRISPR-
267 interference based approaches (14, 23), and the targeted integration of large regions of
268 up to 10 kb of DNA using Tn7-like integrases (24-26). Because of its importance in
269 molecular biology and numerous anticipated applications in medicine, CRISPR-based
270 laboratories are now included in many undergraduate curricula (27).

271 Here we describe an efficient method for engineering the *B. subtilis* 168 genome
272 that bypasses many of the steps that can be rate-limiting during genome engineering. It
273 is relatively easy to move selectable markers from one strain of *B. subtilis* to another by
274 taking advantage of natural competence. As a result, null mutations can be introduced
275 into any desired competent strain using genomic DNA from strains from the BKE
276 collection (9). This collection of nearly 4000 *erm* gene disruptants is commercially
277 available and individual strains can be ordered from the *Bacillus* genetic stock center
278 (BGSC). With a desired recipient strain in hand, genome editing can be done by either
279 cloning the desired repair template into pAJS23 or by providing the repair template as a
280 PCR product or genomic DNA from a donor strain. The efficiency of recovery of the
281 desired mutation is generally highest when the repair template is provided on the
282 plasmid. However, the reduced efficiency when using a PCR product or genomic DNA

283 as a repair template may be preferable when compared to the additional steps required
284 to clone and verify a plasmid construct for each editing application.
285 Genomic DNA is taken up very efficiently by competent *B. subtilis*
286 cells. Mutations that confer a selectable phenotype are routinely introduced by
287 transformation. However, moving mutations and genetic constructions that do not confer
288 a selectable phenotype can be more laborious, often involving screening of colonies for
289 co-transformation of the desired change together with a selectable marker (genetic
290 congression; (28, 29)). Here, we demonstrate that CRISPR-Cas9 mediated genome
291 cleavage can also provide a strong selection for the transfer of mutations or genetic
292 constructions into a recipient strain, even when it does not confer an immediately
293 selectable phenotype.
294 One application of this procedure is for studies involving the genetic basis of
295 phenotypes arising during experimental evolution, or in laboratory selections and
296 screens for particular phenotypes. The advent of cost-effective whole-genome
297 resequencing (WGS) has made it practical to follow evolutionary changes in great detail
298 as bacteria adapt to different environments. We often use WGS to identify mutations of
299 interest by comparison of an evolved strain to the parent strain. Depending on the
300 nature of the experiment, there may be only one or a few changes detected, or the
301 number may be much larger. It can be challenging to know which of the observed
302 genetic changes is causal for the observed phenotype, and it is always possible that an
303 undetected change is responsible. To be rigorous, it is desirable to reconstruct the
304 evolved strain by introduction of the presumptive causal mutation(s) into the parent
305 strain. This type of genetic backcrossing is important, but often non-trivial. Backcrossing

306 of mutations, together with genetic complementation studies, generates assurance that
307 the observed phenotype is correctly linked to a specific genetic change. In previous
308 studies, we have used CRISPR-Cas genome editing with custom gRNAs and repair
309 templates to reconstruct mutations (29-32). For each application, this entailed the
310 design and cloning of an appropriate gRNA, followed by the design and cloning of a
311 repair template. This experience has motivated our current efforts to develop a
312 streamlined procedure that will, in many cases, allow a bypass of all molecular cloning
313 steps and allow strain construction using genetic transformation.

314 *B. subtilis* is a convenient and safe organism for teaching laboratories. This
315 system is appropriate for use as a simple laboratory exercise where students can
316 modify a genome using CRISPR-Cas9. In addition, many undergraduate laboratories
317 may employ *B. subtilis* for use in authentic research experiences, such as genetic
318 selection or screening of mutants with interesting properties. The underlying genotypic
319 changes can then be identified using WGS-based approaches for a modest cost.
320 CRISPR-Cas9 genome editing provides an effective way to move mutations between
321 strains and thereby test the role of individual mutations in the phenotype of evolved
322 strains.

323 While the strategy presented here is specific for *B. subtilis* and for the BKE
324 collection of strains, it is straightforward to extend the idea of using a common gRNA to
325 other collections of bacterial strains with indexed gene disruptions (including the related
326 *B. subtilis* BKK collection). Such collections are available in many model organisms and
327 it is possible in other systems to introduce repair templates as PCR products using
328 electroporation or chemical transformation, as shown for example in *E. coli* (33).

