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***Escherichia coli* vectors having stringently repressible replication origins allow a streamlining of Crispr/Cas9 gene editing**

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Abstract

Readily curable plasmids facilitate the construction of plasmid-free bacterial strains after the plasmid encoded genes are no longer needed. The most popular of these plasmids features a temperature-sensitive (Ts) pSC101 origin of replication which can readily revert during usage and cannot be used to construct Ts mutations in essential genes. Plasmid pAM34 which contains an IPTG-dependent origin of replication largely overcomes this issue but is limited by carrying the most commonly utilized antibiotic selection and replication origin. This study describes the construction of an expanded series of plasmid vectors having replication origins of p15a, RSF1030 or RSF1031 that like pAM34 have IPTG-dependent replication. Surprisingly, these plasmids can be cured in fewer generations than pAM34. Derivatives of pAM34 with alternative antibiotic selection markers were also constructed. The utility of these vectors is demonstrated in the construction of a CRISPR-Cas9 system consisting of an IPTG-dependent Cas9 plasmid and a curable guide RNA plasmid having a streptomycin counterselection marker. This system was successfully demonstrated by construction of point mutations, deletions and insertions in the *E. coli* genome with a very high efficiency and in a shorter timescale than extant methods. The plasmids themselves were readily cured either together or singly from the resultant strains with minimal effort.

1. Introduction

The ability to make sophisticated and precise genome modifications within a practical timescale has made *Escherichia coli* the most comprehensively studied and understood genome of any known organism. Most of the tools available for targeted gene editing in *E. coli* rely on host homologous recombination or a phage-derived homologous recombination pathway (Datsenko and Wanner, 2000; Thomason et al., 2014; Zhang et al., 1998). Group-II

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://doi.org/10.1016/j.plasmid.2019.04.004>.

intron-based homing (Karberg et al., 2001) is also used. Recombinogenic methods based on the λ phage proteins Exo, Beta and Gamma (λ red) (Datsenko and Wanner, 2000; Thomason et al., 2014) catalyze allelic exchange with just 35 bp of flanking homology but typically utilize an antibiotic selection marker and leave a “scar” behind. Achieving scar-less alterations usually requires a counter-selectable marker or multiple rounds of recombination (Thomason et al., 2014). Despite the successful application of these techniques, modification of genes that are either themselves essential or are part of an operon having essential genes downstream remains challenging. Most current tools either require expression of the gene(s) in *trans* or more complex cloning strategies to restore disrupted codons/promoters.

An important recent breakthrough has been the development of a novel RNA-guided endonuclease-based genome editing system called the clustered regularly interspaced short palindromic repeat (CRISPR) (Knott and Doudna, 2018). The most popular of these uses the CRISPR-associated *Streptococcus pyogenes* (Sp) protein 9 (Cas9) (Knott and Doudna, 2018). A CRISPR RNA (crRNA) forms an RNA duplex with a *trans*-acting RNA called tracrRNA and this complex then recruits the Cas9 endonuclease to target a 20 bp protospacer on the genome. The protospacer must match the sequence of the crRNA and be located immediately adjacent to a Cas9 specific protospacer adjacent motif (PAM) (Hille et al., 2018). More recent developments greatly facilitated Cas9-mediated mutagenesis by the demonstration that the tracrRNA:crRNA duplex can be replaced with a single chimeric RNA containing both tracrRNA:crRNA sequences (Jinek et al., 2012) called single guide RNAs (sgRNAs). Thus, by simply changing the 20 bp targeting sequence of a plasmid-encoded sgRNA, the Cas9 endonuclease is reprogrammed. The double strand breaks (DSBs) resulting from CRISPR action are lethal to *E. coli* because the bacterium lacks an efficient nonhomologous end joining (NHEJ) mechanism and thus repair of DSBs is accomplished by homologous recombination catalyzed by the λ Red system (Jiang et al., 2013).

When expressed from a plasmid the crRNA and tracrRNA the CRISPR/Cas9 system has been shown to successfully incorporate point mutations into the *E. coli rpsL* gene with an efficiency of 60% in the presence of λ Red-catalyzed homologous recombination (Jiang et al., 2013). Early three-plasmid versions of the CRISPR/Cas9 tools in *E. coli* provided SpCas9 and its tracrRNA under its native promoter on one plasmid, a crRNA on a second high copy number plasmid and a third plasmid (or the chromosome) provided the λ red genes (Jiang et al., 2013) (Pyne et al., 2015). Later improvements used sgRNAs for ease of cloning and placed the λ red genes on plasmids containing either the Cas9 gene or the sgRNA coding sequence (Jiang et al., 2015; Reisch and Prather, 2015).

