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Coupling the CRISPR/Cas9 system to lambda Red recombineering enables simplified chromosomal gene replacement in *Escherichia coli*

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Running head: CRISPR/Cas9-coupled gene replacement in *E. coli*

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Abstract

To date most genetic engineering approaches coupling the Type II *Streptococcus pyogenes* CRISPR/Cas9 system to lambda Red recombineering have involved minor single nucleotide mutations. Here we show that procedures for carrying out more complex chromosomal gene replacements in *Escherichia coli* can be substantially enhanced through implementation of CRISPR/Cas9 genome editing. We developed a three-plasmid approach that not only allows highly efficient recombination of short single-stranded oligonucleotides, but also replacement of multigene chromosomal stretches of DNA with large PCR products. By systematically challenging the proposed system with respect to the magnitude of chromosomal deletion and size of DNA insertion, we demonstrated DNA deletions of up to 19.4 kb, encompassing 19 non-essential chromosomal genes, and insertion of up to 3 kb of heterologous DNA with recombination efficiencies permitting mutant detection by colony PCR screening. Since CRISPR/Cas9-coupled recombineering does not rely on the use of chromosome-encoded antibiotic resistance, nor flippase recombination for antibiotic marker recycling, our approach is simpler, less labor intensive, and allows efficient production of gene replacement mutants that are both markerless and "scar"-less.

Keywords: Cas9, CRISPR, *Escherichia coli*, Gene replacement, Genome editing, Recombineering

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Introduction

Since its inception in 1998, phage-recombinase-mediated homologous recombination, also known as recombineering, has revolutionized bacterial genetics, synthetic biology, and metabolic engineering (1, 2). Relying on either RecET from the Rac prophage (2) or the bacteriophage lambda Red proteins, Exo, Beta, and Gam (1), recombineering allows simple and efficient construction of gene knockout mutants via homologous recombination of a double-stranded DNA (dsDNA) polymerase chain reaction (PCR) product with bacterial chromosomes (3). This work has led to the construction of an *Escherichia coli* K-12 single gene knockout library [the Keio collection (4)], enabled widespread use of chromosome-encoded expression platforms for metabolic and strain engineering (5-11), and simplified conventional recombinant DNA technology through the advent of ligation-independent DNA cloning (2, 12). Automated, high-throughput, and multiplexed genome editing applications have also been envisioned, including Multiplexed Automated Genome Engineering (MAGE), generating up to 4.3 billion genomic mutants per day (13). Finally, phage-mediated recombineering systems have been identified and exploited for use in an array of bacterial genera (14-19).

Recombineering can be carried out using either single-stranded DNA (ssDNA) or dsDNA substrates. Since recombineering occurs through an entirely ssDNA intermediate (20), ssDNA recombineering requires only a ssDNA recombinase (RecT or Beta) for recombination (21), whereas recombination of dsDNA substrates requires both an exonuclease (RecE or Exo) and its associated recombinase (RecT or Beta). For this reason, recombination of ssDNA substrates, such as oligonucleotides (oligos), is simpler, more efficient, and better understood than dsDNA recombineering (21, 22). Until recently, a fundamental limitation of oligo recombineering lies in

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45 the inability to identify and select cells carrying the target mutation. Recombination of dsDNA
46 PCR products allows the introduction of antibiotic resistance markers directly into the host
47 chromosome (3), while ssDNA recombineering utilizes short synthetic oligos (≤ 90 nt),
48 excluding the prospect of recombination-coupled antibiotic selection. Although successful
49 ssDNA recombinants can be identified in the absence of selection using laborious pooled colony
50 PCR screening (21), recombination efficiency is often too low [e.g., ≤ 1% (23)], rendering oligo
51 recombineering largely impractical. To overcome this limitation, Jiang et al. (24) recently
52 coupled the powerful clustered regularly interspaced short palindromic repeats (CRISPR)
53 selection system from *Streptococcus pyogenes* to lambda Red oligo recombineering, leading to
54 efficient selection of ssDNA recombinants. This CRISPR system, consisting of the CRISPR-
55 associated (Cas) protein, Cas9, a *trans*-activating CRISPR RNA (tracrRNA), and a
56 programmable CRISPR targeting RNA (crRNA), generates a Cas9-mediated double-stranded
57 break (DSB) at almost any target DNA locus (25). Since the DSB is lethal, cells possessing
58 specific modifications generated via homologous recombination of a plasmid, PCR product, or
59 synthetic oligonucleotide can easily be selected (24). Using the CRISPR/Cas9 system for
60 selection of *E. coli* genome editing, up to 65% of cells were found to possess the desired
61 chromosomal mutation specified by the synthetic oligo (24), rendering CRISPR/Cas9
62 recombineering the most powerful methodology for engineering bacterial genomes to date (26).
63 In addition to *E. coli* and *Streptococcus* (24, 27), this technology has recently been adapted for
64 use in lactic acid bacteria (28), *Streptomyces* (29), and *Clostridium* (30).

65 Presently, most reports of *E. coli* CRISPR/Cas9 selection have involved precise mutation
66 of only a few nucleotides using ssDNA oligo recombineering (24). On the other hand,
67 replacement of native chromosomal genes with foreign metabolic pathways or new cellular

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capabilities represents a central facet of metabolic engineering and synthetic biology (31). Owing to the immense selective utility harnessed by the CRISPR/Cas9 system, we hypothesized that it could simplify current protocols for carrying out gene replacement in *E. coli*, the workhorse of molecular biology and a biotechnological host of interest for the production of biological fuels and chemicals (32-34). In the present study, we outline a simplified strategy for chromosomal gene replacement by coupling the powerful CRISPR/Cas9 system with conventional lambda Red recombineering. We perform preliminary gene replacements to demonstrate the utility of this approach, extensively probe its capacity for generating large chromosomal deletions and insertions, and discuss its advantages over traditional recombineering approaches in the absence of CRISPR/Cas9 selection (3, 10). This method overcomes the dependence on Flippase (Flp) recombination, avoids the creation of chromosomal "scar" sites [i.e., Flp recognition target (FRT) scar sites], is adaptable to multiplexing (27), and can be performed in a shorter period of time compared to previous protocols. Accordingly, CRISPR/Cas9 recombineering simplifies the construction of markerless gene replacement mutants of *E. coli* and serves as an effective tool for strain optimization, metabolic engineering and synthetic biology.

Materials and Methods

Bacterial strains, plasmids, oligonucleotides, and growth conditions

Bacterial strains, plasmids, and oligonucleotides utilized in this work are listed in Table 1. *E. coli* DH5α was employed for both vector construction and genome editing purposes. Lambda Red recombineering donor plasmid pKD46 (3) was obtained from The Coli Genetic Stock Center (CGSC) at Yale University (New Haven, CT). Addgene (Cambridge, MA) supplied

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pCas9 (ID 42876) and pCRISPR (ID 42875) donor plasmids (24). Plasmid pTH18cr (35) was kindly provided by Tamotsu Hashimoto-Gotoh. Oligonucleotides were synthesized by Integrated DNA Technologies (IDT; Coralville, IA) at the 25 nm scale using standard desalting.

E. coli strains were grown aerobically in shake-flask cultures at 37 ºC and 250 rpm in lysogeny broth (LB) unless stated otherwise. Recombinant strains were selected with ampicillin, chloramphenicol, or kanamycin at concentrations of 100, 34, and 30 μg ml^{-1}, respectively. Antibiotic concentrations were reduced by half for selection of strains harboring two plasmids. Recombinant strains were stored in 15% glycerol at -80 ºC.

DNA isolation, manipulation, and plasmid construction

Purification, gel extraction, and clean-up of plasmid DNA and PCR products were performed using commercial spin-column kits from Bio Basic Inc. (Markham, ON) according to the manufacturer's instructions. Restriction endonucleases, Quick Ligation Kit, and Phusion and *Taq* DNA polymerases were obtained from New England Biolabs (Beverly, MA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Bio Basic Inc. (Markham, ON). Plasmid pCRISPR::dbpA was constructed by annealing oligos dbpA.crRNA.S and dbpA.crRNA.AS and ligating the resulting product with a 2,398 bp BsaI restriction fragment of pCRISPR. Compatible BsaI cohesive DNA ends were generated upon oligo annealing. Similarly, pCRISPR::ctrl was constructed by annealing oligos ctrl.crRNA.S and ctrl.crRNA.AS and ligating the resulting product with BsaI-digested pCRISPR.

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Preparation of recombinogenic DNA

For ssDNA oligo recombineering experiments, 60 nt recombinogenic oligos (dbpA::STOP.lead or dbpA::STOP.lag) were utilized. Six consecutive point mutations corresponding to all three nucleotides of the *dbpA* Protospacer Adjacent Motif (PAM) and three nucleotides of the protospacer were placed roughly in the middle of the oligo sequences. Oligos were also designed to introduce two consecutive stop codons into the *dbpA* open reading frame (ORF), in addition to a unique AseI restriction recognition sequence to facilitate screening of transformants for successful recombination.