329

330 **Materials and Methods**

331 **Strains, Media, and Growth conditions.**

332 All the strains used in this study are listed in Table S1 and all the oligos used in gene
333 amplification, genetic manipulations, and construct verifications are listed in Table S2.
334 All of the *Bacillus* erythromycin knockout (BKE) strains in 168 background were
335 obtained from BGSC or from the National BioResource Project (NBRP, Japan).
336 Experiments for *E. coli* and *B. subtilis* growth were performed in LB medium (lysogeny
337 broth; 5 ml in 25 ml tube) or agar (20 ml in petri plate) with appropriate antibiotics. We
338 used kanamycin in final concentrations of 30 μg ml^{-1} for *E. coli* and 15 μg ml^{-1} for *B.*
339 *subtilis.* The *erm* gene encodes macrolide lincosamide streptogramin (MLS) resistance
340 and is selected with erythromycin (1 μg ml^{-1}) and lincomycin (25 μg ml^{-1}). 96 well plates
341 with 0.1 ml of LB were inoculated with mid-log (OD 600 nm = 0.4) cultures at 50-fold
342 dilutions in final volume for growth measurements. These aerobic cultures were
343 monitored periodically every 60 min for 37 °C growth at 600 nm in Synergy H1 plate
344 reader (BioTek Instruments Inc, VT).

345 **Erythromycin specific-guide RNA (*erm*-gRNA) cloning.** To target the MLS (*erm*)
346 cassette we selected the 20 nt sequence 5'TTTGAAATCGGCTCAGGAAA3' followed by
347 AGG of PAM sequence (supplemental file S2), which spans the 35th-41st codon of *erm*.
348 We added BsaI compatible ends to this sequence and its reverse complement
349 sequence (Table S1) and oligos were mixed in 1:1 ratio (10 μM each) in TE buffer (pH
350 7.0). This mixture was heated at 95 °C for 10 min. and cooled to room temperature to
351 generate double stranded *erm*-gRNA with BsaI compatible 5' and 3' ends (Fig 1), prior

352 to cloning into pJOE8999 (11) digested with BsaI. The ligation reaction was transformed
353 into *E. coli* DH5α and plated on LB agar with X-gal (40 μg ml^{-1}) plus kanamycin. White
354 clones (pJOE8999 containing *erm*-gRNA) were selected and subjected to PCR
355 verification and sequencing at Cornell Biotechnology Resource Center facility to verify
356 the *erm*-gRNA insertion. Culture of *E. coli* harboring *erm*-gRNA in pJOE8999 plasmid
357 (pAJS23) was stored as HEAS25630 (Table S1).
358 **Genome editing in *Bacillus* with a cloned repair template.** Repair templates were
359 generated using overlap extension (SOEing) PCR or PCR amplification of a suitable
360 region from genomic DNA. The outermost primers were designed to include SfiI
361 restriction sites (see SI Fig 2). The PCR product was digested with SfiI and then ligated
362 into pAJS23 plasmid that had also been digested with SfiI and column purified to
363 remove the small (12 bp) excised fragment. The repair template and vector were
364 incubated with T4 DNA ligase enzyme at room temperature for 1 hr (or 18 hr at 16 °C),
365 and then transformed into *E. coli* DH5α with selection for Amp^R. The desired clone was
366 confirmed with analytical PCR, and then transformed into *E. coli* TG1 to generate
367 multimeric plasmid DNA. The resulting pAJS23 derivative can then be used for genome-
368 editing of the appropriate strain containing an integrated *erm* cassette.
369 For each application, we transformed the appropriate recipient strain from the
370 BKE collection with the corresponding pAJS23 derivative. Recipient cells (5 ml) were
371 grown in competence medium to OD 600 nm ~0.8, 1 ml was removed as a negative
372 control, and 1 μg plasmid was added to the remaining and culture and incubated for 2
373 hrs at 30 °C with shaking (250 rpm). Cells (from 1 ml culture) were plated on LB agar
374 containing 15 μg ml^{-1} kanamycin (to select for plasmid) and 0.2% mannose (to induce