The use of multiple plasmids becomes problematic in that after obtaining the desired changes curing (removal) of the plasmids often requires multiple rounds of growth in selection-free media followed by screening for plasmid loss. Two-plasmid systems typically utilize a temperature-sensitive pSC101(Ts) replication origin plasmid to express the sgRNA plasmid to expedite curing. However, these plasmids cannot be used to generate the temperature-sensitive (Ts) mutations required to study essential genes. Moreover, the mutation in the pSC101(Ts) plasmid is a point mutation in the *rep* gene that often reverts thereby complicating the curing process. Gil and Bouché have described plasmid pAM34, a pBR322-derived ampicillin-resistant vector in which replication is dependent upon addition

of isopropyl β -D-1-thiogalactopyranoside (IPTG) (Gil and Bouche, 1991). Although valuable, plasmid pAM34 has the disadvantage that it utilizes the most widely used plasmid replication origin and antibiotic resistance determinant and these are often included in CRISPR/Cas9 scheme plasmids. To cope with this disadvantage, we have expanded the repertoire of IPTG-dependent plasmids by constructing IPTG-dependent vectors having the replication origins of p15a, RSF1030 and RSF1031. Each of these vectors is compatible with pBR322-derived plasmids and with one another (with the exception of the two RSF plasmids). To our surprise when propagated in the absence of IPTG these new vectors were cured from the host cells significantly more rapidly and efficiently than was pAM34. We have also constructed versions of pAM34 that encode resistance to commonly used antibiotics rather than ampicillin. A CRISPR-Cas9 system was assembled using these vectors that could be used to make rapid iterative changes with ease while leaving the mutated plasmid-free strain

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

E. coli strain BW25113 was employed for genome editing and strain DH5a for cloning. The λ red recombineering donor plasmid pKD46 was obtained from the *E. coli* Genetic Stock Center, Yale University. Plasmids pCas9-CR4 and pKDsgRNA-p15a (Addgene plasmids # 62655 and 62,656) were originally from K. Prather (Reisch and Prather, 2015; Reisch and Prather, 2017). Oligonucleotides were synthesized by Integrated DNA Technologies (IDT). *E. coli* strains (Table 1) were grown aerobically in shake flask cultures at 37 °C with strong aeration in lysogeny broth (LB) unless stated otherwise. Recombinant strains were selected with 100 μ g/ml ampicillin, 300 μ g/ml streptomycin or 10 μ g/ml chloramphenicol, induced with 0.4% arabinose or 100 ng/ml anhydrotetracycline and stored in 40% glycerol at –80 °C. Plasmid assembly details are given in the supplement (Table 1).

2.2. Construction of the pl vector series

The p15a origin was amplified from bacteria carrying vector pdCas9- (Qi et al., 2013) by PCR using primers p15a *KpnI* F and p15a *XbaI* R whereas the RSF1030 and RSF1031 origins were amplified by PCR from plasmids pCY2016 and pCY2017 (Chakravarty and Cronan, 2015) using the restriction site-containing primers RSF *KpnI* F and RSF *XbaI* R. The RSF1030 and RSF1031 plasmids differ from one another by a single nucleotide (Phillips et al., 2000). The amplified fragments and pAM34 were digested with *KpnI* plus *XbaI* and ligated to obtain plasmids pI15, pI1030 and pI1031, respectively (Fig. S2). To construct derivatives of pAM34 having antibiotic resistance cassettes for chloramphenicol, kanamycin and gentamycin the cassettes were amplified from p34s-Cm2 p34s-Km3 and p34s-Gm (Dennis and Zylstra, 1998a; Dennis and Zylstra, 1998b) by PCR using the primers p34C *AatII* F/p34C *XbaI* R, p34K *AatII* F/p34K *XbaI* R and p34G *AatII* F/p34G *XbaI* R, respectively. The resultant fragments were digested with *AatII* plus *XbaI* and ligated to the pAM34 fragment obtained by digestion with the same restriction enzymes. The resulting ampicillin-sensitive plasmids were named pSW436, pSW438 and pSW437 (or pIBRC, pIBRK and pIBRG), respectively (Fig. S3).

2.3. Determination of curing rates and relative copy numbers

Relative copy number determinations were made by growing cultures in LB to an OD of 1.0 and extracting plasmids from 1 ml of culture. The extracted plasmids were digested with XbaI and run on a 1% agarose gel. DNA was quantitated by densitometry calculations using a Biorad Chemidoc XRS.

To measure the rates of curing of plasmids pAM34, pI15, pI1030 and pI1031, strains carrying one of these plasmids were first grown in a selection-free LB medium and then periodically plated on LB agar containing IPTG. Optical density measurements were also made to calculate the number of generations that elapsed since selection pressure was removed. Cultures were diluted when an OD of 1.2 was reached until 25 generations had elapsed. Thirty-six colonies were screened for resistance to ampicillin at each timepoint.

2.4. Construction of pITRCas9 and pSgRNA

A fragment containing *lacI^Q* and part of the p15a origin was amplified by PCR from plasmid pI15 using primers p15a-lacI *SpeI* F and p15a-lacI *AgeI* R. The PCR fragment was digested with *SpeI* plus *AgeI* and ligated to the larger fragment of pCas9-CR4, digested with the same enzymes, to give pSW442. The *μred* genes under arabinose control were amplified from pKD46 using primers LRed *AatII* F and LRed term *AatII* R. A minimal dual direction *tonB* terminator was introduced via these primers having the sequence 5'-CATGGTAATAGTCAAAAGCCTCCGGT CGGAG GCTTTTGACTTAAGCTT-3'. This fragment was digested with *AatII* and ligated to pSW442 digested by the same enzyme. The resultant plasmid was named pITRCas9 (or pSW443; Fig. S4). Correct plasmid assembly was confirmed by restriction mapping plus PCR amplification of the junctions.