PCR primers used to generate recombinogenic dsDNA PCR products were designed to possess 20 nt priming regions (P1 or P2) for amplification of the *lacZ′* cassette of pUC19 or pTH18cr and 40 nt homology regions (H*n*) for homologous recombination with the *E. coli* DH5α chromosome. To generate chromosomal deletions of various sizes encompassing the *dbpA* DSB, a 560 bp *lacZ′* PCR product was amplified from pUC19 using primer sets dbpA::lacZ′.del8bp.H0P1 + dbpA::lacZ′.del8bp.H0P2 (8 bp deletion), dbpA::lacZ′.del818bp.H1P1 + dbpA::lacZ′.del818bp.H2P2 (818 bp deletion), dbpA::lacZ′.del2428bp.H3P1 + dbpA::lacZ′.del2428bp.H4P2 (2,428 bp deletion), dbpA::lacZ′.del5123bp.H5P1 + dbpA::lacZ′.del5123bp.H6P2 (5,123 bp deletion), dbpA::lacZ′.del9590bp.H7P1 + dbpA::lacZ′.del2428bp.H4P2 (9,590 bp deletion), dbpA::lacZ′.del11068bp.H8P1 + dbpA::lacZ′.del11068bp.H9P2 (11,068 bp deletion), and dbpA::lacZ′.del9590bp.H7P1 + dbpA::lacZ′.del11068bp.H9P2 (19,378 bp deletion). To generate PCR products of various sizes for deletion of an 818 bp region of the *dbpA* gene, pUC19 was used as template along with primer sets dbpA::lacZ′.del818bp.H1P1 + dbpA::lacZ′.del818bp.H2P2 (560 bp insertion), dbpA::lacZ′.del818bp.H1P1 +

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dbpA::lacZ′.ins1264.H2P2 (1,264 bp insertion), dbpA::lacZ′.del818bp.H1P1 + dbpA::lacZ′.ins1756.H2P2 (1,756 bp insertion), and dbpA::lacZ′.del818bp.H1P1 + dbpA::lacZ′.ins2492.H2P2 (2,492 bp insertion), or pTH18cr was used as template along with primer sets dbpA::lacZ′.ins550.H1P1 + dbpA::lacZ′.ins550.H2P2 (550 bp insertion) and dbpA::lacZ′.ins550.H1P1 + dbpA::lacZ′.ins3000.H2P2 (3,000 bp insertion). All recombinogenic PCR products were treated with 5 U of DpnI for 2 h prior to gel electrophoresis, extraction, and purification.

CRISPR/Cas9-coupled lambda Red recombineering

E. coli DH5α harboring pKD46 and pCas9 was used as the host strain for all CRISPR/Cas9 recombineering experiments. Cultures for electroporation were grown at 30 ºC in LB medium. Ten mM L-arabinose was added at an OD_{600} of 0.3-0.4 for induction of the lambda Red recombineering operon of pKD46 (3). Cells were harvested at an OD_{600} of 0.5-0.7, washed three times in ice-cold 10% glycerol, and concentrated approximately 250-fold. Cell-DNA suspensions were transferred to pre-chilled 0.1 cm electroporation cuvettes and electroporated at 1.8 kV, 25 µF, and 200 Ω. Cells were recovered in 1 ml SOC medium (36) for 2 hours at 37 ºC and 250 rpm. Outgrowth cultures were serially diluted and 0.002-0.5 µl equivalents were plated onto selective LB agar plates. Selection of pKD46 using ampicillin was not performed following electroporation.

For ssDNA oligo recombineering, approximately 500 ng of recombinogenic (dbpA::STOP.lead or dbpA::STOP.lag) or non-recombinogenic oligo and 100 ng of pCRISPR::dbpA or pCRISPR::ctrl were mixed with 40 µl of electrocompetent DH5α [pKD46 +

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pCas9]. Transformants capable of survival in the presence of the CRISPR/Cas9 machinery (pCRISPR::dbpA + pCas9) were selected using kanamycin and chloramphenicol. For dsDNA gene replacements, 700-1,000 ng of gel-purified PCR product (recombinogenic or non-recombinogenic) and 100 ng of pCRISPR::dbpA or pCRISPR::ctrl were mixed with 40 µl of electrocompetent DH5α [pKD46 + pCas9]. Transformants capable of survival in the presence of the CRISPR/Cas9 machinery (pCRISPR::dbpA + pCas9) were selected using kanamycin, chloramphenicol, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

In all CRISPR/Cas9 recombineering experiments, controls were included to assess plasmid electroporation [pCRISPR::ctrl + non-recombinogenic oligo (ctrl.60mer) or non-recombinogenic PCR product], CRISPR/Cas9-mediated cell death (pCRISPR::dbpA + ctrl.60mer or non-recombinogenic PCR product), and recombineering in the absence of CRISPR/Cas9 selection (pCRISPR::ctrl + recombinogenic oligo or recombinogenic PCR product). At least two replicates were utilized to assess plasmid electroporation and recombination efficiency in each CRISPR/Cas9 recombineering experiment.

Screening of ssDNA oligo recombination and dsDNA gene replacement

Successful recombination of recombinogenic *dbpA* oligonucleotides was screened by colony PCR using kanamycin- and chloramphenicol-resistant transformants with primers dbpA1.Fw and dbpA1.Rv, followed by overnight digestion of the resulting PCR products with 1 U of AseI. Recombination efficiency of ssDNA recombination was assessed by the proportion of correct colonies following PCR screening and AseI digestion. Double-stranded DNA gene replacement of the *dbpA* locus with *lacZ'* was first screened directly on chloramphenicol and

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kanamycin agar plates containing X-Gal. Recombination efficiency of dsDNA gene replacement was assessed by the proportion of blue colonies on selective plates containing 40-400 colonies. Expected gene insertion and orientation was further verified by colony PCR and Sanger sequencing across both chromosome-PCR-cassette junctions using a single blue colony of each mutant type.

Results

Selection of pKD46-based oligo recombination using the CRISPR/Cas9 system

Prior to attempting chromosomal gene replacement using CRISPR/Cas9 selection, we first performed a ssDNA oligo recombination experiment using conventional pKD46-based lambda Red recombineering, as recombination of short oligos is more efficient than recombination of dsDNA PCR products (21, 22). The CRISPR/Cas9 system has recently been coupled to oligo recombineering in *E. coli* by utilizing a host strain possessing a chromosomal copy of the lambda *bet* gene expressed from a temperature-sensitive promoter (24). For metabolic and strain engineering, however, it is preferable to express the *bet* gene *in trans*, rather than carrying out recombineering in a universal recombination host and subsequently transducing the mutant allele to the desired host background. We therefore investigated the use of a three-plasmid system to couple plasmid-encoded recombineering (pKD46) to the CRISPR/Cas9 system (pCRISPR + pCas9).

We selected the gene encoding DEAD-box protein A (*dbpA*) in *E. coli* DH5α for gene disruption (10), since the gene is non-essential and deletion does not negatively affect cell growth (4). We designed recombinogenic *dbpA* disruption oligonucleotides targeting the leading

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(dbpA::STOP.lead) and lagging (dbpA::STOP.lag) strands of DNA replication whereby successful oligo recombination leads to mutation of the *dbpA* PAM sequence and introduces two consecutive stop codons within the *dbpA* ORF, in addition to a unique AseI recognition sequence for mutant screening (Fig. 1a). Cas9 was targeted to *dbpA* through construction of pCRISPR::dbpA, which expresses a 30 nt *dbpA* crRNA. To assess electroporation and oligo recombination in the absence of CRISPR/Cas9 selection, we also constructed pCRISPR::ctrl, expressing a scrambled crRNA devoid of homology to the *E. coli* genome. Following arabinose induction of lambda Red functions in strain DH5α [pKD46 + pCas9], co-electroporation of pCRISPR::ctrl and a non-recombinogenic control oligo (ctrl.60mer) generated approximately 1.6 $\times 10^9$ colony forming units (CFU) μg^{-1} plasmid DNA, indicating efficient electroporation of pCRISPR::ctrl (Fig. 1b). Conversely, co-electroporation of pCRISPR::dbpA and ctrl.60mer led to a drastic reduction in electroporation efficiency (greater than three orders of magnitude), owing to CRISPR/Cas9 selection against unmodified host cells. A small proportion of pCRISPR::dbpA transformants (approximately 0.06% compared to pCRISPR::ctrl) were able to survive Cas9 endonuclease attack, presumably due to spontaneous deletion of the *dbpA* crRNA within pCRISPR::dbpA or mutation of the *cas9* coding sequence within pCas9 (24). Having established effective CRISPR/Cas9 selection against unmodified cells, we next utilized our three-plasmid system to select for oligo recombineering mutants through mutation of the CRISPR/Cas9 PAM sequence within the *dbpA* coding sequence. Co-electroporation of pCRISPR::dbpA with dbpA::STOP.lead or dbpA::STOP.lag oligo led to a 5- and 28-fold increase in electroporation efficiency, respectively, compared to the non-recombinogenic control oligo (ctrl.60mer) through mutation of the *dbpA* PAM sequence (Fig. 1b). In line with previous studies of lambda-Red-mediated oligo recombineering in *E. coli* (21, 37), the oligo targeting the

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223 lagging strand of DNA replication produced substantially more transformants (greater than five-
224 fold) than the leading-strand-targeting-oligo. Using colony PCR followed by AseI digestion, we
225 genotyped a total of 28 and 41 pCRISPR::dbpA transformant colonies generated using the
226 dbpA::STOP.lead and dbpA::STOP.lag oligos, respectively, yielding *dbpA* recombination
227 efficiencies of 50% for the leading strand oligo and 80% for the lagging strand oligo. Genotyping
228 results from 13 representative colonies generated using the dbpA::STOP.lag oligo is shown in
229 Figure 1c. To further demonstrate the efficacy of CRISPR/Cas9-coupled recombineering, we set
230 out to determine oligo recombineering efficiency in the absence of CRISPR/Cas9 selection.
231 Here, we co-electroporated pCRISPR::ctrl and dbpA::STOP.lag, and screened the resulting
232 transformants using colony PCR and AseI digestion. Of a total of 154 pCRISPR::ctrl
233 transformants screened, two mutant colonies were identified, corresponding to a recombination
234 efficiency of 1.3% (Fig. 1b). Collectively, the results presented in this section confirm that our
235 three-plasmid system supports efficient CRISPR/Cas9 recombineering using recombinogenic
236 oligos.

