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Recombineering mycobacteria and their phages

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Abstract

Bacteriophages are central components in the development of molecular tools for microbial genetics. Mycobacteriophages have proven a rich resource for tuberculosis genetics, and the recent development of a mycobacterial recombineering system based on phage Che9c-encoded proteins offers new approaches to mycobacterial mutagenesis. Expression of the phage exonuclease and recombinase substantially enhances recombination frequencies in both fast-and slow-growing mycobacteria, facilitating construction of both gene knockout and point mutants; it also provides a simple and efficient method for constructing mycobacteriophage mutants. Exploitation of host-specific phages thus provides a general strategy for recombineering and mutagenesis in genetically naive systems.

Introduction

Genetic manipulation of *Mycobacterium tuberculosis* is complicated by its pathogenesis, slow growth rate, inefficient DNA uptake, and relatively high level of illegitimate recombination. Construction of gene knockout mutants can be achieved using plasmid-based systems and by phage-mediated specialized transduction, but the simple introduction of a linear double-stranded DNA (dsDNA) substrate by electroporation leads to a high proportion of ectopic integration events regardless of homology between the targeting substrate and the bacterial chromosome[1, 2]. Other types of mutagenesis such as the construction of defined isogenic strains with single point mutations are further complicated by the lack of generalized transducing phages that infect *M. tuberculosis*[3].

A variety of techniques for constructing gene knockout or replacement mutants have been described which are designed to overcome the relatively high level of illegitimate recombination compared to homologous recombination observed with linear dsDNA allelic exchange substrates (AESs). These include the use of long linear dsDNA substrates[4], as well as various plasmid and cosmid-based strategies[4–8], and these are often coupled with the use of counter-selectable genetic markers such as *sacB*[9, 10]. Specialized transducing phages based on conditionally-replicating shuttle phasmids represent an alternative and highly effective approach to mutagenesis[11], including the construction of isogenic *M. tuberculosis* strains differing by a single known point mutation[12]. While most of these methods are effective, they are constrained by the requirements for complex genetic constructions and/or multiple steps of manipulation and screening. Given the extremely slow growth rate of *M. tuberculosis* (doubling time of 24 hours), alternative methods that simplify its genetic manipulation are highly desirable.

Recombineering – genetic engineering using recombination proteins[13] – is a powerful system for mutagenesis in *Escherichia coli* (as well as *Salmonella* and *Shigella*[14, 15]) in

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which recombination systems encoded by the lambda Red system or the RecET genes of the Rac prophage massively enhance the frequencies of homologous recombination. The high recombination efficiencies can be exploited in a variety of ways including the construction of chromosomal gene knockouts, point mutations, deletions, small insertions, *in vivo* cloning, and mutagenesis of bacterial artificial chromosomes and genomic libraries[13, 16–19]. Only relatively short segments (50 bp) of DNA homology are required, and mutagenic substrates constructed by PCR can be readily introduced into *E. coli* strains expressing the phage recombination proteins by electroporation.

Recombineering in *E. coli* using the λ Red system involves three phage-encoded proteins, Exo, Beta, and Gam, whereas the Rac prophage encodes just RecE and RecT, which are functionally equivalent to λ Exo and Beta, respectively[20, 21]. Exo and RecE are 5′→3′ exonucleases that degrade a single strand of a linear dsDNA substrate[22, 23], exposing a 3′ single-stranded DNA (ssDNA) tail to which Beta or RecT can bind[24, 25]. Recombination is then mediated by association of these complexes with resident chromosomal or plasmid targets through pairing of complementary sequences, strand exchange, or strand invasion[26–29]. λ Beta and RecT proteins are members of a large family of single-stranded DNA annealing proteins (SSAPs)[30]; two other families of SSAPs that function similarly are defined by the P22 Arf protein and the eukaryotic protein, Rad52. While the RecET and λ Red systems confer similar levels of recombination in *E. coli*, the Beta and RecT proteins have a strong preference for their cognate Exo and RecE proteins, and it is likely that they function as protein complexes[31, 32]. While *E. coli* dsDNA recombineering requires both an exonuclease and its associated SSAP, recombination using ssDNA substrates requires only the SSAP. The ability to recombineer with short oligonucleotide-derived ssDNA substrates is especially useful[19], and frequencies can be sufficiently high to enable identification of mutants without the need for direct genetic selection, especially when using strains defective in mismatch repair[34]. The third protein in the λ system, Gam, increases recombineering frequencies by binding to the RecB subunit of RecBCD and inhibiting degradation of dsDNA substrates [35, 3618, 19]. While other Gam functional analogues have been characterized, such as Abc2 of bacteriophage P22[37], these proteins are more rare compared to the large superfamilies of SSAPs and their associated exonucleases[30].

