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In vivo recombineering of bacteriophage E by PCR fragments and single-strand oligonucleotides

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Abstract

We demonstrate that the bacteriophage E Red functions efficiently recombine linear DNA or single-strand oligonucleotides (ss-oligos) into bacteriophage E to create specific changes in the viral genome. Point mutations, deletions, and gene replacements have been created. While recombineering with oligonucleotides, we encountered other mutations accompanying the desired point mutational change. DNA sequence analysis suggests that these unwanted mutations are mainly frameshift deletions introduced during oligonucleotide synthesis.

Keywords: Bacteriophage; Homologous recombination; E Red; Oligonucleotide; Recombineering

Introduction

Bacteriophages are astonishingly abundant on our plant. It has been estimated that the number of free phage particles exceeds 10^{30} (Hendrix et al., 2000). In addition to existing free, especially as components of aquatic microbial communities (Wommack and Colwell, 2000), a large number of phages are resident prophages in bacterial genomes. Casjens (2003) estimated the presence of 230 prophages in 51 bacterial genomes whose sequence was deciphered. Some of these prophages code for genes of major medical importance, for example, the cholera toxin carried by CTXA prophage (Davis and Waldor, 2003). Obviously, the genetic analysis of phage genomes is at its infancy (Campbell, 2003). Such analysis can be facilitated by a recently developed efficient in vivo recombination system (recombineering) that uses the functions Exo, Beta, and Gam of bacteriophage E (Yu et al., 2000; Zhang et al., 1998). These functions promote recombination of a double-strand linear DNA into the chromosome using flanking homologies between the linear DNA and the chromosome. Single-strand oligonucleotides (ss-oligos) also promote in vivo recombination in a reaction requiring only Beta protein (Ellis et al., 2001; Zhang et al., 2003). The electroporated ss-oligo may anneal to transiently single-strand regions of chromosomal DNA at the replication fork, resulting in a recombinant (Court et al., 2003; Ellis et al., 2001). We now extend recombineering by targeting PCR generated DNA fragments and ss-oligos to phage. The precision and ease of these experiments open an exciting pathway for new studies. In addition, we analyze in detail some inaccuracies arising from recombineering with synthetic oligonucleotides.

Results

Creation of novel bacteriophage genotypes. We have been creating mutations in phage E using recombineering with a simple protocol. Briefly, *Escherichia coli* harboring a defective E prophage is infected with the phage to be engineered. The partial prophage carries the p_L operon under control of the *cI857* temperature-sensitive repressor. The lysogen is induced to express the Red functions, the induced cells are made competent for electroporation, and the PCR product or oligonucleotide is introduced by electroporation. Following electroporation, a phage lysate is made from the

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electroporation mix. Several of our constructions are described below.

(i) We generated suppressible mutations by introducing UAG termination codons in essential E genes *O*, *P*, *Q*, *S*, and *E*. The target phage E *c*II*68* acquired these amber mutations at a frequency of 1 – 3% in a cross with 70-nucleotide-long ss-oligos with the UAG codon at the center. Amber mutants were easily identified as cloudy plaques with a double-layer bacterial lawn (Campbell, 1971): the lower layer contains the restrictive host W3110 and the top layer contains the infected SupF suppressor host LE392. E *c*II68 lyses both hosts, thereby generating a clear plaque. Amber mutants lyse only the infected LE392 cells and form cloudy plaques because W3110 cells in the lower layer grow to confluence.

(ii) Previous studies of E Cro function were based primarily on the use of one missense mutant, *cro*27. The phage *c*I*857 cro*27 forms clear plaques at 37jC but cannot form plaques at either 32 or 42jC (Eisen and Ptashne, 1971). The Cro protein contains three tyrosine residues, and we independently replaced each tyrosine codon with UAG. Screening plaques at 42jC in a double layer, approximately 2% of total plaques were cloudy. On LE392, the resultant mutants grow at 32, 37, and 42jC, but on W3110 they form plaques only at 37jC.

(iii) We used an 80-nucleotide oligo to generate a 326-bp deletion of the *c*II gene in E c^+. This ss-oligo provides 40 bases of homology at each end of the segment to be deleted. E c^+ normally forms turbid plaques. Clear plaque recombinants were found at a frequency of 2%. Sequencing showed that the resulting clear mutant phage carried a deletion exactly corresponding to the original design. This deletion fuses the *c*II translation initiation codon to the downstream *O* gene, creating a phage with *O* at the normal *c*II location. (Using recombineering, deletions as large as 5 kb have been generated with oligos on the *E. coli* chromosome with good efficiency.)