237

238 *pKD46-based chromosomal gene replacement of* dbpA *with a markerless dsDNA PCR*
239 *product using CRISPR/Cas9 selection*

240 We next aimed to utilize our three-plasmid CRISPR/Cas9 recombineering system to
241 carry out chromosomal gene replacement of *dbpA* with a markerless dsDNA template.
242 Recombination of dsDNA PCR products requires both lambda Exo and Beta proteins, which are
243 co-expressed from plasmid pKD46 (3). We selected the *lacZ'* subunit for gene knock-in, since
244 the resulting PCR cassette is small in length (< 1 kb) and recombinants can be identified directly

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following electroporation using agar plates supplemented with X-Gal for blue-white colony screening. We targeted the same *dbpA* gene region employed in our oligo recombineering demonstration by employing pCRISPR::dbpA. We also devised two different gene replacement approaches by varying the location of H*n* homology arms flanking the chromosomal DSB within the *dbpA* gene, generating an 8 bp (H0 + H0) or 818 bp deletion (H1 + H2) (Fig. 2a). To first assess plasmid electroporation efficiency and Cas9-mediated killing of unmodified host cells, we again utilized pCRISPR::ctrl and pCRISPR::dbpA, respectively, along with a 466 bp non-recombinogenic PCR product. In a similar manner to the CRISPR/Cas9 oligo recombineering demonstration (Fig. 1b), co-electroporation of pCRISPR::ctrl and a 466 bp non-recombinogenic PCR product yielded a substantially higher electroporation efficiency (more than three orders of magnitude) compared to co-electroporation of pCRISPR::dbpA with the same control PCR product (Fig. 2b), again demonstrating efficient selection against cells possessing the *dbpA* PAM sequence. Compared to electroporation of pCRISPR::ctrl, approximately 0.07% of pCRISPR::dbpA transformants were able to evade Cas9 cleavage. Conversely, co-electroporation of pCRISPR::dbpA with a 560 bp recombinogenic *lacZ'* PCR cassette containing 40 bp of homology generated a 1.8-fold increase in electroporation efficiency for both *dbpA* deletion sizes of 8 bp and 818 bp relative to the control non-recombinogenic PCR product (Fig. 2b). This result is in agreement with our ssDNA oligo recombineering data, where high-level recombination produced a dramatic enhancement in electroporation efficiency through efficient replacement of the *dbpA* PAM sequence and subsequent evasion of Cas9-mediated endonuclease attack. Following electroporation of the 8 bp or 818 bp deletion PCR cassette, recombination efficiency can be determined directly by enumerating the proportion of blue transformants. Recombination efficiencies of 39% and 47% were obtained for the respective 8 bp and 818 bp

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268 deletion cassettes, validating effective selection of chromosomal gene replacement. To compare
269 CRISPR/Cas9 recombineering to traditional lambda Red recombination of dsDNA PCR products
270 in the absence of CRISPR/Cas9 selection, we co-electroporated pCRISPR::ctrl with the 8 bp or
271 818 bp *dbpA* deletion PCR product and obtained recombination efficiencies of 0.68% and 0.25%,
272 respectively. Colony PCR and Sanger sequencing of one blue colony of each deletion mutant
273 generated using CRISPR/Cas9-coupled recombineering confirmed successful replacement of the
274 *dbpA* gene regions with *lacZ'* (Fig. 2c).

275

276 *Challenging pKD46-based CRISPR/Cas9 gene replacement by increasing the size of*
277 *chromosomal deletion*

278 Since dsDNA gene replacement using CRISPR/Cas9 recombineering proved to be highly
279 efficient (39% and 47% of colonies possessed the target mutation), we sought to establish the
280 performance of this technique with respect to the magnitude of deletion that can be efficiently
281 introduced to the *E. coli* chromosome. We first examined phenotypic data from the *E. coli* K-12
282 Keio single gene knockout mutant collection (4) to identify essential genes flanking *dbpA* that
283 lead to lethality upon gene knockout. We identified a 20.3 kb non-essential chromosomal region,
284 flanked by the essential genes *insH-4* and *racR* (*ydaR*), which, theoretically, could be deleted
285 without causing cell death. Accordingly, we varied the spacing between chromosomal homology
286 regions (H*n*) within PCR primers and held the size of our recombinogenic *lacZ'* cassette constant
287 at 560 bp to generate PCR products leading to deletions of various sizes of the *E. coli* genome.
288 Specifically, we targeted chromosomal deletions of 818 bp (H1 + H2), 2,428 bp (H3 + H4),
289 5,123 bp (H5 + H6), 9,590 bp (H7 + H4), 11,068 bp (H8 + H9), and 19,378 bp (H7 + H9) (Fig.

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290 3a). To ensure effective CRISPR/Cas9 selection in each gene replacement event, homology
291 regions were designed to flank the *dbpA* PAM sequence, which is essential for Cas9
292 endonuclease attack. The 9,590 bp and 11,068 bp chromosomal deletions both encompass the
293 full *dbpA* gene sequence, including the essential PAM sequence, and together span the entire
294 19,378 bp chromosomal deletion segment. An equal amount of each *lacZ'* PCR cassette was
295 electroporated with pCRISPR::dbpA to *E. coli* DH5α [pCas9 + pKD46]. Electroporation
296 efficiency was found to vary by a factor of 2.7 between the six recombinogenic deletion PCR
297 cassettes assayed (Fig. 3b). Notably, electroporation efficiencies corresponding to the 2,428 bp
298 and 19,378 bp deletion PCR products were 4% and 34% lower, respectively, than that of the
299 non-recombinogenic control PCR product. Based on data from our preliminary ssDNA and
300 dsDNA CRISPR/Cas9-coupled recombineering experiments (Fig. 1b and 2b), a lack of elevation
301 in electroporation efficiency of recombinogenic DNAs relative to the non-recombinogenic
302 control PCR product suggests unsuccessful recombination. Surprisingly, however, target
303 recombination events could be identified for all six chromosomal deletions. Further,
304 recombination efficiency was found to vary dramatically between the chromosomal deletions
305 (recombination efficiencies of 3%-47%; Fig. 3b). Generally, recombination efficiency decreased
306 with increasing size of chromosomal deletion, yet not in a linear manner across the sizes of
307 deletion assessed. A major drop in recombination efficiency (>80%) was observed by increasing
308 the size of deletion from 818 bp to 2,428 bp. Conversely, recombination efficiency was similar
309 across chromosomal deletions between 2,428 bp and 11,068 bp (recombination efficiencies of
310 8%-11%). Deletion of a 19,378 bp region, comprising a total of 19 chromosomal genes, yielded
311 the lowest recombination efficiency of 3%. Since replacement of 8 bp and 818 bp chromosomal
312 regions with *lacZ'* using recombineering in the absence of CRISPR/Cas9 selection could be

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313 detected with blue-white colony screening (Fig. 2b), we also assessed replacement of the 2,428
314 bp chromosomal region with *lacZ'* using traditional lambda Red recombineering. Whereas
315 recombination efficiency using CRISPR/Cas9 selection was 9%, we were unable to identify a
316 blue recombinant colony amongst the background of unmodified cells for the 2,428 bp deletion
317 in the absence of CRISPR/Cas9 selection (Fig. 3b). Colony PCR and Sanger sequencing using
318 one blue colony from each of the six chromosomal deletion mutants generated using
319 CRISPR/Cas9-coupled recombineering confirmed successful replacement of the target
320 chromosomal gene(s) with *lacZ'* (Fig. 3c).

321

322 *Challenging pKD46-based CRISPR/Cas9 gene replacement by increasing the size of*
323 *recombinogenic PCR products*

324 We next aimed to challenge our CRISPR/Cas9 recombineering approach by increasing
325 the total size of recombinogenic PCR products. We again utilized *lacZ'* as the source of
326 recombinogenic DNA to facilitate assessment of recombination efficiency via blue-white colony
327 screening. We held the size of *dbpA* homology arms (40 nt) and the distance between homology
328 regions (818 bp) constant and varied the size of recombinogenic *lacZ'* insertion DNA by PCR-
329 amplifying products of various sizes from plasmids pUC19 and pTH18cr. Specifically, we
330 assessed recombination of *lacZ'* products of sizes 550 bp, 560 bp, 1,264 bp, 1,756 bp, 2,492 bp,
331 and 3,000 bp (Fig. 4a). Plasmids pUC19 (2,686 bp) and pTH18cr (3,064 bp) are nearly
332 represented in their entirety within the 2,492 bp and 3,000 bp PCR products, respectively, where
333 portions of the sequences corresponding to plasmid origins were omitted to prevent interference
334 with chromosomal DNA replication following recombination. An equal amount of each *lacZ'*

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PCR cassette was electroporated along with pCRISPR::dbpA to *E. coli* DH5α [pCas9 + pKD46] and electroporation efficiency was compared to the electroporation (pCRISPR::ctrl + 466 bp non-recombinogenic PCR product; 2.1×10^9 CFU μg^{-1} plasmid DNA) and CRISPR/Cas9 selection (pCRISPR::dbpA + 466 bp non-recombinogenic PCR product; 1.4×10^6 CFU μg^{-1} plasmid DNA) controls (Fig. 4b). When co-electroporated with pCRISPR::dbpA, electroporation efficiency between the six various-sized recombinogenic PCR products varied by a factor of 3.6. In a similar manner to the chromosomal deletion PCR cassettes assayed (Fig. 3b), electroporation efficiencies corresponding to two of the insertion PCR cassettes (1,264 bp and 3,000 bp) were lower than that of the control electroporation. Despite this decrease in electroporation efficiency, recombinant colonies could be detected for all six recombinogenic PCR products, where recombination efficiency varied dramatically (1% to 47%) (Fig. 4b). A substantial decline in recombination efficiency (>90%) resulted from increasing the size of recombinogenic DNA from 560 bp to 1,264 bp. However, recombination efficiency remained relatively constant between PCR products of 1,264 bp to 3,000 bp (1%-4%). We also assessed recombination of the three most recombinogenic PCR products (550 bp, 560 bp, and 2,428 bp) using lambda Red recombineering without CRISPR/Cas9 selection. Whereas PCR products of sizes 550 bp and 560 bp yielded recombination efficiencies of 0.24% and 0.25%, respectively, mutant colonies corresponding to the 1,264 bp insertion could not be identified. Finally, colony PCR and Sanger sequencing of one blue colony from each of the six *lacZ′* gene insertion mutants generated using CRISPR/Cas9-coupled recombineering confirmed successful replacement of *dbpA* with the *lacZ′* PCR cassettes of various sizes (Fig. 4c).

