

# Site-Directed Mutagenesis Using Oligonucleotide-Based Recombineering

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## 1. Introduction

Methods enabling mutational analysis of distinct chromosomal locations, like site-directed mutagenesis, insertion of foreign sequences or in-frame deletions, have become of fast growing interest since complete bacterial genome sequences became available. Various approaches have been described to modify any nucleotide(s) in almost any manner. Some genetic engineering technologies do not rely on the *in vitro* reactions carried out by restriction enzymes and DNA ligases (Sawitzke et al., 2001). Complicated genetic constructs that seem to be impossible to generate *in vitro* can be created within one week using *in vivo* technologies (Sawitzke et al., 2001).

Over several decades, researchers developed and refined various strategies for genetic engineering that make use of the homologous recombination system. Its natural main functions are restoring collapsed replication forks, repairing damage-induced double-strand breaks and maintaining the integrity of the chromosome (Poteete, 2001).

We want to focus on a technique for recombination-mediated genetic engineering ("recombineering", Copeland et al., 2001). Recombineering requires only minimal *in vitro* effort. It has been applied to *Escherichia coli*, *Salmonella*, and a range of other Gram-negative bacteria, as well as to bacteriophages, cosmids and bacterial artificial chromosomes (BACs). It was demonstrated that single-stranded DNA (ssDNA) oligonucleotides can be used as substrates for recombineering in *E. coli* (Ellis et al., 2001, Heermann et al., 2008) and BACs (Swaminathan et al., 2001). However, most commonly linear, double-stranded DNA (dsDNA) has been used as the targeting construct (Maresca et al., 2010), e.g., for chromosomal gene replacement (Murphy, 1998), whole gene disruption (Datsenko et al., 2000) or the development of novel cloning strategies, including subcloning of BAC DNA (Lee et al., 2001).

In the early 1990s, the DNA double-strand break and repair recombination pathway proved to be very efficient for recombining incoming linear DNA with homologous DNA in the yeast *Saccharomyces cerevisiae* (Baudin et al., 1993). For generating *null* alleles of a distinct gene, a PCR-amplified *HIS3* selectable marker flanked by homologous sequences to the ORF (ranging from 35 to 51 nucleotides in length) was used to transform a recipient strain lacking the complete *HIS3* gene or a strain containing the *His3* $\Delta$ 200 allele. Due to the auxotrophic selection marker, transformants bearing the expected mutation were among the *His*<sup>+</sup> clones, with up to 80% efficiency (Baudin et al., 1993).

In contrast to *S. cerevisiae*, *E. coli* fails to be readily transformable with linear DNA fragments because of rapid DNA degradation by the intracellular RecBCD exonuclease activity (Lorenz et al., 1994). Mutants defective in the RecBCD nuclease exhibit no degradation of linear DNA in *E. coli*, but unfortunately these strains are also deficient for any recombination events. This recombination defect can be partially rescued in strains with *recA*<sup>+</sup> background (Jasin & Schimmel, 1984). Other mutants defective in *recBC* (or either *recB* or *recC*) carrying an additional suppressor mutation, *sbc* (suppressor of *recB* and *recC*), possess activation of the RecET recombination pathway (*sbcA*) or enhanced recombination by the RecF pathway (*sbcB*). *recE* and *recT* are found on the defective lambdoid *E. coli* prophage Rac and encode an exonuclease and a ssDNA-binding/strand-exchange protein, respectively (Fouts et al., 1983, Poteete, 2001). Expression of *recET* is induced by few *cis*-acting point mutations, e. g. *sbcA6* (Clark et al., 1994).

One highly applicable RecET-mediated recombination reaction, termed 'ET-cloning', combines a homologous recombination reaction between linear DNA fragments and circular target molecules, like BAC episomes (Zhang et al., 1998). After co-transformation of linear and circular DNA molecules, only *recBC sbcA* recipient strains resulted in the intended recombination products (insertion or deletion). Recombination was more efficient with increasing length of the homology arms, and some constructs showed higher efficiency with increasing distance between the two homology sites (Zhang et al., 1998). For ET-cloning in *recBC*<sup>+</sup> strains, which are commonly used as hosts for P1, BAC or PAC episomes, a plasmid encoding the recombination functions was constructed. In pBAD-ET $\gamma$ , *recE* is under the control of an inducible promoter; and *recT* is expressed from a strong constitutive promoter. To inhibit RecBC-mediated degradation of linear DNA, the  $\lambda$  *gam*, encoding the Red $\gamma$  protein, was incorporated on the plasmid (Zhang et al., 1998). Later, *E. coli* hosts with chromosomally-encoded, inducible recombinases have been developed to allow easy manipulation of BAC DNA (Lee et al., 2001).

### 1.1 The bacteriophage $\lambda$ Red recombination system

Besides the mutagenesis pathway described above, Red recombination is one of the most commonly exploited techniques to foster recombination between the bacterial chromosome and linear dsDNA introduced into the cell (Murphy, 1998). The Red recombination system of the bacteriophage  $\lambda$  leads to a precise and rapid approach with greatly enhanced rates of recombination, compared to those found in *recBC*, *sbcB* or *recD* mutants. Its ability to catalyze the incorporation of PCR-generated DNA species led to an immense spread of the system. Numerous groups have developed various methodologies tailored to a variety of scientific questions. Besides the high recombination rate, the biggest advantage of the  $\lambda$  Red system, compared to previously used recombination systems, is that it accepts very short regions of homologous DNA (stretches of less than 100 nucleotides) for recombination. Because fragments of such size can be readily synthesized, there is a high degree of freedom in designing targeting constructs for recombination.