375 gRNA expression) and incubated for 24-48 hrs at 30 °C. The resulting colonies were
376 passaged three times on LB plates (without any antibiotics) at 45 °C for 18-24 hrs each
377 time. The resulting clones were then tested for loss of the plasmid by patching on LB
378 plates with MLS, kanamycin, or no antibiotics. Alternatively, colonies may be first
379 patched onto LB plates at 50 °C, and then colony purified at 42 °C as previously
380 described (11). Clones that failed to grow when patched onto both MLS and kanamycin
381 test plates were selected. The presence of the desired genome change was confirmed
382 using PCR amplification and Sanger sequencing.
383 **pAJS24 (*rsgA*).** We amplified three overlapping fragments by PCR including *Bacillus*
384 *prkC*-fragment (767bp), the *rsgA* gene (*cpgA* ortholog) from *S. aureus* Newman
385 (876bp), and *rpe* (799bp). These PCR fragments were fused to generate a single PCR
386 fragment consisting with SfiI restriction sites for cloning into pAJS23.
387 **pAJS25 (*sodA*-FLAG).** For this FLAG epitope fusion, we chose LFH PCR based in-
388 frame *sodA* ORF fusion to nucleotides GATTATAAAGATGATGATGATAAA coding for
389 an extended DYKDDDDK fusion tag on Mn-SOD protein.
390 **pAJS26 (YqgC-GFP).** For *yqgC* gene fusion to *gfp*, we generated chimeric PCR by
391 individually amplifying three overlapping fragments. The fragment one consisting of
392 *yqgB*-*yqgC* (1205bp) and second fragment consisting of mostly 712 bp long *yqgC*
393 adaptors-*gfp* (taken form pGFP-star plasmid (34)-*sodA* adaptors and *sodA*-*yqgE*
394 fragment (1467bp).
395 **pAJS27 (*yceF**)**. To introduce a point mutation in *yceF*, we employed LFH primers
396 encoding an Ile206Thr in *yceF* (*yceF**). In this approach we generated and joined two
397 fragments into a single fragment.

398 **pAJS28 (ΔS936).** To delete the S936 RNA feature in the non-coding region between
399 *yqgC-sodA* element, we engineered a 177 bp deletion spanning reference positions
400 from 2586043 to 2586220 in the *Bacillus* genome NC_000964.3. We ordered a gBlock
401 with SfiI recognition sequences at both ends from IDT (Integrated DNA Technologies,
402 Inc. IA, USA), which was cloned into pAJS23 to generate pAJS25.
403 **pAJS29 and pAJS30 (Δ*yqgC*).** To convert *yqgC*::*erm* to Δ*yqgC* we selected primers
404 such that the length of the repair template was either 546 bp or 1594 bp (Table 1).
405 These repair template were PCR amplified from Δ*yqgC* genomic DNA (generated from
406 *yqgC::erm* using pDR244 as described; (9)). The resultant strain has an unmarked
407 *yqgC* deletion (Δ*yqgC*) with a 150 bp scar in place of the *erm* resistance cassette.
408 **Genome editing by co-transformation with a repair template.** 6 ml of recipient
409 *Bacillus subtilis* strain was transformed as above with 1 μg of pAJS23 plasmid DNA
410 mixed with either 1 to 2 μg of PCR DNA or with 1 μg of sheared/sonicated (20 sec. 40%
411 amplitude) genomic DNA. 1 ml of culture was removed as a negative control. The
412 competent cells were incubated with DNA at 30 °C overnight and then split into 5 x 1ml
413 aliquots. Cells were centrifuged for 3 min at 5000 rpm, resuspended in sterile PBS/LB
414 (0.1 ml), and plated onto LB containing kanamycin and 0.2 % mannose for 36-48 hrs at
415 30 °C. Plasmids were cured at 42 °C and the recovered clones confirmed by Sanger
416 sequencing as described above.
417 **Spot dilution assay**. Assay was performed as described in (17). Briefly, cultures were
418 streaked onto LB agar plate and incubated at 37 °C for growth. These cultures were
419 then grown in liquid LB broth (5 ml) till OD at 600 nm was 0.4. Then serial dilution was
420 performed for cells in 96 well microtiter plates. 10 μl of serially diluted cells were spotted