Discussion

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For more than 15 years, recombineering has served as the most powerful means of creating precise genetic changes to bacterial genomes (2, 3, 21). More recently, the CRISPR/Cas9 system has heightened the utility of recombineering by providing a method of selecting chromosomal mutations down to the single nucleotide level (24). Still, the true potential of CRISPR/Cas9 systems for selection of bacterial genome editing has not been fully explored, as most CRISPR/Cas9 genome editing approaches to date have focused on the generation of minor chromosomal alterations using ssDNA oligonucleotides. Traditional protocols for replacing large segments of bacterial genomes with heterologous genes or genetic operons could be vastly improved by implementing the utility of CRISPR/Cas9 selection. Here we are proposing an improvement on such earlier methods (3, 9, 10) by simplifying the protocol for chromosomal gene replacement and abolishing the reliance on chromosomal antibiotic markers and the use of Flp-FRT recombination for antibiotic marker recycling. We have shown that *E. coli* gene replacement can be greatly simplified through application of the CRISPR/Cas9 system.

To couple high-level recombineering with the powerful *S. pyogenes* CRISPR/Cas9 system, we devised a triple plasmid strategy expressing the lambda Red functions, Exo, Beta, and Gam (pKD46) (3), the Cas9 protein and associated tracrRNA (pCas9), and programmable crRNA (pCRISPR) for generating DSBs at any location within the *E. coli* genome (24). Our devised gene replacement approach has three steps. First, Cas9 is directed to the target gene or chromosomal locus by reprogramming crRNA within pCRISPR (24). Next, a recombinogenic dsDNA consisting of a gene or gene operon, including associated regulatory elements, is amplified by PCR using primers possessing homology regions flanking the target chromosomal DSB region. The source of DNA for gene replacement can be of plasmid, genomic, or synthetic

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origin and need not encode an antibiotic resistance marker, nor possess FRT recombination sites. Finally, the recombinogenic PCR product is co-electroporated with retargeted pCRISPR into any *E. coli* host background harboring pKD46 and pCas9. A mixture of target modified cells and unmodified wild-type cells that escaped Cas9 cleavage are obtained via selection of pCas9 + pCRISPR co-transformants and can be easily differentiated using PCR screening. In contrast to traditional recombineering protocols for chromosomal gene replacement (3, 9, 10), CRISPR/Cas9 recombineering is simpler, less labor intensive, and can be carried out in a substantially shorter time frame (Fig. 5). In addition, our approach is easily amendable to multiplexing through the targeting of multiple chromosomal loci (27), and circumvents the use of chromosomal antibiotic markers and Flp-mediated excision for antibiotic marker recycling. Hence, the resulting mutant genome is both markerless and "scar"-less, as no FRT "scar" sites remain following recombination.

Our devised three-plasmid approach was shown to allow selection of recombinant cells generated using either ssDNA oligonucleotides (Fig. 1) or heterologous dsDNA PCR products (Fig. 2). We further probed the performance of the dsDNA gene replacement protocol with respect to the magnitude of deletion and insertion that can be introduced to the host genome. We have shown that deletions up to 19.4 kb, encompassing 19 non-essential chromosomal genes (Fig. 3), and insertions up to 3 kb (Fig. 4) can be introduced with high recombination efficiency (1%-47%). For comparison, gene replacement using the most recombinogenic substrates assayed in this study produced recombination efficiencies of 0%-0.68% in the absence of CRISPR/Cas9 selection, as more challenging gene replacement events could not be detected without incorporation of the CRISPR/Cas9 system (Fig. 3b and 4b). When implemented, CRISPR/Cas9 selection elevated sensitivity of mutant detection by up to 187-fold (Fig. 1b, 2b, 3b, and 4b).

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404 Although we utilized blue-white colony screening for detection of CRISPR/Cas9-coupled
405 recombineering mutants, almost all gene replacements involving insertion of *lacZ′* yielded
406 recombination efficiencies enabling mutant detection via PCR screening [defined by
407 recombination efficiency ≥ 2.5% (requiring screening of ≤ 40 colonies)]. However, attempting to
408 replace an 818 bp region of *dbpA* with a 3.8 kb heterologous PCR product failed to yield the
409 target mutant upon PCR screening of 40 colonies (data not shown), specifying a limitation on the
410 size of DNA that can be inserted into the host chromosome or a sequence-dependent effect on
411 gene replacement. It is also important to note that limitations on chromosomal deletions and
412 insertions were assessed independently in this study, where chromosomal deletions were assayed
413 using a comparatively small PCR product (560 bp) and genome insertions were evaluated using
414 only a modest chromosomal deletion (818 bp). Recombination efficiency was found to drop
415 significantly when the magnitude of chromosomal deletion or insertion was increased beyond
416 these levels (Fig. 3 and 4). Interestingly, Maresca et al. (38) found similar reductions in gene
417 insertion using substrates ≥ 500 bp, yet recombination was largely unaffected by deletion of
418 bacterial artificial chromosome (BAC) regions ranging from 0.5 kb to 50 kb. This discrepancy
419 may arise from the use of different recipient DNAs (i.e., BACs versus the *E. coli* chromosome),
420 or through incorporation of CRISPR/Cas9 selection in this study. Nevertheless, our findings
421 imply that replacing a large chromosomal segment (> 818 bp) with a large recombinogenic PCR
422 product (> 560 bp) should yield poor recombination, resulting in an inability to identify and
423 isolate the target mutant. Therefore, gene replacements using our CRISPR/Cas9 recombineering
424 strategy should target either large multi-gene deletions or chromosomal insertion of large PCR
425 products. Owing to the high recombination efficiencies we obtained when deleting large
426 chromosomal segments (3%-8% for deletions of 9.6 kb to 19.4 kb), our approach could find

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utility in strain optimization and chromosome reduction, whereby genomes are "cleansed" through large-scale deletion of prophages, transposons, and other non-essential host elements (39). In efforts demanding both large chromosome deletion and large heterologous DNA insertion, we recommend utilizing traditional, yet more laborious, lambda Red protocols in the absence of CRISPR/Cas9 selection (3, 10), as rare recombination events can be selected directly via chromosomal antibiotic resistance. Alternatively, increasing the size of homology arms beyond the common 40 nt limit utilized in this study has been shown to enhance recombination (27), which could enable detection of low efficiency events. It has also been reported that homologous recombination varies drastically between genomic loci (40), presumably due to secondary structure and sequence-dependent effects. The chromosomal *dbpA* locus employed in this study potentially represents a recombination "hot spot", as recombination efficiency of the lagging-strand-targeting oligo was 80%, compared to 65% at the *rpsL* locus reported by Jiang et al. (24). It is also worth noting that only one of the plasmids employed in our three-plasmid system, pKD46, possesses a low copy, temperature-sensitive plasmid origin (3). Whereas previous recombineering strategies have relied exclusively on such easily curable plasmids (3, 10), pCas9 and pCRISPR possess the multi-copy p15A and pBR322 origins, respectively (24), thus requiring specialized procedures for plasmid curing. Fortunately, protocols for curing multi-copy plasmids from recombinant *E. coli* strains using a range of artificial plasmid-curing agents [e.g., acridine orange or sodium dodecyl sulfate (41)] are firmly established (42).

Overall, the approach outlined in this study demonstrates that coupling traditional lambda Red recombineering with the powerful CRISPR/Cas9 system leads to simplified construction of markerless chromosomal gene replacement mutants of *E. coli*. We postulate that the methodology outlined herein will pave the way to novel and more ambitious strain engineering

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450 applications through the advent of multiplexed gene replacement strategies and modifying the
451 CRISPR/Cas9 recombineering machinery for use in a selection of biotechnologically relevant
452 bacteria other than *E. coli*.

453

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458

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570

571

572 ## Figure captions

573 **FIG 1** Single-stranded oligo recombineering demonstration using the proposed three-plasmid
574 system. **(A)** Disruption of chromosomal *dbpA* in *E. coli*. A 60-nucleotide recombinogenic oligo

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575 targeting the lagging strand of DNA replication (dbpA::STOP.lag) was utilized to disrupt *dbpA*
576 by introducing six consecutive base pair changes and an AseI recognition sequence (lowercase),
577 generating two consecutive in frame stop codons (underlined). Sequence corresponding to the
578 *dbpA* protospacer and PAM are shown in white. Rep corresponds to 36 bp repeats necessary for
579 crRNA processing. (**B)** Electroporation and *dbpA* recombination efficiency data resulting from
580 electroporation (pCRISPR::ctrl + ctrl.60mer), CRISPR/Cas9 (pCRISPR::dbpA + ctrl.60mer),
581 and recombineering (pCRISPR::ctrl + dbpA::STOP.lag) controls, as well as CRISPR/Cas9-
582 coupled recombineering using leading or lagging strand oligos (pCRISPR::dbpA +
583 dbpA::STOP.lead or dbpA::STOP.lag). Electroporation efficiency is defined as the total number
584 of CFU generated per µg of plasmid DNA (pCRISPR::ctrl or pCRISPR::dbpA) and
585 recombination efficiency was measured by determining the proportion of mutant colonies
586 following PCR screening and AseI digestion. Recombination efficiency at the *dbpA* locus was
587 not determined (ND) for electroporation of the non-recombinogenic control oligo (ctrl.60mer).
588 Results shown are averages of at least two independent experiments and error bars depict
589 standard deviation. **(C)** Colony PCR and AseI digestion screening of oligo-mediated *dbpA* gene
590 disruption. A representative 13 colonies were used as template in colony PCR with primers
591 dbpA1.Fw and dbpA1.Rv, yielding a product of 1,002 bp. Successful oligo recombination
592 generates products of 464 bp and 538 bp upon AseI digestion. Lane 1: marker; lanes 2, 4, 7, 8,
593 and 13: negative, unmodified colonies; lanes 3, 5, 6, 9-12, and 14: positive, *dbpA* gene disruption
594 colonies

595

596 **FIG 2** Double-stranded DNA gene replacement demonstration using the proposed three-plasmid
597 system. **(A)** Replacement of an 8 bp (H0 + H0) and 818 bp (H1 + H2) region of *dbpA* with a 560