(iv) We replaced the phage E *rexA* and *rexB* genes precisely with a *bla* gene conferring ampicillin resistance. The *bla* gene was first amplified by PCR using primers with 5Vhomology to the flanking regions of the *rexAB* genes; the PCR product was then targeted to the E chromosome with recombineering. A phage lysate was grown from the electroporation mix and used to form lysogens. AmpR lysogens were selected and the replacement of the *rexAB* genes by the *bla* gene in such lysogens was confirmed by PCR analysis (Yu et al., 2000) and by the ability of the recombinant lysogens to plate T4*r*II mutant phage (Benzer, 1955).

(v) Using appropriate PCR primers and the gene SOEing technique (Horton et al., 1990), we created a linear DNA product containing an intact copy of the wild-type E *P* gene adjoining a precise deletion of the entire *ren* gene but with homology beyond *ren* in the *ninR* region of the phage. The construct was targeted to an infecting *Pam80* phage; P^+ recombinants were selected and screened for the *ren* deletion. P^+ recombinants were obtained at a frequency of 2%; 20% of these had the deletion.

Analysis of mutations arising from the use of oligonucleotides in recombineering. We have demonstrated that recombineering provides an efficient way to manipulate the bacteriophage genome. However, we found that sometimes oligo recombination has associated unwanted mutations. To understand the origin and nature of these unwanted mutants, we designed a protocol to score for both true recombinants and unwanted changes. Phage E *c*I*857* carries a temperature-sensitive mutation in repressor; thus, the phage forms clear plaques at 37jC and turbid plaques at 30jC (Sussman and Jacob, 1962). We designed two complementary oligonucleotides 82 residues long, with wild-type repressor gene sequence that could generate wild-type E recombinants in a cross with E *c*I*857* (Fig. 1). These oligos cover about 1/10 of the *c*I coding region and are centered on the c*I857* allele. The recombinant lysate was diluted and plated on W3110 at either 37 or 32jC. At 37jC, E c^+ recombinants form turbid plaques. At 32jC, both parent and recombinant should form turbid plaques. When plaques from the recombineering cross were grown at 37jC, most were clear, however, 4 – 13% were turbid as expected of wild-type recombinants (Table 1). When the recombinant lysate was plated at 32jC, most plaques were turbid as expected, however, a significant proportion, 0.5 – 2%, was clear. This number is 10 – 40 times higher than the spontaneous frequency of clear plaques (approximately 0.05%) found in lysates prepared the same way but without the addition of oligonucleotide or with the addition of an oligonucleotide lacking homology.

To understand the source of the unwanted clear mutations, we purified clear and turbid recombinants and sequenced their *c*I gene (Fig. 1). Fourteen turbid E cI^+ recombinants isolated at 37jC had all been corrected for the *c*I*857* mutation without additional mutations. However, all clear plaques identified at 32jC contained other mutations in *cI*. These mutations were about equally produced by the two oligonucleotides. Twenty-four of twenty-five sequenced had mutations in the region covered by the ss-oligo. Among these 24 mutants, 22 had also converted the *c*I*857* allele to wild type. One of these 22 mutants was a GC to TA transversion, the rest were deletions of one or more bases of the *c*I sequence. The one change outside of the oligo region was a GC to TA transversion that retained the *c*I*857* allele and possibly arose spontaneously.

To demonstrate that these mutations were not specific to *c*I*857* or to the oligo sequence, we repeated the experiment using wild-type E cI^+ and complementary ss-oligos from a different region of the *c*I gene in a cross (Fig. 1). These oligonucleotides carried a single silent AT to GC change. As before, clear plaques were found in the lysate following recombineering. We sequenced the DNA from 16 clear