To construct pSgRNA, pKDSgRNA-p15a and an ampicillin resistance cassette amplified from pKD46 (primers Amp *NcoI* F and Amp *XhoI* R) were digested with *NcoI* plus *XhoI* and ligated to give pSW444. This removed the plasmid-encoded *μred* genes and spectinomycin resistance cassette. The resulting plasmid was linearized by PCR using primers Non Ts F and Non Ts R while introducing a point mutation (A to G) in the plasmid origin that restored the wild type *rep* allele to give temperature-independent growth. The linear fragment was phosphorylated using T4 polynucleotide kinase and self-ligated to give pSW445. Plasmid pSW445 was digested with *NcoI* and ligated with a synthetic construct containing a *Photorhabdus luminescens rpsL* gene (codon optimized for *E. coli*) under a constitutive *proD* promoter (Davis et al., 2011). The 20 bp protospacer region of the resulting plasmid, pSgRNA-p15a or pSW446, was modified by PCR to contain a *NcoI* site flanked by two *BbsI* sites to yield plasmid pSgRNA (pSW447; Fig. S5) using the strategy described below.

2.5. Protospacer cloning to form pSgRNAs

All pSgRNA-xxx plasmids (where xxx denotes the gene targeted) were created using a one-step digestion-free PCR based cloning strategy as described below. The entire pSgRNA plasmid was amplified via PCR using a target specific primer pSgRNA-xxx F, a fixed primer pSgRNA-R and pSgRNA as template to yield a linear product. A fifteen-cycle PCR was performed with Q5 polymerase (New England Biolabs, NEB), an annealing temperature of

59 °C and an extension time of 45 s with the remaining steps as per the protocol of the manufacturer. The amplified DNAs were then purified using a Qiagen PCR purification kit and the DNA concentrations were determined using a Nanodrop spectrophotometer. The phosphorylation reaction was composed of 100 ng of purified linear DNA, adjusted to 10 µl with water, combined with 10 µl of 2 × Quick Ligation buffer (NEB) and 1 µl of T4 polynucleotide kinase (NEB). This was allowed to incubate at 37 °C for 30 min and then cooled to room temperature for 5 min. NEB Quick Ligase (1 µl) was then added and the reaction allowed to incubate at 25 °C for another 15–30 min. Two µl of this reaction mixture was used to transform 50 µl of chemically competent *E. coli* DH5α or NEB Turbo cells and selected on plates with 100 µg/ml ampicillin. Successful integration of the 20 bp protospacer sequence would remove one of three *NcoI* sites allowing candidate constructs to first be screened by restriction digestion and subsequently confirmed by sequencing using the primer pSgRNA check.

2.6. Homologous recombination

E. coli strain SW435 carrying plasmid pIRTCas9 was first transformed with a pSgRNA-xxx plasmid by electroporation and transformants were selected for growth on plates containing chloramphenicol, ampicillin and IPTG. Large colonies were selected and grown at 37 °C in LB medium supplemented with chloramphenicol, IPTG and ampicillin to an optical density of 0.4. Small colonies had inconsistent, usually lower, efficiencies of editing. Expression of the *μred* genes was induced by the addition of 0.4% arabinose for 30 min. The cells were harvested by centrifugation and washed thrice with ice-cold 10% glycerol. The cells were then resuspended in 1% of the original culture volume in 10% glycerol and divided into 50 µl aliquots and frozen at –80 °C. Donor DNAs were added to 50 µl of cells at a final concentration of 10 µM and transferred to a 0.1 cm electroporation cuvette. Electroporation was performed on a BioRad Gene Pulser and cells were immediately recovered in 1 ml of SOB containing 1 mM IPTG for 45 min at 37 °C before plating on LB containing 100 ng/ml anhydrotetracycline, which induces the tetracycline promoter. The plates were incubated overnight at 37 °C (or 30 °C for Ts strains). A small portion of the recovered cells were plated on LB as a control for transformation efficiency.

2.7. Plasmid curing

The pSgRNA plasmid was readily cured when manipulations were performed in a streptomycin resistant background. Strains were first grown in liquid culture and then subsequently plated on solid media (both supplemented with 300 µg/ml of streptomycin). Loss of the plasmid was verified by loss of ampicillin resistance. Plasmid pIRTCas9 was cured by growth in IPTG-free media for 10 or more doublings. Both plasmids can be cured simultaneously by growth in media that contains streptomycin and lacks IPTG.

2.8. Efficiency of chromosomal editing

The efficiency of the CRISPR/Cas9 system under different conditions is expressed as the ratio of the number of surviving cells in the presence or absence of anhydrotetracycline. All colony counts were calculated from serial dilutions and are the average of three independent trials.