Mycobacteriophages have played key roles in circumventing the challenges of genetics in *M. tuberculosis*, including the development of shuttle phasmids[38] for transposon mutagenesis[39], reporter gene delivery[40, 41], and specialized transduction[11], as well as integration-proficient vectors for stable introduction of foreign genes[42–45]. Exploitation of these phages is simplified by the availability of more than 50 completely sequenced mycobacteriophage genomes[44–47], and the amazingly high degree of genetic diversity provides at least 1,500 phamilies of unique genes. Because the *E. coli* based systems appear to function less well in distantly related bacteria, we turned to this collection of mycobacteriophage genomes to identify mycobacteriophage-encoded proteins that could be developed for mycobacterial recombineering. Although RecET homologues are rare among mycobacteriophages, Che9c encodes both proteins, and we have exploited them to develop recombineering systems for both fast-and slow-growing mycobacteria[48, 49]. This approach also provides a powerful strategy for constructing mutant derivatives of lytically-replicating mycobacteriophage genomes, including in-frame deletions, point mutations and small insertions.

Identification of mycobacteriophage recombineering functions

Mycobacteriophages are genetically diverse, possess architecturally mosaic genomes[44–47], and are replete in predicted open reading frames of unknown function that have no detectable similarity to known proteins. While constructing shuttle phasmid derivatives of phage TM4, Jacobs and colleagues[38] noted the high prevalence of recombinants following

electroporation of phage libraries into *M. smegmatis*, suggesting the presence of a phage-encoded recombination system. However, bioinformatic analysis of the TM4 genomic sequence[50] provides no clues as to which genes might encode recombination functions.

A search of the predicted open reading frames of all completely sequenced mycobacteriophage genomes reveals a small number of homologues of the *E. coli* RecET proteins but none related to the λ Red proteins. Che9c encodes for homologues of both RecE and RecT, the products of genes *60* and *61* respectively (Fig. 1a)[47, 48]; both are rather distant relatives and share less than 30% amino acid identity. Che9c gp60 (314 aa) is also much smaller than *E. coli* RecE (866 aa) and corresponds to just the C-terminal part encompassing the RecB-family nuclease domain (Fig. 1a); the N-terminal region of RecE that is absent in Che9c gp60 is not necessary for RecE exonuclease activity[51]. Che9c gp61 is clearly a member of the superfamily of SSAPs[30] with 29% amino acid identity to *E. coli* RecT, but with a longer C-terminal extension (Fig. 1a). Although Che9c gp60 and gp61 are distant relatives of the RecET and λ Exo/Beta proteins, they possess similar biochemical functions[48]. Che9c gp60 has exonuclease activity *in vitro* that is dependent on the presence of dsDNA ends, and Che9c gp61 binds short (20 nucleotides) ssDNA as well as dsDNA substrates in the absence of Mg^{2+}. It was also observed by electron microscopy to form toroidal multimers in the presence of ssDNA (Fig. 1b), a property exhibited by other SSAPs[52, 53].

Two other mycobacteriophages, Halo and Giles, encode similar recombination systems. Halo gp42 is 46% identical to Che9c gp60 and 30% identical to RecE, while Giles gp52 contains a domain from the YqaJ family of phage-encoded exonucleases (Fig. 1a). However, while Halo gp43 and Giles gp53 are 30% identical, they are even more distantly related to other phage-encoded RecT homologues than Che9c gp61 (Fig. 1a). Interestingly, a prophage in the sequenced genome of *M. avium* strain 104 also encodes RecE/RecT-like proteins (MAV_0830 and MAV_0829, respectively) that are similar to Che9c gp60 and gp61 (41% and 29% identical) and also related to the Rac prophage proteins RecE and RecT (23% and 40% identity). Thus while the mycobacteriophage putative SSAP proteins are in each case associated with a gene encoding an exonuclease, these systems exhibit the same modularity seen broadly in other phage genomes[54]. None of these mycobacteriophage systems encode homologues of the λ Gam protein, and it is unknown if there are any mycobacteriophage functional analogues that are RecBCD-inhibitors similar to the Abc2 protein of phage P22.

Construction of recombineering strains of mycobacteria

Mycobacterial strains have been constructed for recombineering with both dsDNA and ssDNA substrates. These contain an extrachromosomal plasmid in which the phage recombination genes are under the control of an inducible acetamidase promoter[55], and the most widely used configurations use the Che9c genes *60* and *61* for dsDNA recombineering[48] or just Che9c *61* for ssDNA manipulations[49]. Optimal levels of recombination are obtained using plasmid derivatives expressing Che9c genes *60* and *61* from their endogenous translational signals (e.g. pJV53), though it is noteworthy that while some alternative constructions may give higher levels of expression, this does not necessarily result in higher levels of recombineering[48]. This has also been observed for the λ Red system where recombination activity correlates poorly with expression levels[31].

Plasmid pJV53 serves as a basic recombineering plasmid for regulated expression of gp60 and gp61 (Fig. 2), although other derivatives of this plasmid have been made, including those with different selectable markers or containing a *sacB* cassette to simplify removal of the plasmid by counter-selection following mutagenesis (plasmid pJV48). The acetamidase promoter is somewhat leaky, and there is a detectable level of Che9c gp61 expression in *M. tuberculosis* in some uninduced strains, although recombination frequencies are low without

induction[48]. High expression levels of these proteins are toxic to *M. smegmatis*, and plasmids in which the constitutive *Mycobacterium bovis* BCG *hsp60* promoter is linked to Che9c *60* and *61* do not transform mycobacteria. This toxicity appears to derive largely from the Che9c gp60 exonuclease since plasmids expressing only gp61 constitutively are tolerated, although they grow slowly. For ssDNA recombineering, plasmids such as pJV62 (Fig. 2) have been constructed that express gp61 under its own translation signals and the acetamidase promoter. In preparation for recombineering experiments, *M. smegmatis or M. tuberculosis* plasmid-containing strains in mid-logarithmic growth are induced with acetamide and harvested for electroporation[48, 49].