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598 bp recombinogenic PCR product encoding *lacZ′* (depicted by α). PCR primers possessing
599 different H*n* homology regions corresponding to 8 bp and 818 bp chromosomal deletions were
600 utilized. Successful gene replacement replaces the *dbpA* PAM sequence and prevents Cas9 from
601 generating a chromosomal DSB, which are both located between each set of homology regions.
602 Genomic layout, primer binding sites, and expected PCR product sizes are shown for each *dbpA*
603 gene replacement mutant, in addition to the wild-type strain. Genes corresponding to *dbpA* and
604 *lacZ′* are depicted to scale. Rep corresponds to 36 bp repeats necessary for crRNA processing.
605 **(B)** Electroporation and *dbpA* recombination efficiency data resulting from electroporation
606 (pCRISPR::ctrl + control PCR product), CRISPR/Cas9 (pCRISPR::dbpA + control PCR
607 product), and recombineering (pCRISPR::ctrl + 8 bp or 818 bp deletion cassettes) controls, as
608 well as 8 bp or 818 bp chromosomal deletions using CRISPR/Cas9-coupled recombineering
609 (pCRISPR::dbpA + 8 bp or 818 bp deletion cassette). Electroporation efficiency is defined as the
610 total number of CFU generated per µg of plasmid DNA (pCRISPR::ctrl or pCRISPR::dbpA) and
611 *dbpA* recombination efficiency was measured by determining the proportion of blue colonies.
612 Recombination efficiency at the *dbpA* locus was not determined (ND) for electroporation of the
613 non-recombinogenic control PCR product. Results shown are averages of at least two
614 independent experiments and error bars depict standard deviation. **(C)** Colony PCR screening of
615 *dbpA* gene replacement with *lacZ′*. Primers dbpA1.Fw and dbpA1.Rv were utilized in a colony
616 PCR with one white and one blue colony of each gene replacement mutant type. Lane 1: marker;
617 lane 2: wild-type *E. coli* colony; lanes 3 and 4: 8 bp gene replacement colonies; lanes 5 and 6:
618 818 bp gene replacement colonies; lanes 3 and 5: white (negative) colonies; lanes 4 and 6: blue
619 (positive) colonies
620

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621 **FIG 3** Challenging the proposed strategy of dsDNA gene replacement by varying chromosomal
622 gene deletion size. **(A)** Replacement of various-sized chromosomal regions with a
623 recombinogenic PCR product encoding *lacZ'* (depicted by α). PCR primers possessing different
624 H*n* homology regions corresponding to chromosomal deletions of various sizes (818-19,378 bp)
625 were used to amplify a 560 bp *lacZ'* product from pUC19. In each case, successful gene
626 replacement replaces the *dbpA* PAM sequence and averts generation of a chromosomal DSB,
627 which are both located between each set of homology regions. Genomic layout between the
628 essential *racR* and *insH* genes (white) is shown for each gene replacement mutant. Chromosomal
629 gene orientation is shown based on the *dbpA* coding sequence in the reverse (antisense)
630 orientation. Primer binding sites and expected PCR product sizes are also depicted for each gene
631 replacement mutant, in addition to the wild-type strain. All chromosomal genes and *lacZ'* PCR
632 product are depicted to scale. **(B)** Electroporation and *dbpA* recombination efficiency data
633 resulting from electroporation (pCRISPR::ctrl + control PCR product), CRISPR/Cas9
634 (pCRISPR::dbpA + control PCR product), and recombineering (pCRISPR::ctrl + 818 bp or
635 2,428 bp PCR deletion cassette) controls, as well as six various-sized chromosomal deletions
636 using CRISPR/Cas9-coupled recombineering (pCRISPR::dbpA + PCR deletion cassette).
637 Electroporation efficiency is defined as the total number of CFU generated per μg of plasmid
638 DNA (pCRISPR::ctrl or pCRISPR::dbpA) and *dbpA* recombination efficiency was measured by
639 determining the proportion of blue colonies. A recombination efficiency of 0% reflects an
640 inability to identify blue mutant colonies on agar plates containing up to 400 transformants.
641 Recombination efficiency at the *dbpA* locus was not determined (ND) for electroporation of the
642 non-recombinogenic control PCR product. Results shown are averages of at least two
643 independent experiments and error bars depict standard deviation. **(C)** Colony PCR screening of

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644 gene replacement mutants. Various PCR primers were used to ensure successful genomic
645 organization by screening one white colony and one blue colony resulting from each gene
646 replacement scheme. Lane 1: marker; lanes 2 and 3: 818 bp gene replacement colonies; lanes 4
647 and 5: 2,428 bp gene replacement colonies; lanes 6 and 7: 5,123 bp gene replacement colonies;
648 lanes 8 and 9: 9,590 bp gene replacement colonies; lanes 10 and 11: 11,068 bp gene replacement
649 colonies; lanes 12 and 13: 19,378 bp gene replacement colonies; lanes 2, 4, 6, 8, 10, and 12:
650 white (negative) colonies; lanes: 3, 5, 7, 9, 11, and 13: blue (positive) colonies. White colonies
651 corresponding to lanes 4, 6, 8, 10, and 12 are expected to generate a PCR product that is too
652 large to be amplified under the conditions employed

653

654 **FIG 4** Challenging the proposed strategy of dsDNA gene replacement by varying dsDNA
655 insertion size. **(A)** Replacement of an 818 bp region of the *dbpA* gene with recombinogenic PCR
656 products of various sizes encoding *lacZ′* (depicted by α). Products were PCR-amplified from
657 plasmid pUC19 (560 bp, 1,264 bp, 1,756 bp, and 2,492 bp products) or pTH18cr (550 bp and
658 3,000 bp products) using PCR primers possessing H1 and H2 homology regions. Regions
659 corresponding to the β-lactamase (Ap^R) coding sequence, ColE1 RNA (ColE1), and *repA* coding
660 sequence (pSC101) are truncated in the 1,264 bp, 2,492 bp, and 3,000 bp products, respectively.
661 In each case, successful gene replacement replaces the *dbpA* PAM sequence and avoids creation
662 of a chromosomal DSB, which are both located between H1 and H2 homology regions. Expected
663 genomic layout at the *dbpA* locus is shown for each gene replacement mutant. Primer binding
664 sites and expected PCR product sizes are also depicted for each *dbpA* gene replacement mutant,
665 in addition to the wild-type strain. Genes and plasmid regions corresponding to *dbpA*, *lacZ′*, Ap^R,
666 Cm^R, ColE1, and pSC101 sequences are depicted to scale. Note that size of the *lacZ′* coding

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667 sequence differs between plasmids pUC19 (324 bp *lacZ'*) and pTH18cr (237 bp *lacZ'*). **(B)**
668 Electroporation and *dbpA* recombination efficiency data resulting from electroporation
669 (pCRISPR::ctrl + control PCR product), CRISPR/Cas9 (pCRISPR::dbpA + control PCR
670 product), and recombineering (pCRISPR::ctrl + 550 bp, 560 bp, or 1,264 bp PCR insertion
671 cassette) controls, as well as six various-sized chromosomal deletions using CRISPR/Cas9-
672 coupled recombineering (pCRISPR::dbpA + PCR insertion cassette). Electroporation efficiency
673 is defined as the total number of CFU generated per µg of plasmid DNA (pCRISPR::ctrl or
674 pCRISPR::dbpA) and *dbpA* recombination efficiency was measured by determining the
675 proportion of blue colonies. A recombination efficiency of 0% reflects an inability to identify
676 blue mutant colonies on agar plates containing up to 400 transformants. Recombination
677 efficiency at the *dbpA* locus was not determined (ND) for electroporation of the non-
678 recombinogenic control PCR product. Results shown are averages of at least two independent
679 experiments and error bars depict standard deviation. **(C)** Colony PCR screening of *dbpA* gene
680 replacement mutants. Primers dbpA1.Fw and dbpA1.Rv were utilized in a colony PCR with one
681 blue colony resulting from each gene replacement scheme. Lane 1: marker; lane 2: wild-type *E.*
682 *coli* colony; lane 3: 550 bp insertional colony; lane 4: 560 bp insertional colony; lane 5: 1,264 bp
683 insertional colony; lane 6: 1,756 bp insertional colony; lane 7: 2,492 bp insertional colony; lane
684 8: 3,000 bp insertional colony

685

686 **Fig. 5** Comparison of the proposed CRISPR/Cas9-coupled recombineering methodology with
687 previous strategies of *E. coli* chromosomal gene replacement in the absence of CRISPR/Cas9 (9,
688 10). All technical steps involved in each protocol to obtain the target markerless gene
689 replacement mutant strain are depicted. Previous methods result in a mutant strain possessing an

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690 FRT “scar” site, whereas the mutant derived from the protocol proposed in this study generates a

691 “scar”-less mutant. AB^R corresponds to an antibiotic resistance marker.