3. Results

3.1. Compatible origins of replication

The pBR322, RSF1030 and p15a origins of replication are often found in cloning vectors. Though these plasmids all use the same unidirectional mechanism of replication dependent on an RNAII primer (Fig. SI), they fall into different incompatibility groups and hence multiple plasmid origins can be maintained in the same cell. These origins have an additional benefit of having nominal copy numbers ranging from 10 for p15a, 15–20 for pBR.322 to 100 for RSF1030. Variants of pBR322 (namely the pUC plasmids) or RSF1030 (namely RSF1031) can have very high copy numbers of 500–700 copies/cell. An alignment of the RNA pBR322, p15a and RSF1030 origins of replication allowed us to map the RNAII priming region (Fig. 1). The well-studied ColEI origin (Fig. SI) was included in the alignment for comparison. The RNAI sequence, including its –10 and –35 promoter regions, are identical in all four replication origins. The RNAII sequences, differ between pBR322, p15a and RSF1030 and thereby place the plasmids in different incompatibility groups. Note that ColEI and pBR322 fall within the same group. In each case the native –10 and –35 regions of the RNAII promoter were replaced with an *E. coli lacZYA* promoter under control of the *lacI* overproduction allele, *lacI^Q*. With the inclusion of *lacI^Q* RNAII production (hence plasmid replication) became dependent on induction by IPTG. Plasmids pI15 and pI1030 were constructed accordingly (Fig. 2). pI1031 is a high-copy number variant of pI1030 with a single base pair changed (Phillips et al., 2000). Plasmids pIBRC, pIBRK and pIBRG with alternative antibiotic selection markers (resistance to chloramphenicol, kanamycin or gentamycin, respectively) were also constructed based on pAM34 (Fig. 3).

3.2. Relative copy numbers of different origins

The copy numbers varied as expected with the replication origin. Plasmid pAM34 is a 6 kb plasmid with an IPTG-dependent pBR322 origin of replication having a slightly higher copy number (1.5 fold) than the standard pBR322 origin cloning vector (Cronan, 2016). Plasmid pI1030 was found to have a copy number very similar to pAM34 (Fig. 4) whereas the copy number of the p15a origin of plasmid pI15 was 60% that of pAM34. The RSF1031 derived vector has a copy number about twice of pAM34 and is lower than that seen when the RNAII primer is transcribed from its native promoter (Chakravartty and Cronan, 2015). However, the copy numbers of plasmid constructs can vary greatly depending on their cargo.

Omission of ampicillin selection during the first 6–7 generations of growth had very little influence on copy number with only a 5–10% decrease observed. However, in the absence of IPTG, the copy numbers fell precipitously with pAM34 being the only plasmid that maintained an appreciable copy number (about 30% of the original value) in the absence of IPTG.

3.3. Dynamics of IPTG-dependent plasmid curing

Plasmid pAM34 was shown to require IPTG concentrations of > 500 μ M for effective maintenance. By measuring plasmid yields from cultures grown with differing IPTG concentrations, all plasmids tested showed a 15–20% decrease when grown with 0.1 mM IPTG (Fig. 5). These host strains lack the gene (*lacY*) encoding lactose permease so IPTG

enters by diffusion. Upon streaking on LB plates lacking IPTG, almost all colonies formed by cells from cultures grown with ampicillin and IPTG were found to have lost the plasmid. This was verified by screening the colonies for loss of ampicillin resistance and by attempting plasmid purification from a small subset of colonies. Cultures carrying pI15, pI1030 or pI1031 became plasmid-free within 15 doublings. However, plasmids pI15 and pI1031 showed a more severe decline and became plasmid free in < 5 generations whereas plasmid pI1030 showed a slightly slower rate of plasmid loss. In contrast pAM34 cultures required > 25 generations to become plasmid free. The curing rate of plasmid pAM34 agrees with similar observations made by Gill and Bouche (Gil and Bouche, 1991) in their original report of the plasmid.

3.4. Description and validation of a two-plasmid CRISPR mutagenesis system for *E. coli*

Reisch and Prather (2015, 2017) reported a valuable two-plasmid system consisting of a p15a plasmid encoding a Cas9 tagged with a SsrA degradation sequence expressed under tetracycline control and a second plasmid encoding the *sgRNA* under tetracycline control plus the μ Red system under arabinose control. Replication of this second plasmid is temperature sensitive. To obtain plasmid free strains following introduction of the desired mutation(s) growth at 37 °C (or 30 °C for Ts strains) readily eliminates the temperature sensitive plasmid. However, in the Reisch-Prather system (Reisch and Prather, 2015; Reisch and Prather, 2017) the p15a Cas9 plasmid must be eliminated by a more complex procedure. This is accomplished by transformation with a p15a plasmid encoding an *sgRNA* that targets p15a origins to give in lethal DSBs in both the *sgRNA* plasmid and the p15a Cas9 plasmid. Although this gives reasonably efficient curing of both plasmids, the manipulations needed to remove the Cas9 plasmid add two more days to the protocol. We have streamlined this last step by using the pI15 IPTG-dependent plasmid in place of the native p15a origin. We have also rearranged the Reisch-Prather constructs (Reisch and Prather, 2015; Reisch and Prather, 2017) and added other useful elements. In our system the pITRCas9 IPTG-dependent pI15 origin plasmid encodes the *ssrA*-tagged *S. pyogenes* Cas9 endonuclease controlled by a tetracycline-inducible promoter plus the μ red genes under arabinose control. The second plasmid has a wildtype (temperature-resistant) Rep101 origin and encodes the *sgRNA* under tetracycline control plus a constitutively expressed orthologous *rpsL* counterselection marker to allow for rapid curing of the plasmid in a streptomycin resistant strain. Wildtype *rpsL* (encoding the 30S ribosomal subunit S12) is sensitive to streptomycin and is dominant over mutant versions of *rpsL* that confer resistance to streptomycin (the streptomycin-inhibited ribosomes block mRNA translation) (Fig. 8). New spacers were introduced in the pSgRNA plasmid via a PCR-based cloning strategy. Alternatively, two *BbsI* sites were also used to introduce spacers using the Golden Gate approach (Engler et al., 2009; Engler and Marillonnet, 2011) (Fig. 7). Multiple spacers may also be cloned into either vector using synthesized DNA blocks and/or Gibson assembly to simultaneously target multiple genes. (Fig. 7). The effectiveness of the constructed plasmids was tested in *E. coli* BW25113 by using a guide RNA targeting the essential *dnaG* gene (Fig. 9). Upon induction of Cas9 expression with anhydrotetracycline, a 10⁴-fold reduction in colony formation was observed. However, ~10² colonies escaped killing by Cas9. Given this validation we then used our system to make various types of mutations.