Using dsDNA recombineering to construct mycobacterial gene knockout mutants

Targeted gene replacement mutants can be readily constructed by electroporation of linear dsDNA substrates into either *M. smegmatis* or *M. tuberculosis* recombineering strains (Fig. 2). The AESs typically contain regions of homology upstream and downstream of the target gene flanking a cassette for antibiotic resistance, such as hygromycin-resistance (Hyg^R). Although SSAP recombinases such as gp61 can bind short regions of ssDNA, the frequency of recombineering is considerably lower with 50 bp than with 500 bp of homology, and with shorter lengths the DNA uptake frequency becomes limiting; we therefore recommend using a minimum of 500 bp of homology[48]. A simple protocol entails transformation of a linearized AES (100 ng) into electrocompetent mycobacterial cells containing pJV53 and induced for gp60/gp61 expression[4856]. This typically yields 50–200 drug-resistant colonies of which >90% contain correctly targeted gene replacements; control transformations using 50 ng plasmid DNA with the same competent cells typically yields about 10^5 colonies. Similar frequencies are observed when targeting extrachromosomal plasmids, emphasizing the need to ensure that DNA with potential similarity to the pJV53 plasmid backbone (such as the *E. coli* origin of replication) is removed from the AES substrate. AESs can also be designed for subsequent unmarking of mutants, such as including γδ *res* sites flanking the antibiotic resistance cassette and a *sacB* cassette for counter selection[11].

The dsDNA recombineering frequencies appear to be primarily limited by the relatively poor mycobacterial DNA uptake efficiencies rather than poor protein expression or degradation by host nuclease systems. With either *M. tuberculosis*[57] or a high efficiency transformation *M. smegmatis* strain[58] competencies as high as $1–5 \times 10^6$ transformants/μg plasmid DNA can be achieved[48, 59], but only 1 in 1000 of all viable cells productively takes up DNA. Inactivation of the *M. smegmatis recBCD* system only modestly increases dsDNA recombineering frequencies, and expression of λ Gam does not influence the frequencies in any strain background that we have tested (our unpublished observations); mycobacterial recombineering is also RecA-independent as expected[49]. In practice, the recombination and DNA uptake frequencies with the Che9c system yield sufficient recombinants for constructing targeted gene replacement mutants in both *M. smegmatis* and *M. tuberculosis*.

Using ssDNA recombination to construct point mutations in mycobacteria

Recombineering using ssDNA substrates offers a simple method for constructing point mutations in mycobacterial genomes[49]. The overall efficiency of ssDNA recombineering is substantially higher than with dsDNA substrates for both chromosomal and plasmid targets. Moreover, the exonuclease function (Che9c gp60) is not required for ssDNA substrates, and strains containing plasmid such as pJV62 (Fig. 2) are suitable[49].

In ssDNA recombineering, a choice needs to be made as to which DNA strand to target. In mycobacterial recombineering this is a critical issue, since ssDNA substrates targeting the same sequence but on different strands can differ in recombineering efficiencies up to 10,000-fold[49]; this far exceeds the 2–50 fold strand biases observed in the λ Red

system[19, 31]. Recombination frequencies are always greater using a ssDNA substrate that anneals to the template for discontinuous DNA synthesis (lagging strand), presumably due to greater availability of ssDNA chromosomal DNA for pairing with gp61-ssDNA substrate complexes. It is not clear why the biases are so much larger in mycobacteria than in *E. coli*, but it may reflect fundamental differences in the DNA replication systems or in how gp61 interacts with the replication machinery. Substrates as short as 48 nt provide optimal recombination frequencies, and this recombination is also independent of host RecA functions[49].

A simple experiment to test for ssDNA recombineering is using an oligonucleotide substrate to introduce a chromosomal point mutation conferring drug resistance. In a typical experiment using an oligonucleotide targeting the lagging strand, 100 ng ssDNA yields a similar number of drug resistant colonies as obtained using 100 ng extrachromosomal plasmid DNA (~10^5). Thus, in contrast to dsDNA recombineering, a high proportion of cells taking up ssDNA undergo recombination. The question then arises as to whether the overall frequency is high enough to construct point mutations that cannot be directly selected. The challenge with doing this arises not with the recombination frequencies *per se*, but because 99.9% of cell do not take up DNA.