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692 **Table 1**

693 **Strains, plasmids, and oligonucleotides employed in this study.**

Strains and plasmids	Relevant characteristics	Source or reference
Strains		
Escherichia coli DH5α	F$^-$ *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17($r_K^-m_K^+$), λ$^-$*	Lab stock
Plasmids		
pTH18cr	*E. coli* cloning vector (pSC101 ori; Cm^R; *lacZ′*)	35
pUC19	*E. coli* cloning vector (pMB1 ori; Ap^R; *lacZ′*)	Lab stock; 43
pKD46	*E. coli* lambda Red recombineering expression vector (pSC101ts; Ap^R; *araC*-P_{araB}-*gam-bet-exo*)	Coli Genetic Stock Center; 3
pCas9	*E. coli* vector containing the *S. pyogenes cas9* gene and tracrRNA (p15A ori; Cm^R; *cas9*; tracrRNA)	Addgene; 24
pCRISPR	*E. coli* vector containing a crRNA expression cassette for Cas9 targeting (pBR322 ori; Kn^R; crRNA)	Addgene; 24
pCRISPR::dbpA	Derived by targeting the pCRISPR crRNA region to the *E. coli dbpA* gene	This study
pCRISPR::ctrl	Derived by inserting a non-homologous control crRNA sequence into pCRISPR	This study
Oligonucleotide	**Sequence (5'-3')***	
dbpA.crRNA.S	AAACCAGGCTGTCTGCGACGCGCTGAATGAAGTAG	
dbpA.crRNA.AS	AAAACTACTTCATTCAGCGCGTCGCAGACAGCCTG	
ctrl.crRNA.S	AAACAGTTTTCAATCTGATGAAGCTAATACAGATG	
ctrl.crRNA.AS	AAAACATCTGTATTAGCTTCATCAGATTGAAAACT	
dbpA::STOP.lead	gctgtctgcgacgcgctgaatgaagATTAATaaagtgcattgtcattacacggcgatttg	
dbpA::STOP.lag	caaatcgccgtgtaatgacaatgcactttATTAATcttcattcagcgcgtcgcagacagc	
ctrl.60mer	CAGATTGTACAAATGTGGTGATAACAGATAAGTCCTTTTGCATAACTTACCTTTCTTTGT	
dbpA1.Fw	AACCGTTCGGTATGCAGCGTGATT	

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dbpA1.Rv	CGCCCTGTAACTGTTTCCATGCTT
dbpA::lacZ′.del8bp.H0P1	accaaaaaagattgccaggctgtctgcgacgcgctgaatgGAGTCAGTGAGCGAGGAAGC
dbpA::lacZ′.del8bp.H0P2	cgcgttgctccaaatcgccgtgtaatgacaatgcactttgCGGCATCAGAGCAGATTGTA
dbpA::lacZ′.del818bp.H1P1	gcatattatcgtggcaacgccggggcgtttgctggatcacGAGTCAGTGAGCGAGGAAGC
dbpA::lacZ′.del818bp.H2P2	cggatgcacggcgattttgccaatatctgcgccatcaagcCGGCATCAGAGCAGATTGTA
dbpA::lacZ′.del2428.H3P1	ccaatttgccccatttagtaccagagaactgaaataatgcGAGTCAGTGAGCGAGGAAGC
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dbpA::lacZ′.del11068.H8P1	ctgtaactgtttccatgctttatgagcaacagcctgacggGAGTCAGTGAGCGAGGAAGC
dbpA::lacZ′.del11068.H9P2	gtgtgaacaagctggcacaaattgtttaatgtttacagcaTAACTATGCGGCATCAGAGC
ttcA.Fw	CGACGAACAGATAGCCATGTTGAATGCG
dbpA2.Fw	GTGAGCGTAGATCTGCGAGGATACG
intR.Fw	GCAAACGAGGGATTATCTGGTACAACTATCC
ydaN.Fw	GGGTGATGAGATGTTGCGTAACAGGG
sieB.Rv	CCGATGGTTGGAGAAGCACACAGTAAG
dbpA2.Rv	GGCAATCCCGAATAAGATGTTTACTCTTGC
ogt.Rv	AAGCAAACCTTAACAGCAGAGAATTCCG
dbpA::lacZ′.ins550.H1P1	gcatattatcgtggcaacgccggggcgtttgctggatcacGGCTCTTGTATCTATCAGTG
dbpA::lacZ′.ins550.H2P2	cggatgcacggcgattttgccaatatctgcgccatcaagcAGTGTGACCGTGTGCTTCTC
dbpA::lacZ′.ins1264.H2P2	cggatgcacggcgattttgccaatatctgcgccatcaagcTCTTACTGTCATGCCATCCG
dbpA::lacZ′.ins1756.H2P2	cggatgcacggcgattttgccaatatctgcgccatcaagcCAGTGAGGCACCTATCTCAG
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dbpA::lacZ′.ins3000.H2P2	cggatgcacggcgattttgccaatatctgcgccatcaagcGTGAGCCATGAGAACGAACC

*Underline: restriction recognition sequence or overhang; Lower case: homology regions

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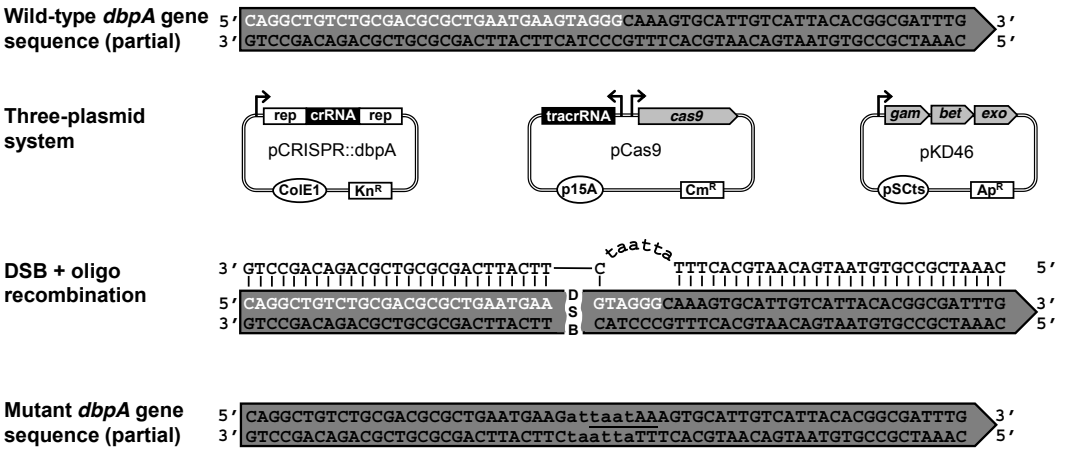
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696 **Fig. 1a)**



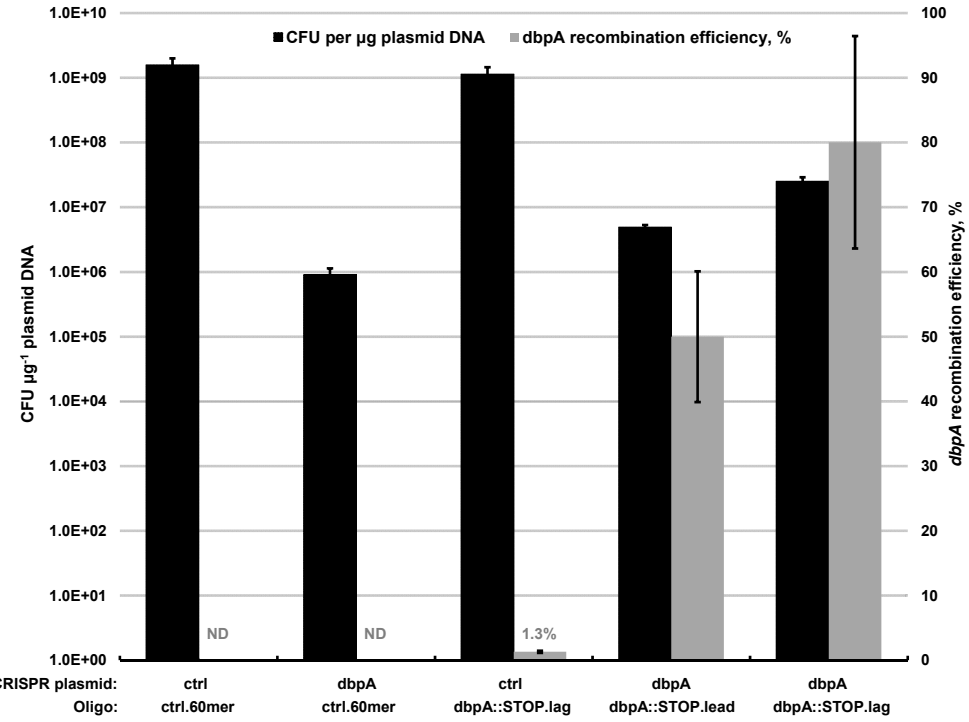
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700 **Fig. 1b)**



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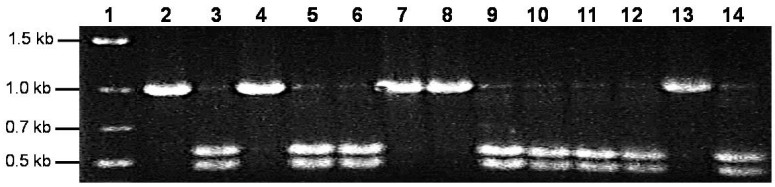
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702 **Fig. 1c)**



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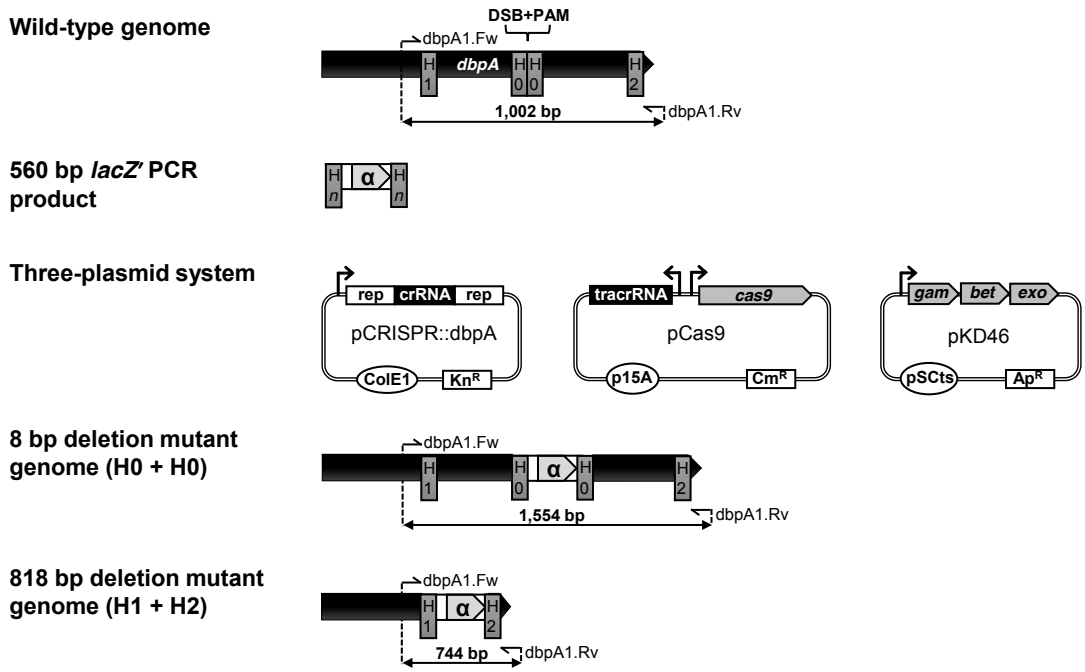
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705 **Fig. 2a)**