421 onto LB agar and allowed to aseptically air dry. These plates were then incubated at
422 either 37 or 30 °C. Plates were imaged after 18 hours.
423 **Western blot.** The *sodA*-FLAG tagged HBYL1249 cells were grown in LB at 37 °C and
424 0.5ml of cell culture was harvested at 0.4, 0.6, 0.8 and 1.0 OD 600nm by centrifugation
425 at 13,000 rpm for 5 min. Cell pellets were suspended with 50 μl of PBS and 1.0 μg of
426 lysozyme and incubated at 37 °C for 20 min. This culture lysate was centrifuged at
427 13,000 rpm for 1 min and soluble fraction was collected, protein concentration was
428 determined by Bradford reagent (BioRad) and separated by SDS-PAGE. Proteins were
429 transferred to PVDF membrane using a Trans-Blot Turbo transfer system (BioRad). The
430 membrane was blocked (for 1 hr) and incubated for 1 hr with primary rabbit anti-FLAG
431 antibody (Sigma-Aldrich). Following buffer wash the membrane was incubated for 1 hr
432 with HRP-conjugated IgG mouse anti-rabbit antibodies (ThermoFisher Sci.). The blot
433 was stained with 1:1 enhancer:substrate reagents (BioRad) for 5 min, visualized with
434 ChemiDoc MP imaging system (BioRad) and ImageLab software.
435 **Epifluorescence microscopy.** Candidate overnight grown cells were collected from
436 the plate and mixed in LB to get 0.1 OD. These cells were spotted onto agarose pad
437 and gently covered with a cover-slip and observed using Olympus BX61
438 epifluorescence (for GFP) microscope and images were captured.
439

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443 *Bacillus* genome editing.

Competing Interest.

Authors declare there is no competing interest.

Author contribution

A.J.S performed all the experiments, J.D.H and A.J.S conceptualized and designed experiments, A.J.S and A.A drafted the manuscript. J.D.H supervised, edited, and finalized the manuscript.

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537

538 **Table 1. Summary of efficiency results for *cis* and *trans* repair templates**
539

Base pairs of homology in repair template (left +right)	Total length of repair template	Plasmid	Kan^R colonies	MLS^S clones	% MLS^S	% efficiency for MLS^S	Purpose
Cloned repair template							
1534 (750+784)	2410*	pAJS24	48	43	89%	100% (10/10)	*cpgA*::*erm*-->*rsgA* (Sau) heterologous complementation
2599 (1028+1571)	3893	pAJS25	197	131	66%	100% (10/10)	*yqgC*::*erm*-->*yqgC-sodA*-FLAG epitope fusion
2188 (725 +1463)	4281**	pAJS26	53	21	40%	100% (15/15)	*yqgC*::*erm*-->*yqgC-gfp* translation fusion
1405 (633+772)	2170	pAJS27	92	82	89%	100% (20/20)	*yceF*::*erm*-->*yceF**(Ile206Thr)
1719 bp (1028+691)	2202	pAJS28	56	46	82%	100% (46/46)	*yqgC*::*erm*-->deletion of intergenic *S936* element
546 (259+287)	696	pAJS29	855	186	22%	100% (25/25)	*yqgC*::*erm*-->Δ*yqgC* (markerless)
1594 (725+869)	1744	pAJS30	110	97	85%	100% (12/12)	*yqgC*::*erm*-->Δ*yqgC* (markerless)
Co-transformation with repair template‡							
1405 PCR (633+772)	2170	N/A	63	28	44%	18% (5/28)†	*yceF*::*erm*-->*yceF**(Ile206Thr)
yceF(Ile206Thr)* gDNA	N/A	N/A	96	26	27%	11% (3/28) †	*yceF*::*erm*-->*yceF**(Ile206Thr)
yceF(Ile206Thr) gDNA*	N/A	N/A	50	38	76%	13% (5/38)†	*yceF::erm-->yceF*(Ile206Thr)*