This problem can be conveniently circumvented using a co-selection strategy, in which a plasmid is co-transformed with the ssDNA and plasmid transformants selected; this effectively counter selects against non-competent cells in the population. The transformants can then be examined by PCR, and we find the mismatch amplification mutation assay PCR (MAMA-PCR)[60, 61] that selectively amplifies the mutant allele to be especially effective. In our experience, 5–10% of the plasmid transformants also contain the newly introduced point mutation. A similar outcome can be achieved using a double-oligonucleotide configuration, in which two ssDNA substrates are used in the same electroporation; one introduces the desired point mutation in the chromosome, while the other repairs a mutant Hyg^R gene present on the recombineering plasmid. The proportion of Hyg^R colonies containing the point mutation is somewhat lower (~3%), but can still be readily identified by MAMA-PCR; the advantage is that only a single plasmid needs to be removed subsequently from strains for further analyses. A plasmid (pJV128) has been constructed containing Che9c gp61 fused to the acetamidase promoter, a Kan^R selectable marker, a mutant Hyg^R gene and a *sacB* counter-selectable gene for this purpose[49]. This co-selection strategy can also be used with dsDNA substrates, and we have successfully constructed an unmarked deletion of the *M. smegmatis leuD* gene using a 200 bp dsDNA substrate with 100 bp *leuD* homology on each side of the deletion (Fig. 3a).

An intriguing use of the ssDNA recombineering technology is the study of mutations that confer antibiotic resistance. This is illustrated by introduction of several well characterized point mutations conferring resistance to isoniazid, rifampicin, streptomycin, and ofloxacin in *M. smegmatis*, as well as rifampicin and streptomycin in *M. tuberculosis*[49]. Recombineering with ssDNA thus offers a simple approach to determine whether any point mutation identified in a clinical strain of *M. tuberculosis* contributes to its drug susceptibility profile.

Identification of other phage recombinases

Although homologues of known recombinases are rare in mycobacteriophages, the Halo gp43 and Giles gp53 proteins are likely candidates for recombination activity (Fig. 1a). Introduction of drug-resistant point mutations by ssDNA recombineering provides a simple assay for testing these as well as SSAPs from non-mycobacteriophage sources including λ Beta and RecT (Fig. 3b). Halo gp43 is somewhat less active than Che9c gp61, and Giles gp53 even less so, further illustrating the utility of a large reservoir of phage genomes to optimize potential genetic tools[49]. Anecdotal reports that the λ Red system functions poorly

in mycobacteria are confirmed by the low activity of λ Beta, although RecT works remarkably well in *M. smegmatis* (Fig. 3b), while still reduced in activity compared to Che9c gp61. Related studies report that Che9c gp61 performs relatively poorly in *E. coli* ssDNA recombineering assays[62]. The molecular basis for differing activities of SSAPs is not clear, although an intriguing possibility is that they act by interacting directly with the host DNA replication machinery.

Construction of mycobacteriophage mutants by recombineering

Constructing mutant derivatives of bacteriophages is often more difficult than manipulating the host chromosome, mostly because drug selection is not useful in lytically propagated viruses. The most powerful current method of manipulating mycobacteriophages is through the construction of shuttle phasmids[63], and while these have many applications, they are of only limited use for determining phage gene functions. In one example, TM4 shuttle phasmids were used to make mutations in the TM4 tape measure gene, performing the mutagenesis in *E. coli* (by λ Red recombineering) and recovering the mutant phage in *M. smegmatis*[64]. However, most mycobacteriophage genomes are too large for shuttle phasmid construction, and this approach is not broadly applicable for functional genomic studies on mycobacteriophages. A homologous recombination approach was used to construct a firefly luciferase recombinant of mycobacteriophage L5[65], although the frequency of host-mediated recombination is sufficiently low that construction is inefficient and time-consuming. Mycobacterial recombineering can be employed for manipulating prophages, but since relatively few mycobacteriophages form stable lysogens, this is also not generally applicable.

The mycobacterial recombineering systems described above offer potentially powerful approaches to constructing mutants of any lytically growing mycobacteriophage. Recombineering of phage genomes has been described for *E. coli* phages such as lambda, using a strategy in which bacterial cells are infected with phage, competent cells are prepared, dsDNA or ssDNA substrate is introduced by electroporation and plaques analyzed for the presence of the mutation[66]. Since the frequency of DNA uptake in mycobacteria is substantially lower than the efficiency of phage infection, we have adopted an alternative approach based on the co-selection methods described above. While still under development, initial experiments suggest that this is a highly effective approach for mycobacteriophage functional genomics (LJM, JCV and GFH, manuscript in preparation).

The scheme we have developed for mycobacterial mutagenesis is shown in Figure 4. An *M. smegmatis* strain carrying a recombineering plasmid such as pJV53 or pJV62 (Fig, 2) is induced with acetamide and electrocompetent cells prepared. Electroporation is performed with two DNAs; phage genomic DNA of the virus to be manipulated and a PCR-generated or synthetic DNA substrate. Plaques are recovered in an infectious center assay (i.e. prior to lysis) such that cells that have taken up phage DNA give rise to plaques on a lawn of *M. smegmatis* plating cells; ~100 plaques are obtained from 50–100 ng of phage DNA in such an experiment. All plaques contain wild-type phage DNA, but 10–40% also contain the mutant allele and can be easily identified by PCR screening of 12–18 individual plaques. Provided that the deleted region is non-essential for phage growth, then purified mutant phage derivatives can be isolated by plating serial dilutions of the mixed plaques and PCR analysis of the purified plaques (Fig. 4). Typically, analysis of 12–18 isolated plaques from this plaque-purification is sufficient to identify a homogenously mutant strain.