3.5. Oligonucleotide-mediated temperature-sensitive (Ts) point mutations

CRISPR/Cas9 has been shown to provide an effective scar-less counterselection for generating point mutations using single stranded donor DNAs (Reisch and Prather, 2015; Wang et al., 2009). We demonstrated the particular utility of this tool in generating a point mutation in an essential gene cluster that results in temperature sensitive growth. Most genes in the *E. coli* fatty acid biosynthetic pathway are essential and have generally been studied using temperature-sensitive (Ts) mutants that grow at lower temperatures but lose function at higher temperatures. (Srinivas and Cronan, 2017). Using plasmid pSgRNA-*fabG* encoding a single guide RNA that targeted base 714 of *fabG* (Table 2) and an oligonucleotide targeted at the sequence of the lagging strand, we successfully introduced the E233K mutation into *fabG* with an efficiency of 99% to give the expected Ts strain (Table 3).

3.6. Short deletions

Expression of the *μred* genes has been successfully used in the past for ssDNA mediated deletions of up to 10 kb of the *E. coli* genome (Ellis et al., 2001; Sawitzke et al., 2013). We used a sgRNA and an oligonucleotide to target and delete a short 32 bp sequence from the *fabG* gene that corresponds to an 8 residue in-frame deletion in the gene. This deletion results in severe temperature sensitivity with growth seen only at 23 °C (Srinivas and Cronan, 2017). Of the 72 colonies tested 94% contained the deletion (Table 3). This was first done by screening for temperature-sensitive growth and confirmed by sequencing several candidates.

3.7. Short sequence insertions

The system to insert short sequences into the *E. coli* genome using *μRed*- mediated recombination to repair the DS breaks generated by Cas9 has been used to make both large and small insertions using just 50 bp regions of homology in the donor dsDNA. However, to introduce short tags such as the hexahistidine tag (His₆), ssDNA has been used to insert up to 30 bp. We used a 95 bp ssDNA to insert a 24 bp hexahistidine tag with a Gly-Gly linker at the C-terminus of *fabG* and obtained the altered gene at an efficiency of 90% (Table 3). Colonies (nine tested) were first tested for the insertion via PCR and subsequently confirmed by sequencing.

3.8. Speed and efficiency of plasmid curing

Due to the limited availability of compatible origins of replication and commonly used genetic markers for strain manipulations, recombineering methods that result in strains free of plasmids are generally preferred. The pITRCas9 construct is based on the pI15 vector backbone (Fig. 7). Vector pI15 was shown to be cured within 12–15 generations (Fig. 6). Given the size of the *cas9* gene, the copy number of pITRCas9 was expected to be much lower than that of the parent plasmid. Indeed, pITRCas9 was completely cured in < 6–7 generations and thus curing was easily achievable in a single culture grown to saturation (data not shown). Moreover, the pSgRNA plasmid can be cured by growing the strain in liquid culture in the presence of streptomycin (300 μg/ml) followed by plating on LB agar plates containing the same streptomycin concentration. Thus, two independent curing mechanisms can cure either or both plasmids.

4. Discussion

CRISPR/Cas systems were originally identified by their role in bacterial adaptive immunity (Hille et al., 2018) but have recently taken the scientific world by storm due to their remarkable ability to target genomes using only a small guide *sgRNA*. In many bacteria, recombineering using phage-derived recombination machinery remains one of the most effective methods of genetic engineering. However, the major limiting aspect of this remains the process of selecting mutant cells over wild-type cells. The combination of recombineering and CRISPR/Cas9 based counter-selection systems has greatly enhanced the effectiveness of this selection process and have since been incorporated into a number of tools for microorganisms into which plasmids can be introduced such as *E. coli* and *Pseudomonas* species. However, recombineering is not needed in highly recombinogenic bacteria such as *Streptococcus pneumoniae*. Although single-plasmid systems containing the guide RNA, the endonuclease and/or the donor DNA have been devised for other bacteria and eukaryotes, in the specific case of *E. coli* single plasmid systems the generally used cloning host are problematical. Jiang and coworkers (Jiang et al., 2013) first demonstrated the utility of a two-plasmid system in *E. coli* consisting of a plasmid encoding Cas9 and another with a CRISPR array targeting a gene of interest. This arrangement requires expression of the *μred* genes from either the genome or a third helper plasmid in order to use ssDNA or short dsDNA as recombination templates. Further iterations have combined different combinations of Cas9, *μred* and guide RNA (*sgRNA* or *crRNA*) in a two-plasmid format under either constitutive or inducible promoters. More recent approaches also utilize temperature sensitive origins of replication to facilitate guide RNA plasmid curing at higher temperatures but require time-consuming manipulations to cure the Cas9 encoding plasmid (Reisch and Prather, 2017).