540
541 N/A: not applicable
542 Column 1: Length of UP and DO homology is depicted in parenthesis.
543 **rsgA* (*cpgA* homolog) of 876 bp was amplified from *S. aureus* Newman strain.
544 ** 715bp of GFP coding sequence was amplified from pGFP-star plasmid DNA.
545 † represents the fact that of 28 clones only 5 clones (for PCR-based) and 5 out of 38 clones (for
546 gDNA-based) showed PCR products.
547 ‡ co-transformation had different size transformants after 2 days on Kan+0.2% mannose
548 plates, and we found large colonies that appear rapidly usually did not show successful CRISPR
549 event.
550

551 **Figure Legends**

552

553 **Figure 1. CRISPR-Cas9 Editing with an *erm* gRNA.**

554 Plasmid pJOE8999 (7.8 kb; (11)) expresses Cas9 nuclease under a mannose-inducible
555 promoter and is temperature sensitive for replication in *B. subtilis.* Plasmid pAJS23
556 contains a cloned gRNA directed against the MLS resistance cassette (*erm* gene). This
557 *erm*-guide RNA (pink) can be used to target and cleave specific genome regions
558 containing integrated *erm* cassettes as present in the BKE collection of gene disruptants
559 (9). Repair templates can be provided in *trans* (left) by co-transformation of recipient
560 cells with pAJS23 and a repair template (either a PCR product of genomic DNA) or in
561 *cis* (right) by cloning of the repair template into pAJS23. Note that repair templates
562 should include DNA from the regions upstream (UP) and downstream (DO) of the
563 deleted gene in the targeted BKE strain. Engineered strains are cured of the
564 temperature-sensitive plasmid, and MLS^S (erm^-) colonies recovered for verification by
565 DNA sequencing.