Which components make up the  $\lambda$  Red system? The genes of the Red system, *exo*, *bet*, and *gam*, cluster together in the P<sub>L</sub> operon, which is expressed in the early transcriptional program of bacteriophage  $\lambda$  (Poteete, 2001). The three resulting  $\lambda$  Red gene products, Red $\alpha$ , Red $\beta$  and Red $\gamma$ , are necessary to carry out homologous recombination of dsDNA. Red $\alpha$ , whose monomer has a Mr of 24 kDa, is responsible for a dsDNA-dependent exonuclease

activity. It degrades one strand at the ends of a linear dsDNA molecule in the 5' to 3' direction. This generates 3' ssDNA overhangs, which are substrates for recombination (Little, 1967). The ring-shaped trimer of Red $\alpha$  passes dsDNA through its center at one end, but only ssDNA emerges from the other end (Kovall et al., 1997). The Red $\beta$  protein, whose monomer has a Mr of 28 kDa, acts as a ssDNA-binding protein that anneals complementary single strands and mediates strand exchange, thus finishing the recombination process (Li et al., 1998). When bound to ssDNA, Red $\beta$  forms large rings and makes a complex with Red $\alpha$  to promote recombination (Passy et al., 1999). Therefore, the Red $\alpha$ /Red $\beta$  pair has a function analogous to that of the RecE/RecT pair. Muyrers et al. (2000b) could highlight that there are specific interactions between the partners of each recombination system. The exonuclease RecE does not form complexes with the strand exchange protein Red $\beta$  or vice versa; Red $\alpha$  does not interact with RecT. Finally, the Red $\gamma$  polypeptide, whose Mr is 16 kDa, protects linear dsDNA from degradation by binding the RecBCD protein and inhibiting its nuclease activities (Murphy, 1991).

## 1.2 Use of $\lambda$ Red recombination for manipulation of bacterial genomes

The basic strategy of the  $\lambda$  Red system is the replacement of a chromosomal sequence with a (e.g., PCR-amplified) selectable antibiotic resistance gene flanked by homology extensions of distinct lengths. For genetic engineering in the *E. coli* chromosome, two efficient  $\lambda$  Red-mediated methods were developed by two independent groups. The first method utilized *E. coli* strains containing the P<sub>L</sub> operon of a defective  $\lambda$  prophage (e.g., deletion of *cro* to *bioA* genes, which includes the lytic genes) under control of the temperature-sensitive  $\lambda$  cI-repressor (allele *cI857*, Yu et al., 2000). To express high levels of the P<sub>L</sub> operon, cultures were shifted from repressing conditions at 32°C to inducing conditions at 42°C for 7.5 to 17.5 minutes. This was optimal for achieving maximal recombination levels. By shifting the cells back to 32°C, further expression of the P<sub>L</sub> operon was repressed; and cell death was prevented. Furthermore, the absence of the Cro-repressor enabled P<sub>L</sub> operon expression to be fully derepressed when the cI-repressor was inactivated under heat induction (Yu et al., 2000). Electroporation of this transiently induced  $\lambda$  lysogen with a short linear, PCR-generated dsDNA segment resulted in efficient recombination events (Poteete, 2001).

The second very efficient  $\lambda$  Red-mediated recombination approach, involved a low-copy plasmid with  $\lambda$  *gam*, *bet*, and *exo* under control of an arabinose-inducible promoter (pKD46, Datsenko et al., 2000). The plasmid pKD46 yielded greatly enhanced recombination events and was preferable to the similar pKD20. However, both harbor the Red system under a well-regulated promoter to avoid undesired reactions under non-inducing conditions and a temperature-sensitive replicon to allow for easy curing of the respective plasmid after recombination (Datsenko et al., 2000). For generating gene disruptions within the *E. coli* chromosome, the R6K $\gamma$  *ori*-based suicide plasmids pKD3 and pKD4 were used as templates to amplify the chloramphenicol (*cat*) or kanamycin (*aph*) resistance gene cassettes, respectively, in a PCR. Using primer pairs with site-specific homology extensions to amplify the resistance cassette, the resulting PCR product was used to transform a freshly competent *E. coli* expressing  $\lambda$  Red from pKD46. After successful recombination, the resistance gene cassette can be excised by F<sub>1</sub>p recombinase supplied *in trans* (1.2.3, Fig. 1).

One example of a possible refinement of the  $\lambda$  Red procedure promotes high-frequency recombination using ssDNA substrates. It has been discovered that only  $\lambda$  Red $\beta$  is

absolutely required for ssDNA recombination (Ellis et al., 2001). Neither *exo* nor *gam* causes a dramatic effect on the recombination efficiency. Only a minor 5-fold reduction was observed in the *gam* deletion strain, most likely attributed to the single-stranded nuclease activity of the RecBCD protein complex, which is not inhibited in a *gam*-deficient strain (Ellis et al., 2001, Wang et al., 2000).  $\lambda$  Red $\beta$  bound to ssDNA is able to protect the DNA segment from nuclease attack, which might explain the recombination events that occurred despite the *gam* deletion (Karakousis et al., 1998).

These methods offer a technology for studying bacterial gene functions or even for introducing mutations or markers in the chromosomes of eukaryotic cells, e.g., to provide special “tags” in the DNA of living cells (Ellis et al., 2001).

### 1.2.1 Gene deletion

$\lambda$  Red recombination has been successfully used for convenient generation of gene deletions in *E. coli* (Datsenko et al., 2000), *Salmonella enterica* (Hansen-Wester et al., 2002), *Pseudomonas aeruginosa* (Liang et al., 2010, Qu  n  e et al., 2005), *Streptomyces coelicolor* (Gust et al., 2004), *Shigella* spp. (Ranallo et al., 2006), *Yersinia pseudotuberculosis* (Derbise et al., 2003) and *Y. pestis* (Sun et al., 2008).