We have used phage recombineering to manipulate several different mycobacteriophage genomes and to generate gene knockouts, in-frame internal deletions, point mutations, and small insertions. Thus far, we find that when constructing deletion mutants a higher proportion of primary plaques contain the desired mutant allele when using dsDNA

substrates than when using ssDNA substrates. Larger dsDNA substrates (200 bp) also appear to work better than shorter ones (100 bp) as with mycobacterial chromosomal recombineering[48]. We have also adapted this approach to determine whether mycobacteriophage genes are required for lytic growth. In one experiment, we used a 200 bp dsDNA substrate to generate a deletion in the lysin A gene of mycobacteriophage Giles that seemed likely to be an essential function. PCR analysis showed that approximately 30% of the initial plaques recovered contained mutants, but when lysates were prepared from re-plating of these mixed plaques, the mutant allele could not be identified even using a sensitive MAMA-PCR approach (see above). We conclude that the gene is essential for Giles growth, and that the mutation could be constructed and the mutant propagated in the presence of wild-type helper phage in the primary plaque, even though single particles do not give rise to plaques in the secondary plating. The full utility of these approaches for mycobacteriophage mutagenesis has perhaps yet to be realized, but the ability to modify mycobacteriophage genomes using mycobacterial recombineering will, for the first time, allow us to use broad functional genomic studies on mycobacteriophages.

Further development of mycobacterial recombineering systems

The ability to stimulate the levels of homologous recombination in both slow- and fast-growing mycobacteria by expression of mycobacteriophage-encoded recombination systems has the potential to be exploited in a variety of ways. First, it seems likely that the same approaches will work for manipulation of a variety of mycobacterial species other than those in *M. smegmatis* and *M. tuberculosis* described here, and possibly in other bacterial species that are closely related to the mycobacteria. Secondly, the relatively high efficiencies of chromosomal recombineering could facilitate construction of ordered gene knockout or replacement mutants, especially since identification of mutants by PCR is compatible with genome-wide robotic strategies. Lastly, the coupling of efficient point mutagenesis with mycobacterial nonsense suppressor strains[67] offers the prospects of a relatively simple method for making conditional lethal mutants in mycobacterial genomes and their phages.

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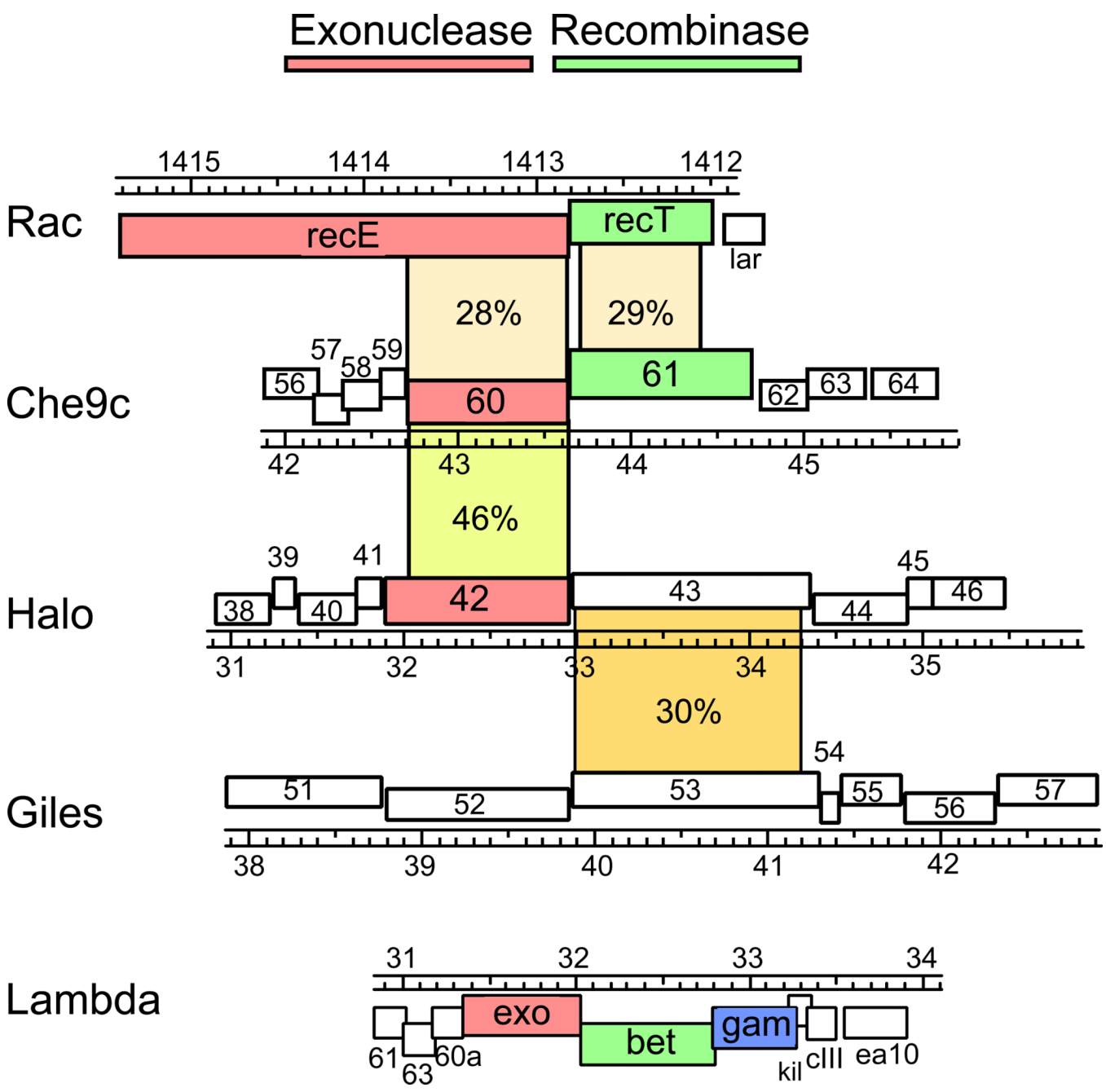