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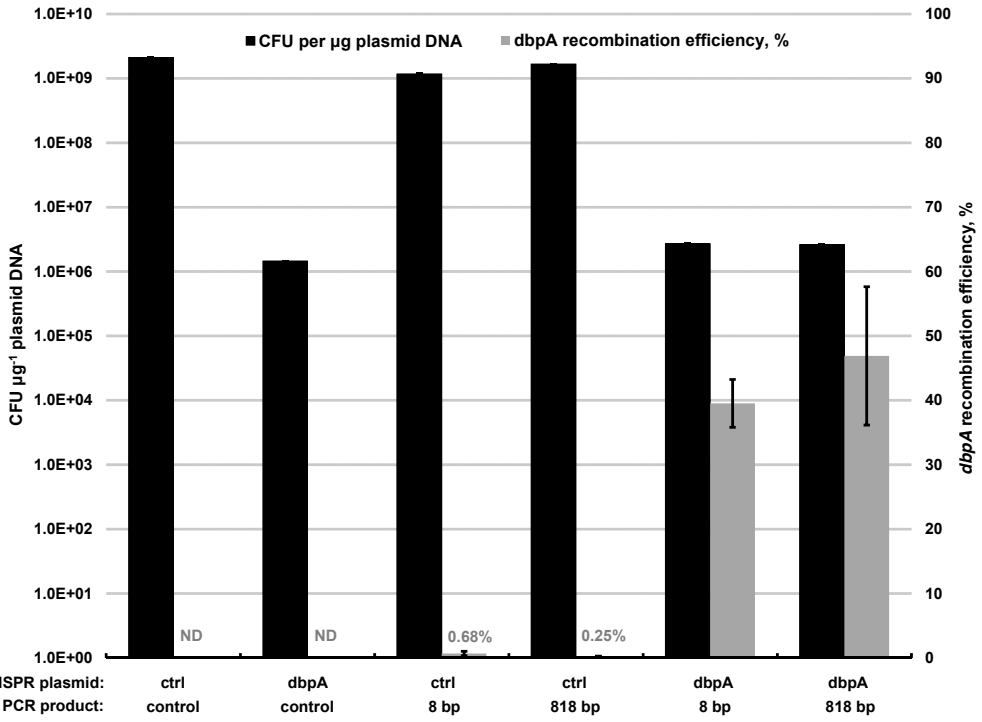
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708 **Fig. 2b)**



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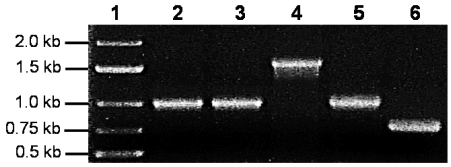
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710 **Fig. 2c)**



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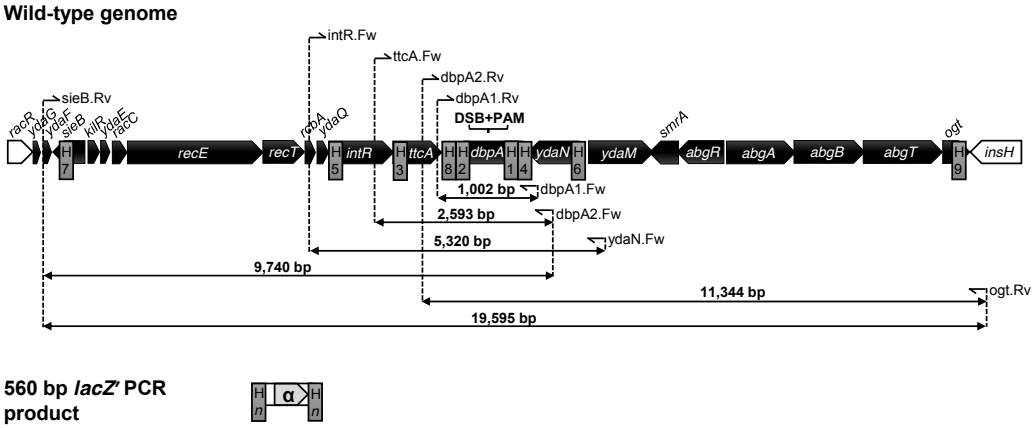
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713 **Fig. 3a)**

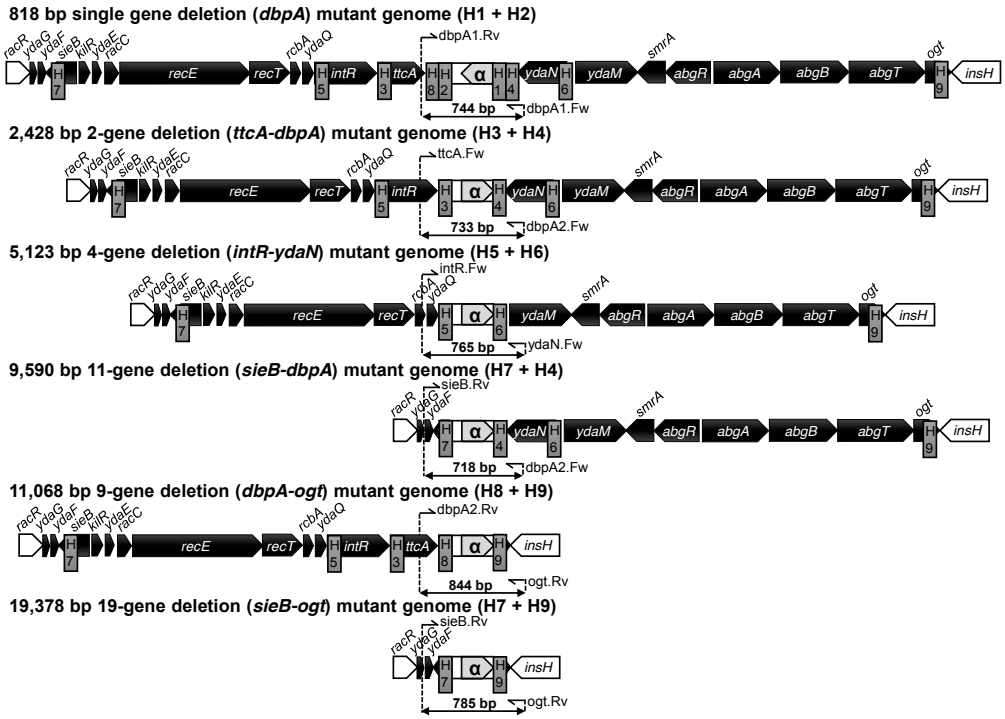


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716 **Fig. 3a) cont'd**



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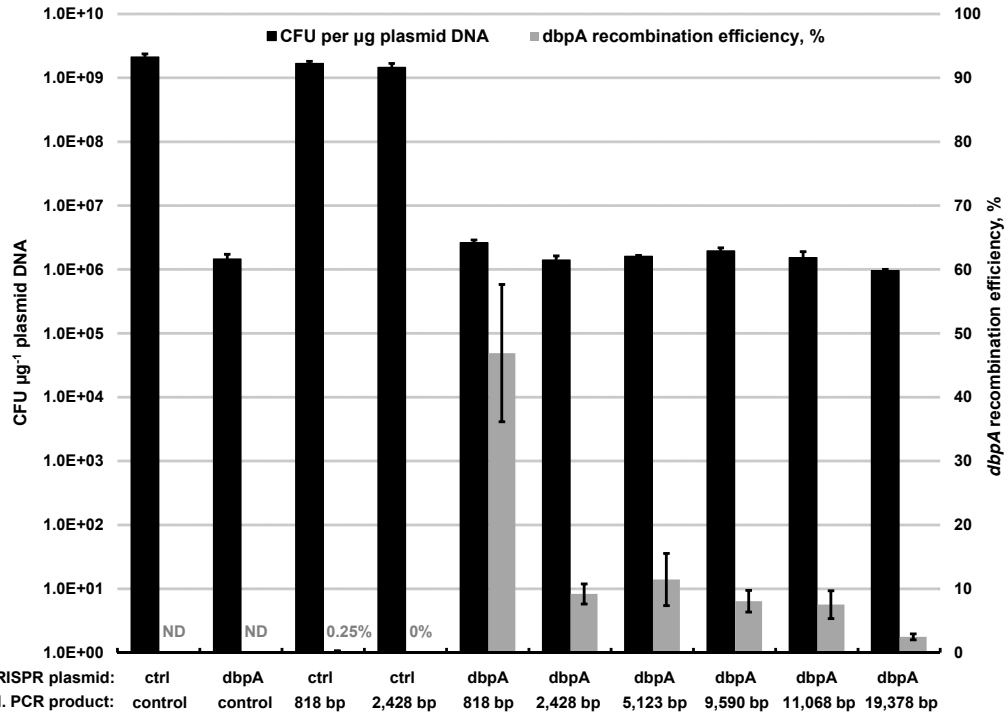
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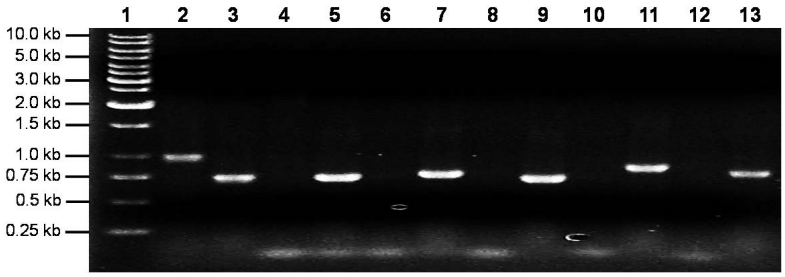
718 **Fig. 3b)**