The first step in generating gene deletions is creating a linear targeting construct which consists usually of a resistance gene (“*res*” in Fig. 1) flanked by terminal extensions homologous to the sequences surrounding the deletion site. In Fig. 1, the homologous regions used for deletion of *orf2* are depicted in blue and red. In the simplest approach, substrates for Red recombination can be generated by adding short homology extensions to PCR primers and using them to amplify a resistance cassette (Datsenko et al., 2000, Yu et al., 2000, Zhang et al., 2000). The length of the homology arms required for efficient recombination depends on the organism. For example, 36-50 bp (base pairs) were shown to work for *E. coli* or *Salmonella enterica* (Datsenko et al., 2000, Hansen-Wester et al., 2002) and 50-100 bp for *Pseudomonas aeruginosa* (Liang et al., 2010). Much longer homology arms were required for efficient Red recombination in *Y. pseudotuberculosis* and *Y. pestis* (~500 bp, Derbise et al., 2003, Sun et al., 2008) and *Vibrio cholerae* (100-1000 bp, Yamamoto et al., 2009). For the latter examples, multiple PCRs have to be done to generate functional targeting constructs.

In the next step, the PCR product is used to transform bacteria expressing  $\lambda$  Red proteins. Homologous recombination results in insertion of the cassette at the precise position determined by the homology extensions (Fig. 1). Transformants can be selected using their acquired antibiotic resistance. Target regions for site-specific recombinases (Fig. 1, yellow triangles) provide the option for subsequent removal of the resistance cassette (see also 1.2.3).

### 1.2.2 DNA insertion

In addition to removing DNA from bacterial genomes (1.2.1),  $\lambda$  Red recombination can also be applied to precisely insert any DNA within a genome. This approach has been widely used for analyzing bacterial gene expression via the generation of reporter gene fusions (Gerlach et al., 2007a, Lee et al., 2009, Yamamoto et al., 2009) or epitope tagging (Cho et al., 2006, Lee et al., 2009, Uzzau et al., 2001). In a similar approach, promoter sequences can be inserted or exchanged within the genome (Alper et al., 2005, Wang et al., 2009).

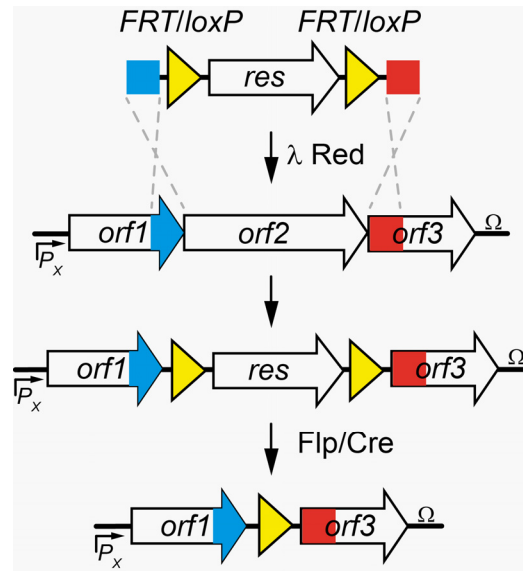


Fig. 1. Deletion of *orf2* using  $\lambda$  Red recombination and subsequent removal of resistance marker (*res*) via Flp or Cre recombinase. Red and blue regions denote regions of homology. The yellow region denotes the target for the site-specific recombinase.

In these cases, the targeting construct includes besides a selectable marker the DNA to be inserted. Using primers with homology extensions, these targeting constructs can be amplified by PCR from sets of template vectors available for different reporter genes (e.g.,  $\beta$ -galactosidase, luciferase, green fluorescent protein (*gfp*) and epitope tags (e.g., Flag®, haemagglutinin (HA), 8xmyc, 6xHis). An interesting alternative that obviates the need for multiple template vectors was developed by Gust et al. (2004). The resistance cassette and the reporter gene were amplified in separate PCRs, using in each reaction only one primer with the homology extension for Red recombination. The other primers included regions of homology to each other to allow them to anneal. The joint molecule was then used in a second round of PCR to generate a fusion cassette.

Depending on the scientific question to be answered, different integration strategies for reporter genes are available. For transcriptional fusions, a promoterless reporter gene is inserted downstream of a promoter of interest. The reporter gene may have optimized translational signals, including an optimized ribosome binding site (RBS) at the optimal distance from the start codon. If such a construct is inserted within an operon, hybrid operons are generated (Gerlach et al., 2007a). We have introduced so-called “start codon fusions,” in which the reporter gene is inserted behind the native RBS and start codon of the gene under study, so that expression is assessed in the native genomic context (Gerlach et al., 2007a, Wille et al., 2012). This gene fusion strategy is closely related to translational fusions. The classical Red recombination protocol enables the easy generation of C-terminal fusion proteins, in which the reporter gene or epitope tag is inserted in-frame at any position in an open-reading-frame (ORF). For the generation of N-terminal fusions, a “scarless” recombination protocol (see 2.) has to be applied.

### 1.2.3 Site-specific recombination for removal of antibiotic resistance genes

Several methods, involving various site-specific recombination systems, have been developed for the removal of unwanted marker sequences from the chromosome. The most frequently used site-specific recombinases for subsequent excision of antibiotic resistance genes are Flp and Cre. Flp and Cre recombinases recognize 34-bp long sequences with palindromic elements (*FRT* and *loxP*, respectively). Both sites, *FRT* and *loxP*, consist of two 13-bp inverted repeats flanking an 8-bp non-palindromic core sequence. The asymmetry of the core sequence determines the polarity of the recombination site and has extensive consequences for the outcome of the recombination. Recombination between directly orientated recombination sites leads to excision of the intervening sequence (Fig. 1, yellow triangles), while recombination between inverted recombination sites results in an inversion of the intervening sequence. Recombination continuously takes place as long as the recombinase is present (Schweizer, 2003, Zhang et al., 2002). However, removal of resistance genes by either one of the site-specific recombinases leaves a *loxP* or *FRT* “scar” sequence within the genome (Datsenko et al., 2000, Lambert et al., 2007).