We report a convenient and facile two-plasmid Cas9 CRISPR system. The first plasmid (pITRCas9) encodes *Sp. Cas9* under a tetracycline inducible promoter, the *μred* genes under arabinose inducible expression and an IPTG-dependent origin of replication. The second plasmid contains a single guide RNA (pSgRNA) also under tetracycline control together with a *rpsL* counterselection system. This combination provides some unique advantages. These are ease of use, rapid turnaround and temperature independent curing. The pSgRNA plasmid is small (~4 kb) and thus new *sgRNA* encoding sequences can readily be introduced via a single PCR reaction followed by circularization and transformation into a cloning host. The empty vector also contains *BbsI* sites that may be used with short annealed oligos for Golden Gate (Engler et al., 2009; Engler and Marillonnet, 2011) cloning. Multiple spacers may also be inserted into either vector using synthesized DNA blocks and/or Gibson assembly (Gibson et al., 2009) products to simultaneously target multiple genes. Since the two plasmids can be independently cured, loss of either one or both plasmids can be achieved in a single step depending on whether or not Cas9 is required for subsequent edits.

A typical workflow (Fig. 8) for introducing a single change takes about 4 days from start to finish. Our system has faster turnaround than the Reisch-Prather system and rivals that of Jiang and coworkers (Jiang et al., 2015) and has the advantage that it avoids curing by high temperature growth and thereby allowing Ts mutants in essential genes to be constructed. The *rpsL* counterselection system uses a wild-type *rpsL* gene from *Phototrhabdus*

luminescens that confers a dominant sensitivity to streptomycin in a streptomycin resistant background (Bird et al., 2011). The sequence of the *P. luminescens rpsL* gene was optimized for use in *E. coli* and has no significant stretch of homology to the endogenous *rpsL* gene (Bird et al., 2011). The only requirement for *rpsL* based counterselection is a streptomycin-resistant mutation which is present in common strains such as Top10, MC4100, MC1061, EPI300, DH10B and their derivatives or by simply transforming a short oligonucleotide to introduce a point mutation in the wild type *rpsL* gene and selecting for streptomycin resistance, as shown by constructing SW435 from BW25113. Moreover, spontaneous streptomycin resistant strains are easily isolated by plating concentrated cultures on plates containing streptomycin (Miller, 1972).

Most colonies that escape mutagenesis can be attributed to mutations in the Cas9 nuclease, a large mutational target, or (much less likely) the spacer sequence. When iterative changes are required, this can prove to be cumbersome since curing the mutant Cas9 encoding plasmid and exchanging it for a new plasmid could be rate-limiting. This problem can be mitigated by including elements such as LacI^Q, TetR or AraC on the host genome to decrease Cas9 expression. However, the largest improvement will probably come from discovery of smaller Cas9 or other CRISPR system nucleases.

Note that plasmid pITRCas9 is itself based on plasmid pI15 which has an IPTG-dependent p15a origin of replication. p15a has a low copy number of 10–15 and thus might be useful to reduce any potential toxicity that Cas9 might have prior to induction. The differing copy numbers provided by pI1030, pI1031 or pAM34 could also be of use in regulation of the expression of other CRISPR nucleases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Comparison of RNAI and RNAII binding sequences of different replication origins.

Alignment was performed with ClustalW. Conserved sequences are highlighted in black.

The -10 and -35 boxes of the RNA II and RNAI are indicated in red. The known RNA II primer sequence of pBR322 is indicated in green while the RNAI sequence is in blue. Strong conservation is observed between the -10 and -35 boxes of the p15a, pBR322 and ColE1 promoters. However, the RNAII primer sequences are sufficiently different to place the plasmids in different incompatibility groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Maps of plasmids pI1030 (pSW440) and pI15 (pSW439). pI15 and pI1030 are smaller than pAM34 due to deletion of *rop* together with the pBR322 origin. The p15a origin of replication is not *rop* sensitive (nor are the pI1030 and pI1031 origins). Other elements including the multiple cloning site (MCS), *aadA* gene and the direct repeat regions found in pAM34 are intact. Antibiotic resistance markers are in yellow and replication origins are in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Maps of plasmids pIBRC, pIBRG and pIBRK. The plasmids are considerably smaller than pAM34 and are resistant to chloramphenicol, gentamycin and kanamycin respectively. They lack the *aadA* gene and the pAM34 multiple cloning site. The restriction sites of the new multiple cloning site are shown. Note: the BamHI site is duplicated and flank the XbaI site. The *AccI* site is also not unique. pIBRC, pIBRG and pIBRK are also referred to as pSW436, pSW437 and pSW438, respectively.

Fig. 4.

IPTG-dependent replication of plasmids pAM34, pI15, pI1030 and pI1031. Plasmids were extracted from 1 ml of an LB culture grown to OD 1.0 from OD 0.01 with the following supplements: AI represents addition of ampicillin (100 μg/ml) and IPTG (1 mM), I represents addition of IPTG (1 mM) only whereas the minus sign represents the absence of IPTG supplementation. Relative plasmid amounts are indicated in the bar graph below with pAM34 set as 100. All four strains show plasmid loss in the absence of IPTG whereas omission of ampicillin selection has minimal or no effect in 6–7 generations. Marker: NEB

2-log ladder. pI15, pI1030 and pI1031 are systematically named pSW439, pSW440 and pSW441, respectively.