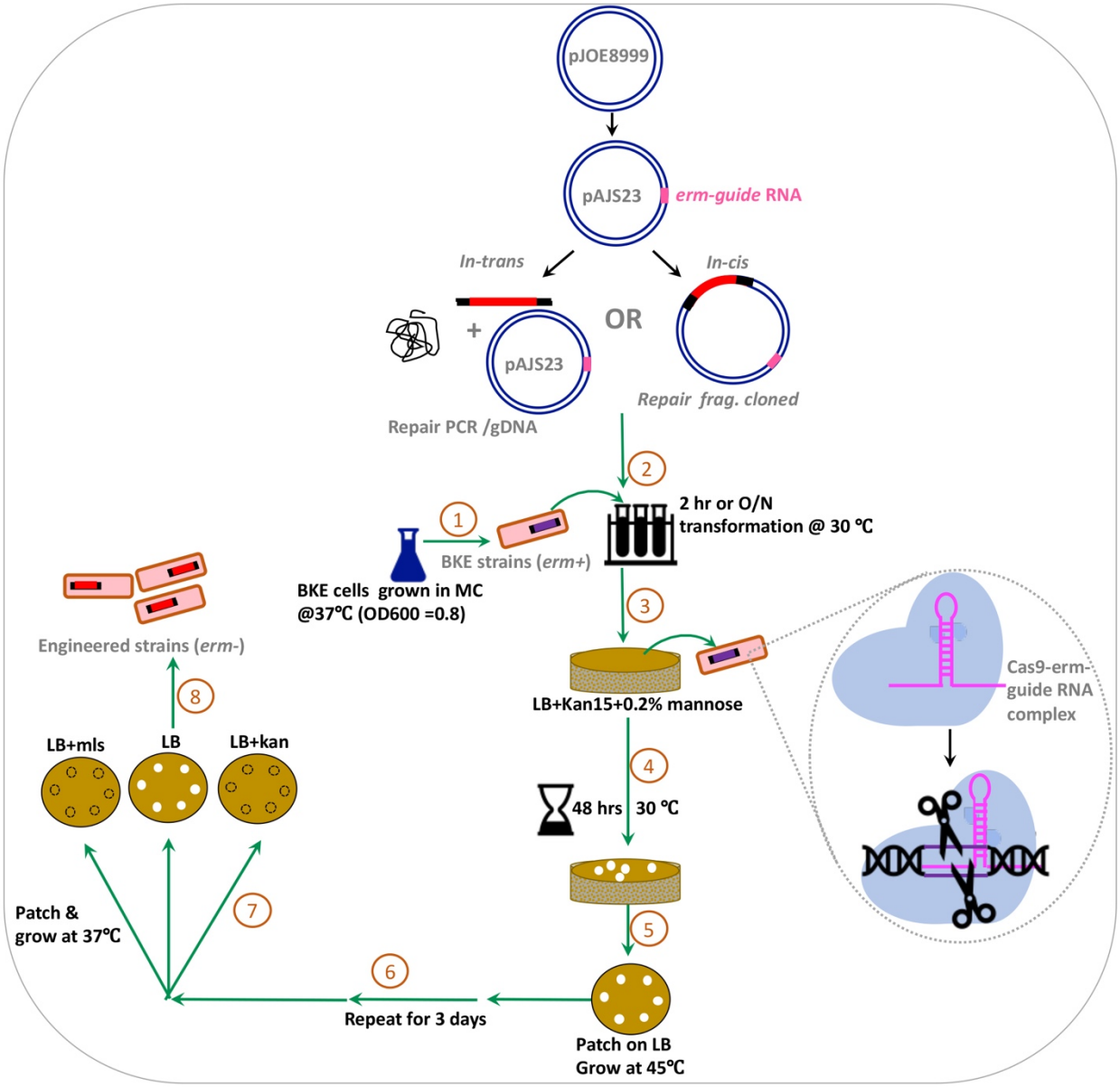
566

567 **Figure 2. Representative applications of CRISPR-Cas9 editing of BKE strains.**

568 Recipient strains from the BKE collection (with specific genes replaced by an integrated
569 *erm* cassette) were transformed with a pAJS23 derivative carrying a cloned repair
570 template (see Table 1). **A.** The *cpgA::erm* recipeient was edited to replace the missing
571 *cpgA* gene with the ortholog from *S. aureus* (*Sau-cpgA* = *rsgA*) shown in pink. The *rsgA*
572 gene complements the cold-sensitivity of the *cpgA* mutant strain as shown by serial
573 dilution on LB plates incubated at 30 ˚C (inset). **B.** The *yqgC::erm* strain was used to
574 introduce a sequence encoding a FLAG tag (DYKDDDDK) at the C-terminus of SodA
575 and simultaneously re-introduce a functional *yqgC* gene. Western blot analysis of cells
576 harvested at the indicated cell density confirms the presence of the 23.5 kDa SodA-
577 FLAG protein (inset). **C.** The *yqgC::erm* strain was edited to express a C-terminal YqgC-
578 GFP (green fluorescent protein, org. *Aequorea victoria*) translational fusion. The
579 resultant strains were confirmed by DNA sequencing and the expression of GFP
580 confirmed by fluorescence microscopy of cells grown in LB to stationary phase (scale
581 bar = 10 microns). **D.** The *yceF::erm* strain was edited to express a mutant allele

582 (*yceF**) encoding the YceF Ile206Thr protein. This strain was generated using a cloned
583 repair template (pAJS27; Table 1) or by providing repair template DNA as a PCR
584 product or genomic DNA (Table 1). **E.** We used a *yqgC*::*erm* recipient strain (as shown
585 in panels B and C) to edit the operon by removing the intervening intergenic region,
586 including the RNA feature S936. The desired deletion was confirmed by visualization of
587 correctly sized (582 bp) PCR products (middle inset), which showed a reduction in size
588 relative to the 760 bp control product. **F.** A clean deletion of *yqgC*. The *yqgC::erm* locus
589 was replaced by a markerless deletion generated using plasmid pDR244 as described
590 (9).
591