Although there is limited homology between the scar sequences themselves, they might serve as hotspots for recombination in successive recombination steps, representing a risk for unwanted deletions or chromosomal rearrangements (Datsenko et al., 2000). In addition, these scars might have influence on gene functions when operon structures or intragenic regions were modified (Blank et al., 2011). Mutations within the inverted repeats of *loxP*, resulting in the *lox66* and *lox71* alleles, can minimize genetic instability. Recombination using either generates *lox72*, which has a strongly reduced binding affinity for Cre (Lambert et al., 2007).

## 2. Site-directed mutagenesis using oligonucleotides

Precise insertion of chromosomal mutations has been established as the “gold standard” for analysis of bacterial gene function. Generation of point mutations, seamless deletions and in-frame gene fusions without leaving selectable markers or a recombination target site (e.g., *loxP* or *FRT*) requires reliable counterselectable markers. The published protocols are usually based on two successive rounds of recombination: (I) integration of a positively selectable marker (e.g., an antibiotic resistance gene) in the first step and (II) selection for marker loss (counterselection) in the final recombination step (Reyrat et al., 1998). Some of the counterselection methods have been shown to function together with the recombination of short synthetic DNA fragments. This circumvents the tedious and time-consuming requirement for PCR-based mutagenesis and cloning to generate mutant alleles for the second recombination step.

### 2.1 Counterselection with SacB

The *Bacillus subtilis sacB* gene is widely used as a genetic tool for counterselection purposes. SacB confers sucrose sensitivity to a wide range of Gram-negative bacteria (Blomfield et al., 1991, Kaniga et al., 1991). The levansucrase SacB catalyzes transfructorylation from sucrose to various acceptors, hydrolysis of sucrose and synthesis of levans (Gay et al., 1985). The molecular basis of its toxic effect on many Gram-negative bacteria is still not completely understood. It is thought that accumulation of levans in the periplasm or transfer of fructose

residues to inappropriate acceptors, subsequently generating toxic compounds, might play an important role (Pelicic et al., 1996).

Linear targeting constructs harboring *sacB* combined with a kanamycin resistance gene (*neo*, Zhang et al., 1998), an ampicillin resistance gene (*bla*, Liang et al., 2010) or a chloramphenicol resistance gene (*cat*, Sun et al., 2008) were used for the first recombination rounds. An interesting marker combining the ability for positive selection and counterselection in one fusion gene is *sacB-neo*. The resulting protein SBn confers kanamycin resistance, as well as sucrose sensitivity, to Gram-negative bacteria.

The latter two methods were used to generate gene deletions within the chromosomes of *P. aeruginosa* and *Y. pestis*, respectively. In these organisms, the  $\lambda$  Red system was used to recombine the targeting constructs with homology extensions of 50-100 bp (Liang et al., 2010) or ~500 bp (Sun et al., 2008) flanking the cassettes.

In the homologous recombination step, clones were selected for the respective antibiotic resistance. Recombinants were selected on medium plates containing 5-7% sucrose to select for loss of the cassette. Exact timing of counterselection is a critical issue when working with SacB or SBn, since *sacB*-inactivating mutations were shown to accumulate. For example, only 10-15% (not 100%) of *sacB*-clones lost their kanamycin resistance after counterselection of SBn on sucrose plates (Muyrers et al., 2000a). Nevertheless, Muyrers and coworkers successfully used this selection scheme to introduce a point mutation within a BAC. In these experiments, the altered base was included in one of the homology arms of both targeting constructs. Interestingly, the investigators observed that the homologous arms for the second targeting construct should be at least 500 bp in length. This excludes the use of a completely synthetic oligonucleotide at that step (Muyrers et al., 2000a).

## 2.2 Dual selection of recombinants with GalK or ThyA

Besides the fusion protein SBn, *E. coli* galactokinase (GalK) and thymidylate synthase A (ThyA) can each be used in the dual role of both a positive selective marker (selection for the gene) and a negative selective marker (selection for the absence of the gene). Depending on the substrates used for growing recombinants, gain or loss of the markers can be selected very efficiently. Both systems were established for the manipulation of BACs in appropriate *E. coli* host strains that are deficient for *galK* or *thyA* and have the  $\lambda$  Red recombination system present within their genomes (Wong et al., 2005, Warming et al., 2005). These methods cannot be used for site-directed mutagenesis of genomes of bacteria with functional *galK* or *thyA*.

The *galETKM* operon enables *E. coli* to metabolize D-galactose (Semsey et al., 2007). In the initial step, GalK catalyzes the phosphorylation of D-galactose to galactose-1-phosphate. Recombinants were selected after the first recombination step with D-galactose as the sole carbon source. A functional GalK is absolutely required for growth under these conditions. Besides D-galactose, GalK can efficiently phosphorylate a galactose analog, 2-deoxy-galactose (DOG). Because the product 2-deoxy-galactose-1-phosphate cannot be further metabolized, it is enriched to toxic concentrations (Alper et al., 1975). In the second recombination step, an oligonucleotide harboring the desired mutation was used as substrate for Red recombination. Loss of GalK was selected on plates containing glycerol as sole carbon source and DOG (Warming et al., 2005).