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Fig. 5.

Effects of varying IPTG concentrations on plasmid levels. Plasmids were extracted from 1 ml of LB cultures grown to OD 1.0 from OD 0.01. Cultures were supplemented with varying IPTG final concentrations as indicated above each lane. Relative plasmid amounts are indicated in the bar graph below with pAM34 set as 100. All four strains show plasmid loss in the absence of IPTG while the lack of ampicillin selection has minimal to no effect in 6–7 generations. Marker: NEB 2-log ladder.

Fig. 6.

Rates of curing of pAM34, pI15, pI1030 and pI1031. Plasmid curing was measured over 25 generations in LB with no IPTG supplementation. Cultures were started at OD 0.01 and samples were withdrawn periodically and plated on LB agar with 1 mM IPTG. OD measurements were used to calculate number of generations grown. Samples were diluted to OD 0.01 when an OD of 1.0 was achieved. Ratio of ampicillin resistant colonies to total number of colonies is represented against number of generations. Thirty-six colonies were analyzed for each time point. Strains were cured of plasmids pI15, pI1030 and pI1031 within 15 generations, a significantly faster rate than pAM34 curing which was not completely cured even after 25 generations. Hence, the pI1030 and pI1031 derivatives are more unstable than the pI15 derivative, pI15, pI1030 and pI1031 are also referred to as pSW439, pSW440 and pSW441, respectively.

Fig. 7.

Plasmid maps of the CRISPR/Cas9 vectors. Plasmid maps showing the key features of both plasmids. Plasmid pITRCas9 (pSW443) contains the *cas9* gene under a tetracycline-inducible promoter with the tetracycline repressor expressed from its native promoter. The *λred* genes are under arabinose control with AraC expressed from its native promoter. The plasmid has an is IPTG-dependent p15a origin. Plasmid pITRCas9 confers resistance to chloramphenicol. Plasmid pSgRNA has a native pSC101 origin, ampicillin resistance and is designed to contain a tetracycline-inducible single-guide RNA. The empty vector has an arbitrary protospacer that contains a *NcoI* site (one of three *NcoI* sites in the plasmid) for quickly verifying insertion of the sgRNA-encoding sequence, (depicted below the map). Two *BbsI* sites for Golden Gate cloning were also included. Two strategies for inserting protospacer sequences are depicted. These are a PCR-based method that includes the protospacer sequence on one primer followed by circularization and transformation into a host strain and a Golden Gate approach requiring specific overhangs to be included in the sgRNA encoding sequence (green and blue) during oligo synthesis. The Cas9 binding site encodes the chimeric tracrRNA and crRNA hairpin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 8.

Schematic for single or iterative mutations. Plasmids pITRCas9 and pSgRNA are sequentially transformed into the host strain. Δred genes are induced during competent cell preparation and 10 μ mol of donor DNA is transformed. IPTG is added during recovery but can be omitted while curing of pITRCas9. For iterative changes, the pSgRNA plasmid could potentially be cured selectively by growing on media containing streptomycin (Strep) provided the host is streptomycin resistant followed by introduction of a new pSgRNA by transformation.

Fig. 9.

Efficiency of killing by Cas9 cleavage of the essential *dnaG* gene. Ten microliter of 10-fold serial dilutions of a culture of strain BW25113 transformed with pITRCas9 and pSgRNA-*dnaG* (pSW448) was plated on LB agar plates with or without 100 ng/ml anhydrotetracycline. Greater than four logs of killing is observed upon induction of the CRISPR system.

Table 1

Bacterial strains used in this study.