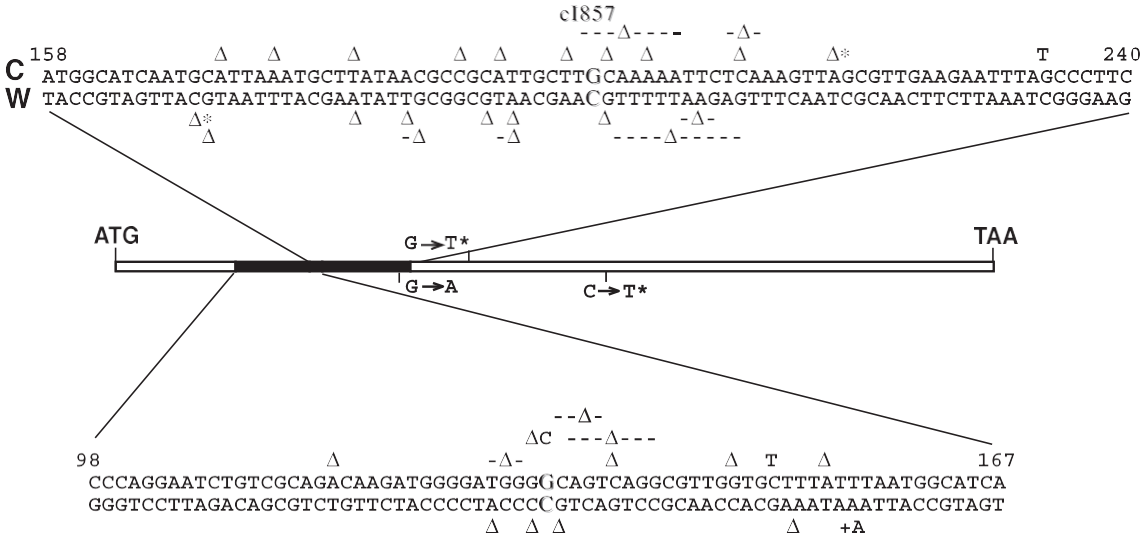

Fig. 1. Distribution of mutations generated by recombineering ss-oligo. The central rectangle represents the entire *c*I-coding region, with the two regions targeted for recombineering with oligonucleotides indicated by filled boxes. Sequences of the corresponding oligonucleotides are expanded and positions of the mutations generated are indicated. A D represents a single nucleotide deletion and dashes indicate the extent of events with greater than a single nucleotide deletion. All mutants with the exception of those marked by asterisks (*) were converted from *cI857* to *cI*$^+$ or carried the silent mutation. The asterisk (*) represents those alleles in which co-conversion of the silent allele or the *cI*$^+$ allele of *cI857* did not occur. Missense mutations and a single addition mutation (+A) are indicated by the nucleotide change. Note that three of the changes are located outside the region covered by the oligonucleotide used. These are marked on the central rectangle (the mutant above the rectangle was obtained in experiments with *cI857* phage and two mutations marked below were found in experiments with *cI*$^+$ phage). Individual clear plaques were purified and then PCR amplified with oligonucleotides corresponding to the ends of the *c*I gene, and both strands of the PCR products were sequenced. The sequence in the upper portion of the figure represents the oligonucleotides covering the *cI857* allele (enlarged GC pair). The *cI857* mutation is a G to A transition in the coding strand (Daniels et al., 1983), resulting in an alanine to threonine change at residue 67 (A67T) in the DNA binding domain. Mutations above the sequence represent those generated by transformation with the top strand (C), and those below the sequence were generated with the bottom strand (W) as per Table 1. The lower portion of the figure indicates experiments with E *cI*$^+$ in which a silent mutation (GC pair indicated in italics) was introduced with the oligonucleotide.

plaques. Fifteen carried the silent mutation indicating that they had undergone recombineering. Nine had a single base pair deletion, three had longer deletions, one mutant had an added AT base pair, one showed a CG to TA transition, and one had a GC to AT base substitution mutation located outside the region covered by the ss-oligo. The one mutant lacking the signature change had a CG to TA transition outside the region covered by the ss-oligo and may have been a spontaneous clear mutant.

Table 1
Recombineering oligonucleotides into phage E

Exp. #	Host	Oligo	E*c*$^+$ (%)	E *cI*$^-$ (%)
1	DY433	W	6	2
2	HME31	W	6	1.5
3	HME31	C	13	2
4	HME31 *rec*A	W	4	0.5
5	HME31	–	<0.05	<0.05
5	HME31	#100	<0.05	<0.05
6	HME31	C*	3.2	0.1

The experiments presented in this table were all carried out using E *cI857 ind*$^-$. The E*c*$^+$ and E *cI*$^-$ columns detail the percentage of recombinants and mutants among total plaques scored as follows: for E*c*$^+$, 500 – 2000 total plaques were scored at 37jC; for % *cI*$^-$, 1000 – 2000 total plaques were scored at 30jC. A detailed description of the strains and methods used can be found in Ellis et al. (2001). ss-oligos (0.05 nmol) were used in electroporations. W, template strand; C, coding strand (see Fig. 1, top). Oligo #100 is a 70-base oligonucleotide containing the wild-type *galK* sequence used by Ellis et al. to repair a *galK*$_{am}$ mutation. C*, gel purified oligonucleotide.

The results presented above suggest that most of the mutations were introduced during synthesis of the ss-oligos. Based on the results and chemistry of synthesis, one would expect that at each position of an oligonucleotide there would be an equal chance of not incorporating the added base (Hecker and Rill, 1998; Temsamani et al., 1995). Examination of the sequence changes among the frameshifts shows that they cluster toward the center of the ss-oligo. The terminal regions lack mutations, suggesting that complete base pairing at the termini may be important for efficient annealing to the phage DNA.