A *thyA* mutation results in thymine auxotrophy of *E. coli*, since the enzyme is required for dTTP and, therefore, DNA *de novo* synthesis. Integration of the *thyA*-containing targeting construct in the first recombination step was selected on minimal medium in the absence of thymine. For its function, ThyA requires tetrahydrofolate (THF) as a cofactor. During the process THF is oxidized to dihydrofolate (DHF). The pool of THF is replenished from DHF by dihydrofolate reductase (DHFR). The action of DHFR can be inhibited by the antibiotic trimethoprim. Using a growth medium supplemented with trimethoprim and thymine, loss of *thyA* was selected in the second round of recombination. At this step, a PCR product containing a mutated allele was applied as targeting construct (Wong et al., 2005).

### 2.3 Counterselection using streptomycin resistance

Several mutants of the ribosomal protein S12 (RpsL) were shown to confer streptomycin resistance (Sm<sup>R</sup>, Springer et al., 2001). Strains harboring such an *rpsL* allele (e.g., *rpsL150*) can be used as hosts in another counterselection method. The main principle is based on the fact that streptomycin resistance-conferring mutations are recessive in merodiploid strains (Lederberg, 1951). In the first step, the wild type (wt) allele of *rpsL* was inserted within the gene of interest using  $\lambda$  Red or RecE/T recombination (1.1). A cassette containing an *rpsL-neo* fusion gene was used. Clones exhibited Km<sup>R</sup> and Sm<sup>S</sup> (Heermann et al., 2008). The desired point mutation for the gene of interest had already been introduced into one of the two 50-bp homology arms of the *rpsL-neo* cassette. The *rpsL-neo* cassette was then deleted in a second round of recombination. For this step, a single-stranded oligonucleotide containing no *rpsL* allele but containing arms of homology to the gene of interest were used. To maintain the mutation, one of the homology arms again harbored the desired mutation. Recombinants that deleted the wt *rpsL* could be selected by their resistance to streptomycin. After successful recombination, the wt copy of *rpsL* was removed, and the gene of interest was re-established, now containing the desired mutation (Heermann et al., 2008).

### 2.4 Selection with the fusaric acid sensitivity system

A counterselection technique developed by Bochner et al. (1980) enables direct selection of tetracycline sensitive (Tc<sup>S</sup>) clones from a predominantly tetracycline resistant (Tc<sup>R</sup>) bacterial population. The method is based on the hypersensitivity of lipophilic Tc<sup>R</sup> cells to chelating agents, like fusaric acid or quinaldic acids. The precise mechanism of tetracycline exclusion is so far unknown and the subject of much speculation. The hypersensitivity seems to be caused by alterations of the host cell membrane, which are evoked from the expression of the tetracycline resistance gene. These alterations interfere, on one hand, with tetracycline permeation to confer tetracycline resistance, but, on the other hand, also to increase susceptibility to other toxic compounds (Bochner et al., 1980). This effect was exploited by using a medium that was effective for the selection of Tc<sup>S</sup> revertants. The counterselection was successful in *Salmonella*; but it was much less effective with most, especially fast-growing, *E. coli* strains (Bochner et al., 1980). Decreasing the nutrient concentration of the selection plates significantly minimized the background of Tc<sup>R</sup> colonies of fast-growing bacteria (Maloy et al., 1981).

The counterselection of Tc<sup>R</sup> clones on Bochner-Maloy plates was sometimes used as the final step in recombineering protocols. Point mutations were inserted in BACs using a combination of  $\lambda$  *gam* (1.1) with RecE/T (1.) to integrate the gene for tetracycline resistance.



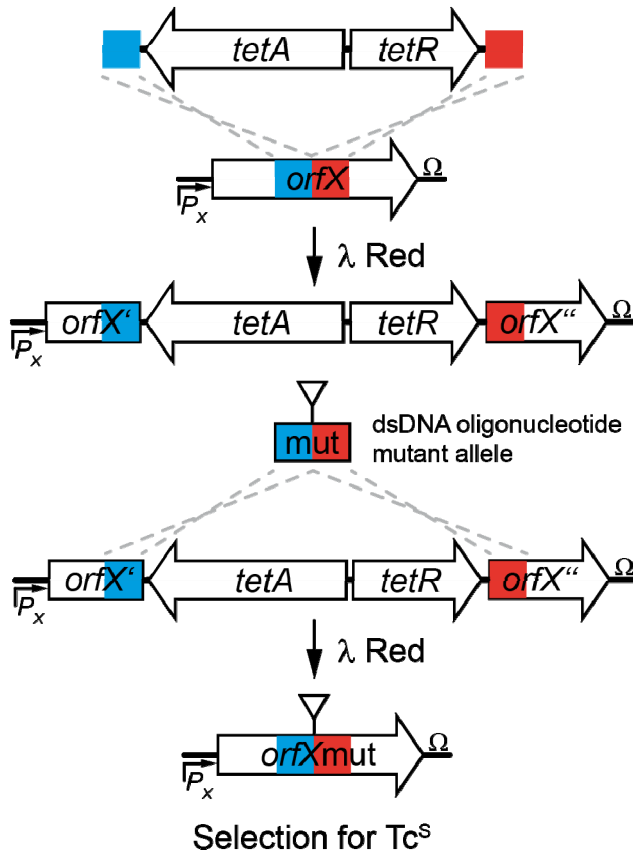


Fig. 2. Use of a *tet* cassette in conjunction with Bochner-Maloy Tc<sup>S</sup>-selective medium for counterselection. Blue and red regions denote regions of homology. "mut" indicates a mutation. The mutation is first in the targeting construct, then recombination transfers it to "*orfX*" ("*orfX*" mut). "*P<sub>x</sub>*" designates the promoter for *orfX*; " $\Omega$ " indicates a transcriptional terminator.