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720 **Fig. 3c)**



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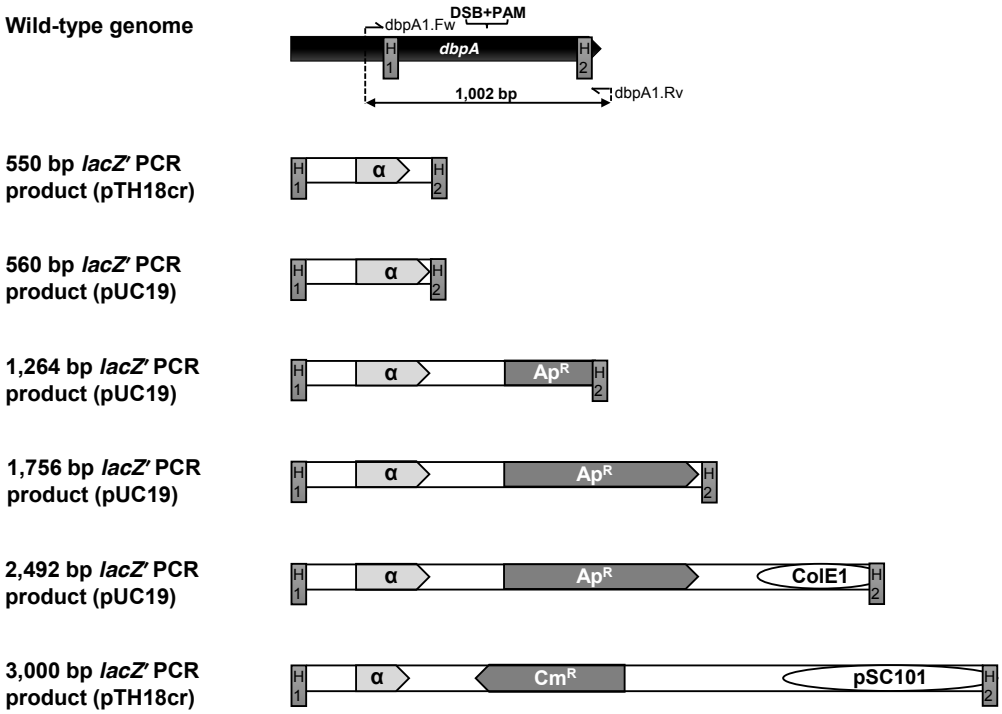
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723 **Fig. 4a)**



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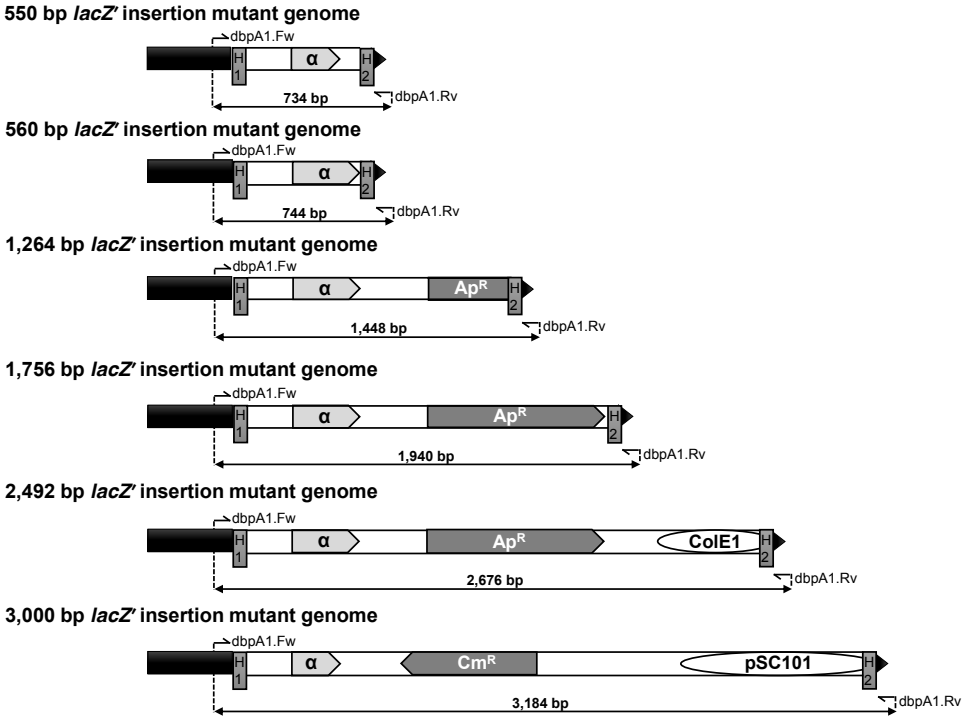
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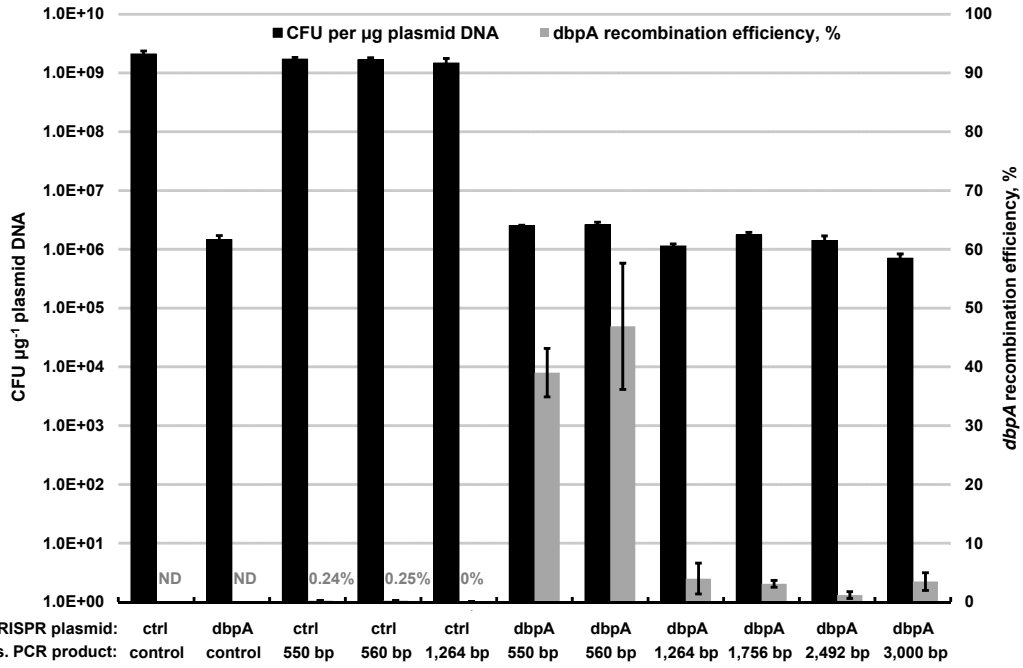
725 **Fig. 4a) cont'd**



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727 **Fig. 4b)**

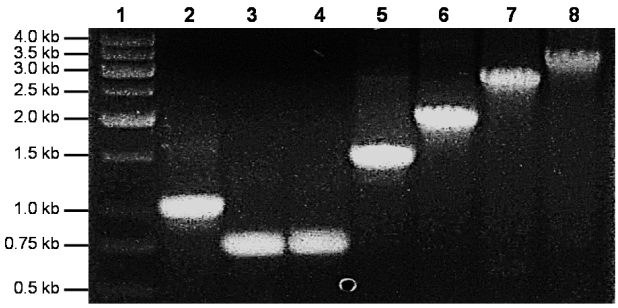


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730 **Fig. 4c)**



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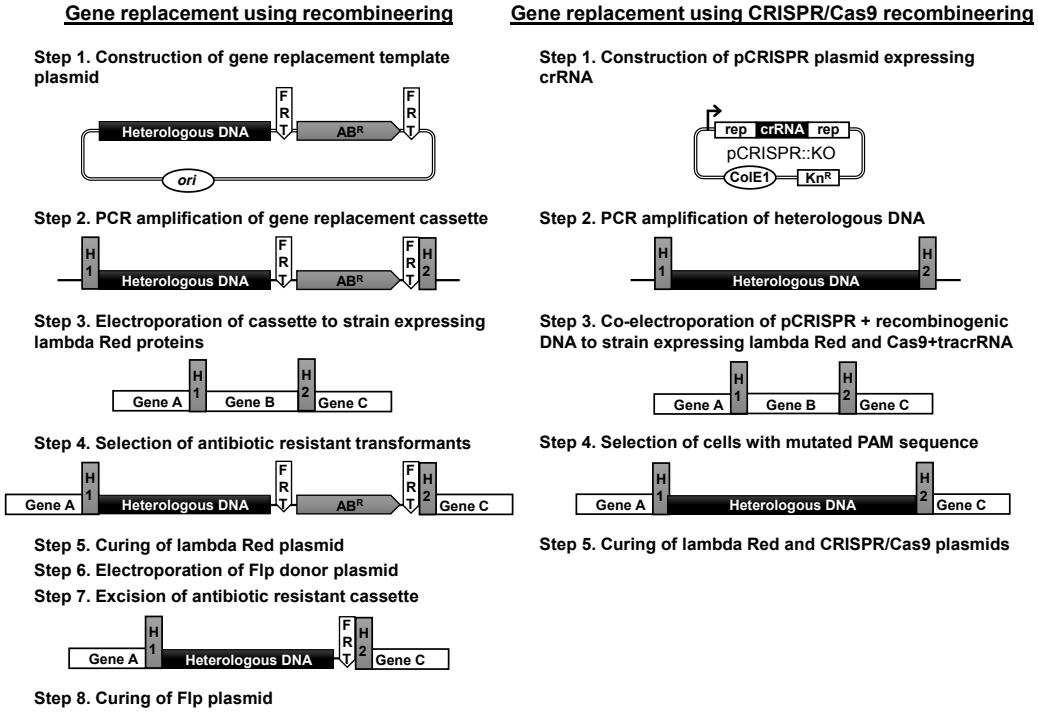
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734 **Fig. 5)**



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