Figure 1. Mycobacteriophage-encoded recombination proteins

A. Mycobacteriophages Che9c gp60 and gp61 are *E. coli* Rac prophage RecE and RecT homologues, respectively. Mycobacteriophage Halo encodes a RecE homologue that is 46% identical to Che9c gp60, while Giles gp52 belongs to the YqaJ family of exonucleases. Halo gp43 is similar to Giles gp53, but these are only distantly related to other phage RecT-like proteins. The λ Red recombination proteins are not closely related to the mycobacteriophage proteins or RecET.

B. Electron micrograph of Che9c gp61 protein multimers in the presence of ssDNA. Reactions containing gp61 protein (1.2 μM) incubated with ssDNA (100 nt; 2 μM) were absorbed to glow discharged 400 mesh formvar carbon coated copper grids, stained with 2%

uranyl acetate, and examined by transmission electron microscopy. Images were collected at a magnification of 140,000×; four examples of toroid structures are shown alongside a size bar.

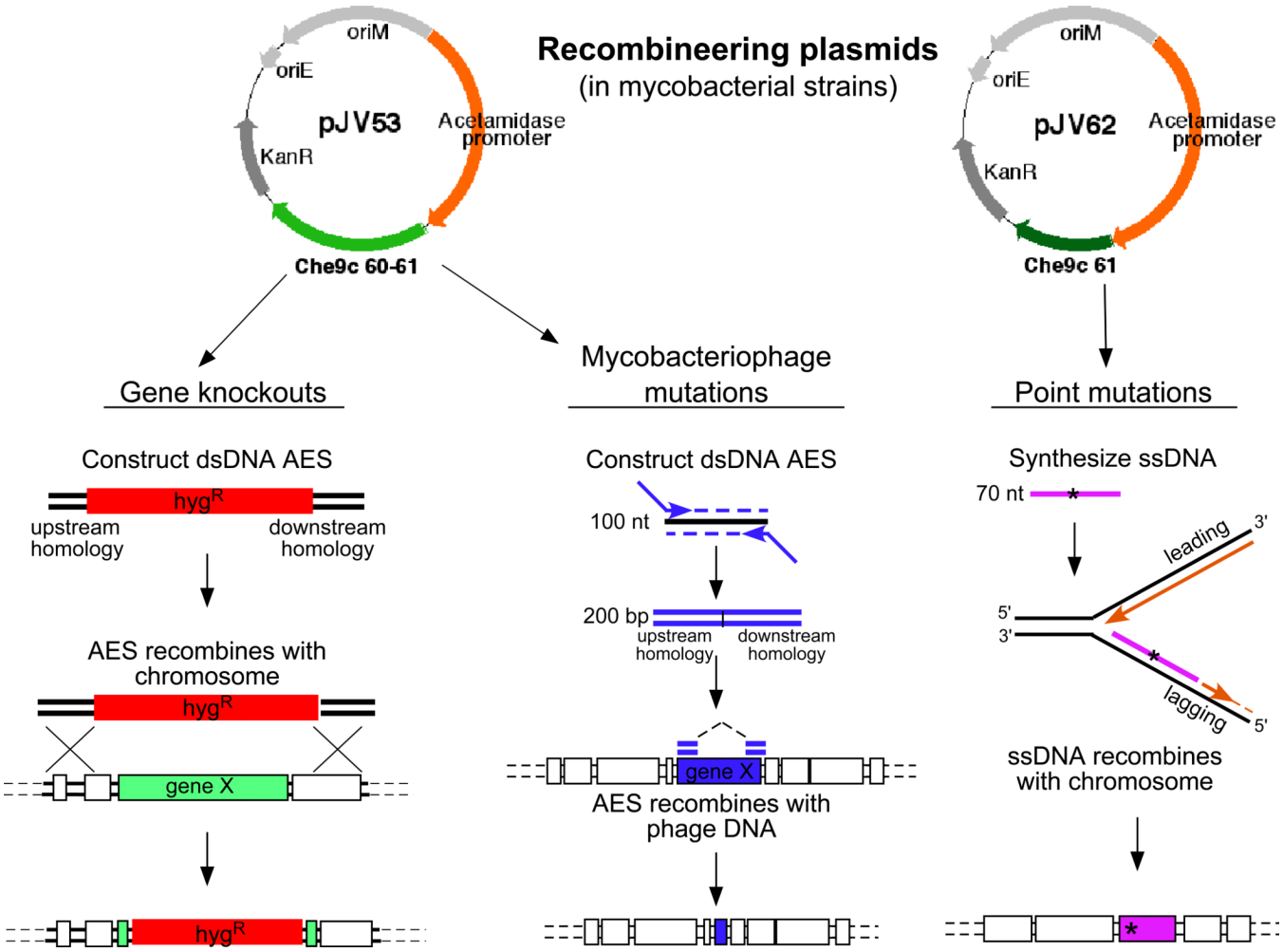

Figure 2. Strategies for mycobacterial recombineering

Plasmids pJV53 and pJV62 express Che9c gp60 and gp61 or just gp61 to facilitate dsDNA and ssDNA recombination respectively. *Left*: gene knockouts are made by targeted gene replacement with a linear dsDNA allelic exchange substrate (AES) that contains 500 bp of homology to the target locus flanking an antibiotic resistance gene (e.g. hygromycin; *hyg*^{R}). *Middle*: Mycobacteriophage mutants (either point mutants or deletion mutants) are made by synthesizing a 200 bp dsDNA AES by PCR using a 100 nt template and 75 nt extender primers. *Right*: point mutants constructed by synthesis of a 50–70 nt ssDNA substrate, centering the desired point mutation that will anneal to the lagging strand for DNA replication to undergo recombination.