A PCR product carrying the desired mutation was used in the second recombination step to exchange *tet*. Recombinants (Tc<sup>S</sup>) were selected on Bochner-Maloy plates (Nefedov et al., 2000). Similar two-step recombination approaches were also used to manipulate the genome of *Salmonella enterica* serovar Typhimurium ("*S. Typhimurium*") (Gerlach et al., 2009, Karlinsky, 2007). For this technique, a *tetAR* cassette, which encodes Tc<sup>R</sup>, was inserted in the first step within a target gene ("*orfX*") with the help of homology extensions (blue and red, Fig. 2) resulting in tetracycline resistant clones. In the second recombination step, either PCR-derived mutant alleles (Karlinsky, 2007) or synthetic oligonucleotides (Gerlach et al., 2009) were used as targeting constructs to remove the *tetAR* cassette. For the latter rationale, no further cloning steps to generate the mutant allele (Fig. 2 "mut") were required. The applicability of this approach was proven with a *Salmonella* virulence-associated gene as an example. *siiF* encodes the putative ATPase of a type I secretion system (T1SS) located within

the *Salmonella* Pathogenicity Island 4 (SPI-4, Gerlach et al., 2007b). Previous work on a homologue demonstrated, that a single amino acid exchange within the Walker Box A of the ABC (ATP-binding cassette) motif disrupted the function of the transport ATPase (Koronakis, 1995). For our mutagenesis of *siiF*, we designed oligonucleotides to introduce a silent mutation resulting in a novel *NlaIV* site (2.6.1), as well as a change of Gly at position 500 to Glu (G500E) or a change of Lys at position 506 to Leu (K506L). Both amino acid positions are within the ABC motif. As a control, we introduced a silent mutation to generate a new *SacI* restriction site (2.6.1) within *siiF*. After growth selection on Bochner-Maloy medium plates that favor the growth of Tc<sup>S</sup> bacteria, clones were screened for the newly inserted restriction sites by the relevant restriction enzymes. Positive recombinants were subjected to functional analyses. The experiments showed the expected results: (I) no influence of the silent mutations and (II) loss of substrate secretion from the amino acid exchanges within Walker Box A (Gerlach et al., 2009).

The selection efficiency of Bochner-Maloy plates was reported not to exceed 50% (Podolsky et al., 1996). Therefore, the selection procedure was not very stringent. Exact timing of all incubation steps was necessary; but still high background might be observed, making purification of positive clones difficult. Highly increased selection efficiencies were obtained with plates containing 5-7 mM NiCl<sub>2</sub>, which led to 80-100% positive Tc<sup>S</sup> revertants (Podolsky et al., 1996).

## 2.5 Double-strand breaks introduced by I-SceI can be used to select recombinants

The endonuclease I-SceI of the yeast *Saccharomyces cerevisiae* is a novel tool for counterselection. I-SceI is an endonuclease with a long recognition sequence of 18 bp, thus ensuring the statistical absence of natural I-SceI recognition sites within bacterial genomes (Monteilhet et al., 1990). Counterselection with I-SceI is based on the induction of lethal double-strand breaks (DSB) within the genome or BAC, thus inhibiting DNA propagation. The Red system promotes enough recombination that recombinants can be obtained by screening survivors.

Several methods for site-directed mutagenesis of BACs and/or bacterial genomes utilizing I-SceI expression have been published. Usually the methodologies are based on the insertion of an I-SceI recognition sequence together with a positively selectable marker near the sequence to be modified. Furthermore, a system allowing for transient expression of the I-SceI restriction enzyme in a coordinated fashion after expression of the  $\lambda$  Red recombination system is required. For the manipulation of BACs, a special *E. coli* host strain was developed to facilitate the independent expression of  $\lambda$  Red and I-SceI. *E. coli* GS1783 harbors within its genome  $\lambda$  Red under control of a temperature-sensitive repressor and I-SceI under control of an arabinose-inducible promoter (Tischer et al., 2010). However, for modification of bacterial genomes the components for recombination and I-SceI have to be provided on one or two plasmid(s). The single plasmid solutions allow for independent inducible expression of both functions using arabinose and tetracycline (pWRG99 and pGETrec3.1; Blank et al., 2011, Jamsai et al., 2003) or arabinose and rhamnose (pREDI, Yu et al., 2008).

For mutagenesis of the genomes of *Salmonella enterica* serovar Enteritidis (*S. Enteritidis*, Cox et al., 2007) and *E. coli* (Kang et al., 2004), setups with the Red components and I-SceI encoded on two separate plasmids were used. Targeting constructs consisting of an I-SceI

recognition site, a Km<sup>R</sup> cassette and long flanking homology regions (>200 bp) were constructed in a two-step PCR approach. After chromosomal integration of the linear DNA via  $\lambda$  Red-mediated recombination, clones were selected with kanamycin (Cox et al., 2007). A two-step PCR approach was used to combine long extensions homologous to *lamB* with sequences encoding antigen epitopes in the second targeting construct. For production of antibodies against foreign proteins, epitopes were inserted into the outer membrane protein LamB to facilitate surface presentation. This linear PCR product was used in a co-transformation with pBC-I-SceI at a molar ratio of 40:1 (PCR product:plasmid) into  $\lambda$  Red-expressing *S. Enteritidis*. I-SceI was constitutively expressed from plasmid pBC-I-SceI (Kang et al., 2004). Screening for the desired recombinants was based on the inability to grow on kanamycin-containing plates (Cox et al., 2007). Because there is no convenient possibility for plasmid curing, the pBluescript (Stratagene)-based pBC-I-SceI remains in the host strains (Cox et al., 2007, Kang et al., 2004).