592



593
594
595 **Figure 1**

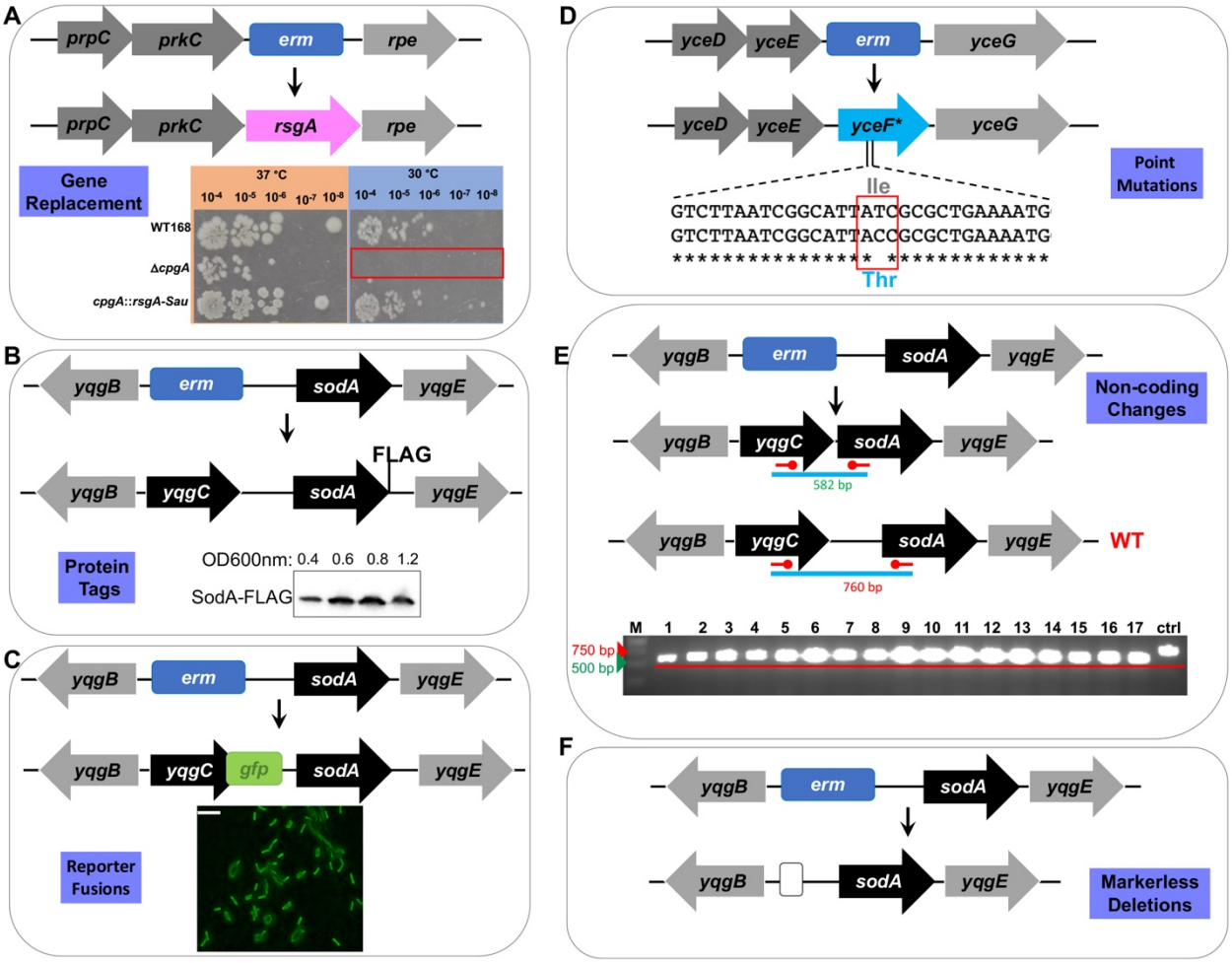

A
prpC
prkC
erm
rpe
prpC
prkC
rsgA
rpe
Gene Replacement
37 °C
30 °C
WT168
ΔcpgA
cpgA::rsgA-Sau
B
yqgB
erm
sodA
yqgE
FLAG
yqgB
yqgC
sodA
yqgE
Protein Tags
OD600nm: 0.4 0.6 0.8 1.2
SodA-FLAG
C
yqgB
erm
sodA
yqgE
yqgB
yqgC
gfp
sodA
yqgE
Reporter Fusions
D
yceD
yceE
erm
yceG
yceD
yceE
yceF*
yceG
Ile
GTCTTAATCGGCATTATCGCGCTGAAAATG
GTCTTAATCGGCATTACCGCGCTGAAAATG
Thr
Point Mutations
E
yqgB
erm
sodA
yqgE
Non-coding Changes
yqgB
yqgC
sodA
yqgE
582 bp
yqgB
yqgC
sodA
yqgE
WT
760 bp
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 ctrl
750 bp
500 bp
F
yqgB
erm
sodA
yqgE
yqgB
sodA
yqgE
Markerless Deletions

Figure 2