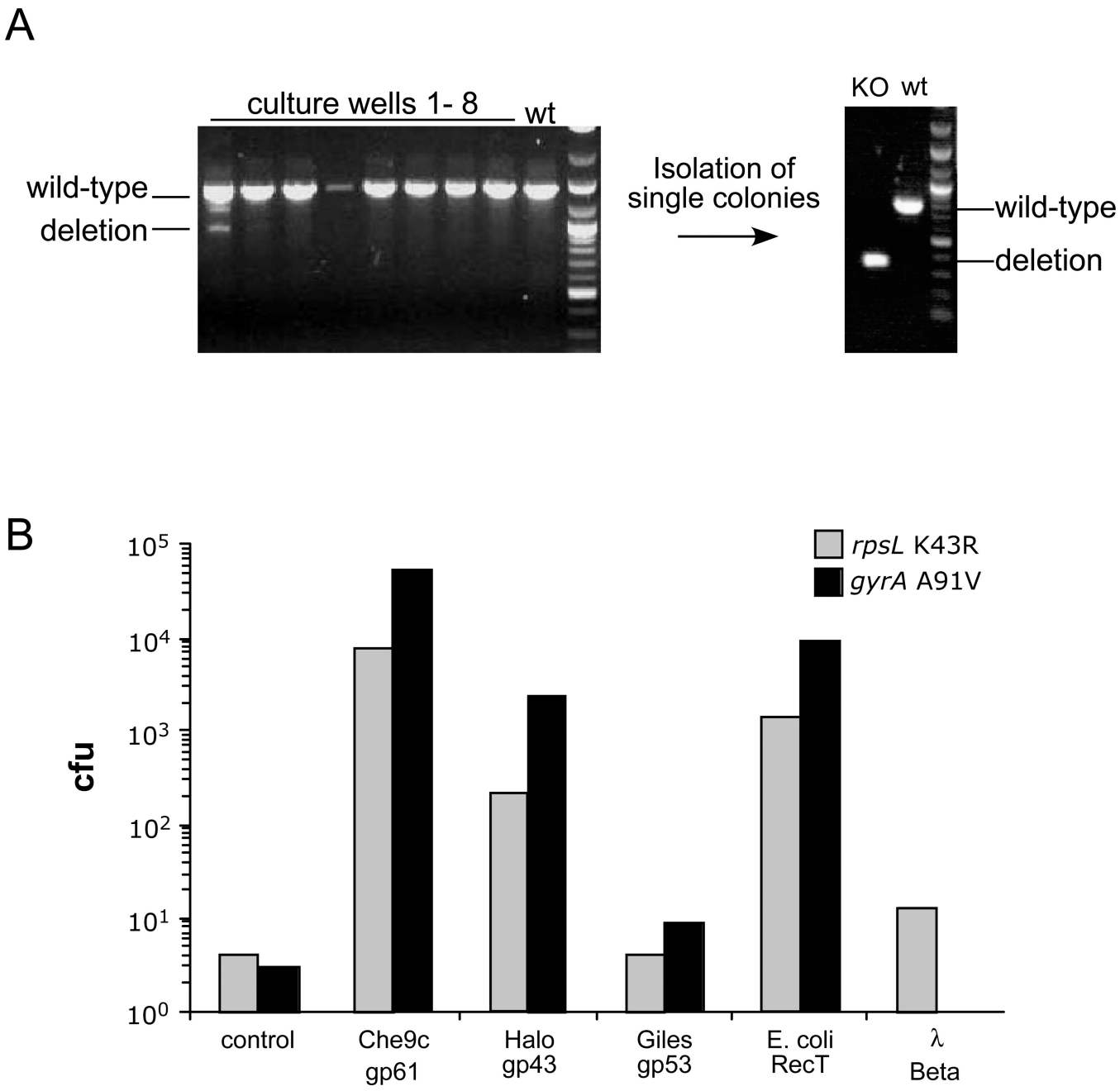

Figure 3. Mutagenesis of mycobacterial genomes

A. Constructing an unmarked deletion of the *M. smegmatis leuD* gene using recombineering. Induced electrocompetent *M. smegmatis*/pJV76amber cells (pJV76amber is a derivative of pJV53 containing a mutant Hyg^R gene) were co-electroporated with a 200 bp *leuD* deletion substrate along with an oligonucleotide that repairs the mutant plasmid Hyg^R gene. After dilution to approximately 10 cells per sample, hygromycin selection and growth, at least one sample (#1) contained the mutant allele, and a leu^- strain was readily recovered and verified by PCR.

B. Recombinases (SSAPs) were assayed for ssDNA recombineering activity in *M. smegmatis* by their ability to introduce streptomycin-resistant (*rpsL* K43R) and ofloxacin-resistant (*gyrA* A91V) mutations. SSAP genes are expressed from the acetamidase promoter