*GET recombination* is a method developed for the manipulation of BACs. It employs  $\lambda$  Gam and RecE/T for recombination (1.1) and I-SceI for counterselection (Jamsai et al., 2003). In a study by Jamsai et al. (2003), the I-SceI endonuclease gene downstream of a repressed promoter, together with a constitutive gene for Km<sup>R</sup> and an I-SceI recognition site, was inserted within the gene of interest in the first recombination step. As targeting construct for the second recombination step, a 1708-bp PCR product carrying the desired mutation was inserted in exchange for the I-SceI-kanamycin resistance cassette. I-SceI expression was induced for 30 minutes with addition of heat-treated chlorotetracycline. Expression was induced from both the inserted I-SceI-kanamycin resistance cassette and plasmid pGETrec3.1 (Jamsai et al., 2003). The kanamycin resistance cassette, with its I-SceI recognition site, was successfully removed from 23.6% of the colonies surviving expression of I-SceI (Jamsai et al., 2003).

Because no specific mechanisms were implemented in pGETrec3.1 and pBC-I-SceI to promote convenient plasmid curing, it might be difficult to get plasmid-free host strains after site-directed mutagenesis. We solved that problem by integrating a tetracycline-inducible I-SceI expression cassette from pST98-AS (Pósfai et al., 1999) in the temperature-sensitive  $\lambda$  Red expression plasmid pKD46 (Datsenko et al., 2000) to generate pWRG99 (Blank et al., 2011). In a first recombination step, a Cm<sup>R</sup> cassette (*cat*) together with an I-SceI recognition site (dark green, Fig. 3A) was integrated within *phoQ* in the genome of *S. Typhimurium*. For that, extensions of 40 nt length homologous to the regions surrounding the intended mutation site within *phoQ* were added to the primers (Fig. 3A, blue and red). PhoQ is the histidine sensor kinase of the *Salmonella* virulence-associated two-component signaling system PhoPQ. Successful recombinants were selected using chloramphenicol, and correct integration of the resistance cassette was checked by colony PCR and sequencing. For the second round of recombination, 80mer dsDNAs, derived from oligonucleotides, were introduced into pWRG99-harboring cells expressing  $\lambda$  Red recombination genes. These 80mer dsDNAs were designed (I) to delete the *phoQ* gene (not shown) or (II) to introduce a threonine to isoleucine exchange at position 48 (T48I) of PhoQ, together with a new *SacII* restriction site (Fig. 3A). Expression of I-SceI was induced with addition of anhydrotetracycline (AHT) leading to lethal DSBs in the clones still harboring the I-SceI recognition site. Surviving clones were screened by PCR, restriction analysis using *SacII* as well as phenotypically. The single amino acid exchange T48I within the periplasmic domain

of PhoQ results in constitutive activation of the response regulator PhoP (Miller, 1990). Besides virulence attenuation, constitutive PhoP activation leads to overexpression of the nonspecific acid phosphatase PhoN. Successful recombinants could therefore be screened phenotypically by forming blue colonies on 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt (BCIP) plates due to increased PhoN activity. Correct recombination events could be further confirmed by a macrophage infection model, which showed the predicted virulence-attenuated phenotype (Blank et al., 2011).

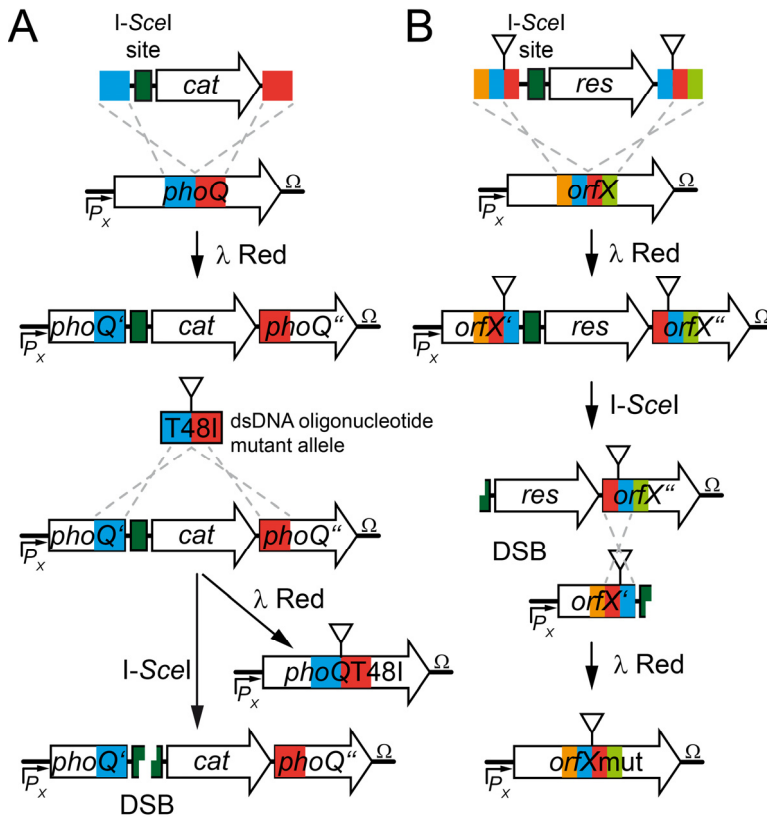


Fig. 3. Methods for single nucleotide exchange using I-SceI counterselection. Details for A and B are in the text. The symbols and colors are the same as those described in Fig. 2. However in A *orfX* is *phoQ*. The antibiotic resistance is Cm<sup>R</sup>; the resistance gene is *cat*. The mutation (inverted triangle) is a point mutation that changes threonine at position 48 in PhoQ to isoleucine (T48I). The green rectangle is the recognition site for the I-SceI endonuclease; “DSB” is the “double-strand break” induced by I-SceI. In B, the symbols are the same as those in A and Fig. 2. Unique to B are “*res*” (antibiotic resistance) and the modular extensions for the primers (orange, blue, red, and light green). All are homologous to *orfX*. The two internal primers (relative to the intact gene) have the blue and red extensions, which will become the duplicated region. The “left” primer has unique homology (orange); the “right” primer has unique homology (light green).