Strains	Relevant genotype	Source
MC1061	<i>araD139 (araA-leu)7697 (lac)X74 galK16 galE15 mcrA0 relA1 rpsL150 spoT1 mcrB1 hsdR2</i>	Casadaban et al. (1980)
DH5α	<i>endA1 recA1 #80dlacZ M15 (lacZYA-argF)U169, hsdR17</i>	Hanahan et al. (1985)
BW25113	<i>lacI^r rrmBT14 lacZWJ16 hsdR514 araBADAH33 rhaBADL78 rph-1</i>	CGSC #7636
NEB Turbo	<i>F⁺ proA + B+ lacIq lacZM15 /fluA2 (lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 (hsdS-mcrB)5</i>	New England Biolabs
SW435	BW25113 <i>rpsL135 (strR)</i>	This work
Plasmids	Relevant characteristics	Source
pAM34	pBR322 <i>ori</i> /IPTG-dependent plasmid	Gil and Bouche (1991)
pCas9-bacteria	dCas9 vector with p15a <i>ori</i>	Qi et al. (2013)
pCY2016	pBAD plasmid with RSF1030 <i>ori</i> ; medium copy	Chakravarty and Cronan (2015)
pCY2017	pBAD plasmid with RSF1031 <i>ori</i> ; high copy	Chakravarty and Cronan (2015)
pKD46	Amp ^R , pSC101(Ts) origin, arabinose-inducible expression of <i>μred</i> genes	Datsenko and Wanner (2000)
pCas9CR4	Cas9 nuclease under pTet promoter and constitutive TetR	Reisch and Prather (2015)
pKDSgRNA-p15	pSC101(Ts) origin; Tet-inducible protospacer targeting the p15a <i>ori</i> , Ara-inducible <i>μred</i> , Spec ^R	Reisch and Prather (2015)
pSW436	pIBRC; Amp ^S , Cm ^R of pAM34	This work
pSW437	pIBRG; Amp ^S , Gm ^R of pAM34	This work
pSW438	pIBRK; Amp ^S , Kan ^R of pAM34	This work
pSW439	p115; p15a <i>ori</i> /IPTG-dependent plasmid	This work
pSW440	p11030; RSF1030 <i>ori</i> /IPTG-dependent plasmid	This work
pSW441	p11031; RSF1031 <i>ori</i> /IPTG-dependent plasmid	This work
pSW442	pCas9CR4 with IPTG-dependent replication	This work
pSW443	pITRCas9; IPTG-dependent replication, Ara-inducible <i>μred</i> , Cas9 under promoter pTet; Cm ^R	This work
pSW444	pKDsgRNA-p15a w/o <i>μred</i> , pSC101(Ts), Amp ^R	This work
pSW445	pSW443 mutated to temperature resistance, Amp ^R	This work
pSW446	pSgRNA-p15a; Protospacer targeting p15a origin; ctaicgtctgagtcacac	This work
pSW447	pSgRNA; pSC101 native origin; Amp ^R ; non-targeting protospacer sequence with BbsI sites, Tet-inducible guide RNA production, <i>rpsL</i> counterselection.	This work
pSW448	pSgRNA- <i>dnaG</i> ; Protospacer targeting <i>dnaG</i> ; ggagctctggacattaaacc	This work
pSW449	pSgRNA- <i>fabG</i> ; Protospacer targeting <i>fabG</i> ; gggtgaacttgcattgaa	This work

Strains	Relevant genotype	Source
pSgRNA-450	pSgRNA- <i>fabG2</i> ; Protospacer targeting <i>fabG</i> ; tggaacggcgggattaca	This work

Table 2

Plasmids and oligonucleotide primers used in this study.

Oligonucleotides	Sequence
p15a KpnI F	ATGCCAGGTACCTTGCCTGAAAAACGAAAAAACCGC
p15a XbaI R	GATCCATCTAGAACTAGAGTCACACTGGCTCAC
RSF KpnI F	ATGCCAGGTACCTCATATAATAAGAAAAACCCCGC
RSF XbaI R	GATCCATCTAGACTCTTTTGTATTATTTTCTAAATACATTC
p34C AatII F	ATCGAGACGTCTTGAATAAGATCACTACCGGG
p34C XbaI R	ATGCCATCTAGAGGATCCGCATGCCCTGCAGGTGACAAAGCTTCCCGGGTACCGAGCTCGCGAATTTCTGCCAATTCATC
p34K AatII F	ATCGAGACGTCCCACGTTGTGTCTCAAAATCTCTG
p34K XbaI R	ATGCCATCTAGAGGATCCGCATGCCCTGCAGGTGACAAAGCTTCCCGGGTACCGAGCTCCGCTGAGGTCTGCCTCG
p34G AatII F	ATCGAGACGTGCGAATTGACATAAGCCCTGTTTCGGTTTCG
p34G XbaI R	ATGCCATCTAGAGGATCCGCATGCCCTGCAGGTGACAAAGCTTCCCGGGTACCGAGCTCGAATTTGGCCCGCGCGCT
p15a-lacI SpeI F	TCTAGAACTAGTGAACCATTATATATCATGACATTAAACC
p15a-lacI AgeI R	CGGTTTACCGGTGTCATTC
LRed AatII F	ATGCCAGACGTCCATCGATTATATATGACAACTTGACGG
LRed term AatII R	GATCAGGACGTCAAGCTTAAGTCAAAAGCCTCCGACCGGAGGCTTTTGACTATTACCATGGTCATCGCCATTGCTCCC
Amp NcoI F	ATGACACCAATGGTAAATACATTCAAATATGTATCCGCTC
Amp XhoI R	GCAATGACTCGAGGTAAACTTGGTCTCTGACAGTTAC
Non Ts F	GCTTACTTTGCATGTCACTC
Non Ts R	ATGATCTCAATGGTTTCGTTTC
pSgRNA- <i>dnaG</i> F	GGAGCTCTGGACATTAAACCGTTTTTAGAGCTAGAAATAGCAAG
pSgRNA- <i>fabG</i> F	GGTGAAACTTTGCATGTGAAGTTTTTAGAGCTAGAAATAGCAAG
pSgRNA- <i>fabG2</i> F	TGTGAACGGCGGATGTACAGTTTTTAGAGCTAGAAATAGCAAG
pSgRNA R	GTGCTCAGTATCTCTATCACTGA
pSgRNA check	GTCTGCTATGTGGTGCTATC

Table 3

Efficiency of the pITRCas9/pSgRNA system.

Target	Mutation	CFU	Positives
Control	None	5.2×10^7	N.a.
<i>dnaG</i>	None	2.7×10^3	N.a.
<i>fabG</i>	Ts (point mutation)	4.5×10^5	99 $\pm$ 1%
<i>fabG</i>	6 $\times$ His at C-terminal	5×10^5	90 $\pm$ 5%
<i>fabG</i>	Ts (24 bp deletion)	1.7×10^4	94 $\pm$ 3%