and translationally-fused to the pLAM12 vector[48] rather than using their native translation initiation signals[49]. The number of drug resistant colony forming units (cfu) obtained for each drug target are shown.

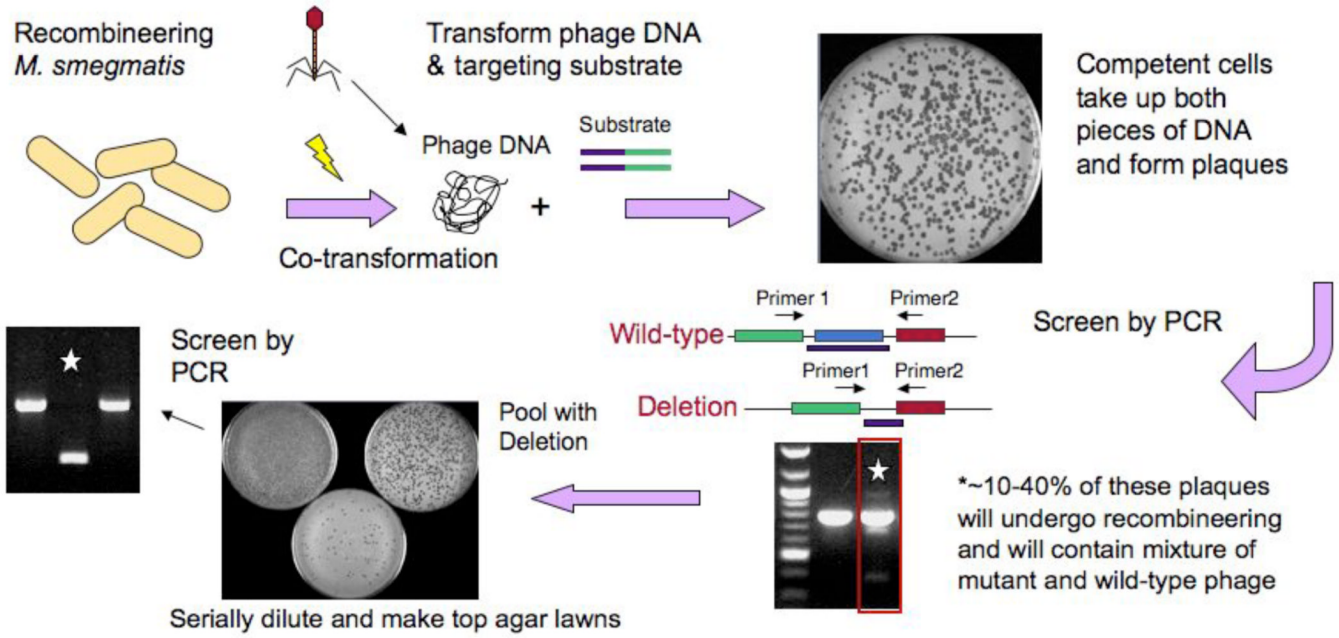

Figure 4. Mycobacteriophage recombineering

Schematic illustration of the strategy utilized to adapt mycobacterial recombineering for use on mycobacteriophages. Induced electrocompetent *M. smegmatis*/pJV53 cells are co-electroporated with phage DNA (50–100 ng) and a 200bp dsDNA recombineering substrate (50–500 ng), mixed with *M. smegmatis* plating cells, and plated for individual plaques. Approximately 10–40% of plaques are mixed, containing both the mutant and wild-type alleles, and are distinguishable by PCR. A mixed plaque can be diluted and plated for analysis of individual plaques or for preparation of a lysate. If a non-essential gene is deleted then a mutant phage derivative can be readily identified by PCR, but if the gene is essential, then the mutant allele is no longer present in the lysate.