The Red recombination system can anneal single-stranded DNA derived from dsDNA substrates into replicating homologous target sequences (1.1). Usually homologous sequences for recombination are supplied with the homology extensions flanking the targeting construct. In contrast, homologous regions flanking a DSB generated by *I-SceI* can also act as substrates for Red recombination. This strategy has been used for scarless site-directed mutagenesis of BACs (Tischer et al., 2010, Tischer et al., 2006) and the *E. coli* genome (Yu et al., 2008). These approaches require the integration of a duplicated sequence stretch in the first recombination round to serve as a substrate for recombination in the second round. For seamless deletions or insertion of point mutations, the duplications can be readily incorporated within the primers used to amplify the positive selection marker. In one study, modular sequence extensions, each ~20 nt in length (Fig. 3B; orange, blue, red and light green), were added to the primers used to amplify a resistance cassette (*res*). The two primers were unique but shared about 40 nt of sequence. The desired mutation (Fig. 3B, inverted triangles) was included in the duplicated sequence. This resulted in a ~40-bp sequence duplication (red and blue) after integration of the targeting construct into the gene of interest (*orfX*) via Red recombination (Fig. 3B, Tischer et al., 2010). The duplicated sequences were separated by the *I-SceI* recognition site and after induction of DSB, the Red-mediated recombination between these sequences led to the reconstitution of *orfX* in its mutated form *orfXmut* (Fig. 3B). Figure 3B shows a generalization of this strategy. If longer DNA sequences need to be integrated (1.2.2), a preceding cloning step is required to insert the selectable marker together with the *I-SceI* recognition site and a sequence duplication into the DNA to be inserted (Tischer et al., 2010). Combining *I-SceI*-induced DSB with *SacB*-mediated sucrose sensitivity (2.1) was shown to improve selection for loss of resistance marker within the *E. coli* genome (Yu et al., 2008).

One major problem of the *I-SceI* counterselection approach is the accumulation of point mutations within or deletion of the *I-SceI* recognition site during the selection process. This effect demands tight regulation of *I-SceI* expression to minimize selection pressure before the final (markerless) recombination takes place. It was important to optimize the procedures to maximize counterselection after the 2<sup>nd</sup> round of recombination.

## 2.6 Screening methods for recombinants

An underestimated problem is the screening effort needed to identify correct recombinants when using seamless recombination techniques. Although PCR fragment length polymorphism can be used in case of deletions and insert-specific PCRs in case of DNA insertion, successful single nucleotide exchanges are hard to detect. Direct phenotypical screening or the parallel introduction of novel restriction sites together with the nucleotide exchange are solutions of the problem.

### 2.6.1 Introduction of silent mutations to generate novel restriction sites

A screening problem arises if mutations introduced via recombineering have no direct or indirect impact on the phenotype or if the phenotypic test required is very time-consuming. Introduction of a novel restriction site adjacent to the mutation was proven to be very useful for colony screening. Designing the oligonucleotides for  $\lambda$  Red recombination offers the prospect of introducing silent mutations in the target region. Identification of silent

mutations generating novel restriction sites can be done *in silico* using WatCut [an online tool for restriction analysis, silent mutation scanning and SNP-RFLP analysis (<http://watcut.uwaterloo.ca/>)] or other DNA analysis software (e.g., Clone Manager). As mentioned before, this screening method has been used successfully [e.g., for screening *siiF* recombinants by introducing a new *NlaIV* and/or *SacII* restriction site (2.4), as well as for the *phoQT48I* mutation by generating a novel *SacII* restriction site (2.5)].

### 2.6.2 Phenotypical screening

If available, phenotypical screening is the fastest way for selecting recombinants with the desired mutation. The screening is based on phenotypic differences between the mutant and the wt. In the simplest case, activity of an integrated reporter gene like *gfp* or *lacZ'* might be detected (Gerlach et al., 2007a). Another possibility for screening is the ability (gain of function) or inability (loss of function, auxotrophy) of the mutant to grow under specific conditions. In the latter case it is necessary to define permissive conditions in which the mutant can grow and restrictive conditions in which it cannot. Moreover, mutations might lead to differences in cell morphology that can be identified by microscopic examination. Last but not least, there may be a difference in the ability to utilize a chromogenic substrate, like BCIP, *p*-nitrophenyl-phosphate (pNPP) or 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal). These could be used for screening of positive recombinants. All of the phenotypes can originate directly from the activity of the mutated gene product or indirectly by influencing the activity and/or expression level of other proteins. One example is the PhoQ T48I mutant, which causes overexpression of PhoN. The overexpression can be monitored using the chromogenic substrate BCIP (2.5, Blank et al., 2011).

## 3. Conclusion

Two successive recombination steps catalyzed by the phage  $\lambda$  Red or phage Rac RecE/T recombination systems in combination with a negative selection procedure provide a venue for scarless mutagenesis within bacterial genomes and BACs. The outstanding ability of these enzymes to use homologous sequences as short as 35 bp as substrates for recombination allows the use of linear DNA derived from synthetic oligonucleotides as targeting constructs. The limiting step of this rationale is the availability of a reliable counterselection method. Here we gave an overview about recombination and the counterselection techniques successfully applied to the manipulation of bacterial genomes, as well as BACs.

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