To reduce the frequency of frameshift mutations, we further purified the ss-oligos. Purification by HPLC did not reduce the mutation frequency (data not shown) probably because HPLC does not efficiently separate oligos of this length, whereas PAGE-purified oligonucleotides yielded efficient recombineering with fewer frameshifts (Table 1). This result supports the notion that base deletions originating during chemical synthesis of the oligonucleotides are responsible for generating mutations. Single base frameshift deletions occur rarely as spontaneous mutations (Schaaper and Dunn, 1991). In our examples, deletion mutations formed usually also carried the designed change present on the ss-oligo, suggesting that the frameshifts were

conferred by the synthetic ss-oligo. Thus, the experimental approach described here provides a simple and sensitive assay for oligonucleotide quality. We note that recombineering with unpurified synthetic oligonucleotides could also be used to provide an efficient way to introduce random single base deletions at specific sites in genes or regulatory regions. Our results do not suggest that the act of recombineering causes random mutagenesis.

When recombineering with the bacterial chromosome, one of two complementary ss-oligos gives more recombinants (Ellis et al., 2001; Zhang et al., 2003). This strand bias depends upon the direction of replication through the recombining region with the lagging strand being the more recombinogenic. In the phage crosses, both complementary oligos were equally efficient in promoting recombination at E *c*I (Table 1). We believe this is due to the rolling circle mode of phage DNA replication, which can roll in either direction (Takahashi, 1975). Thus, replication forks pass through *c*I in both directions and neither strand is exclusively leading or lagging.

In the cross with E *c*I*857*, we observed mottled plaques at 37jC, which suggested that the E DNA was packaged with a heteroduplex allele in *c*I (Huisman and Fox, 1986). We purified six independent mottled plaques and found that they gave rise to a mixture of turbid and clear plaques. Sequence analysis showed that in all cases the turbid plaques had incorporated the wild-type allele, whereas the clear plaques retained the original *c*I*857* mutation, indicating that the oligonucleotide paired with the phage chromosome and was incorporated without mismatch correction. These heterozygous phages are generated in *recA* mutant crosses, which suggests that the ss-oligo is annealed by Beta protein to single-strand gaps at the replication fork (Court et al., 2003; Stahl et al., 1997).

Discussion

The discovery of new phages and the identification of prophages in pathogenic bacteria yield genes that have no homolog in the database. With slight modifications, the techniques we have developed for E can be readily adapted for use with other less studied phages. We have successfully introduced mutations onto heteroimmune E, such as E*imm434* and E*imm21*. We anticipate that recombineering will permit rapid and complete genetic and functional analysis of newly identified phage genomes as well as more intricate study of well-known phages like E.

Materials and methods

Creating mutations with recombineering. The strains used for recombineering carry a defective E prophage containing the p_L operon under control of the temperature-sensitive repressor *c*I*857*. The genotype of one commonly used strain, DY330, is W3110 D*lacU169 gal490 pgl*D8 kcI*857* D*(cro-bioA)*. Others are listed in Ellis et al. (2001) and Yu et al. (2000); all are available from D. L. Court. The strain of choice is grown in a shaking water bath at 32jC in LB with 0.4% maltose to mid-exponential phase, A_{600} 0.4–0.6 (30 ml is adequate for several recombineering reactions). The culture is harvested by centrifugation and resuspended in 1 ml TM (10mM Tris base, 10mM $MgSO_4$, pH 7.4). The phage to be engineered is added at a multiplicity of infection of 1–3 phages/cell (we assume cell density of approximately 1 × 10^8/ml before concentration) and allowed to adsorb at room temperature for 15 min (this step would need modification for other phages, i.e., adsorption on ice). Meanwhile, two flasks with 5-ml broth are prewarmed to 32 and 42jC in separate shaking water baths. The infected culture is divided and half-inoculated into each flask; the cultures are incubated an additional 15 min. The 42jC heat pulse induces prophage functions; the 32jC uninduced culture is a control. After induction, the flasks are well chilled in an ice water bath and the cells transferred to chilled 35-ml centrifuge tubes and harvested by centrifugation at approximately 6500 × *g* for 7 min. The cells are washed once with 30-ml ice-cold sterile water; the pellet is quickly resuspended in 1-ml ice-cold sterile water and pelleted briefly (30 s) in a refrigerated microfuge. The pellet is resuspended in 200-Al cold sterile water and 50 – 100 Al aliquots are used for electroporation with 100 – 150 ng PCR product or 10 – 100 ng oligonucleotide. We use a BioRad *E. coli* Gene Pulser set at 1.8 mV and 0.1-cm cuvettes. Electroporated cells are diluted into 5 ml 39jC LB medium and incubated to allow completion of the lytic cycle. The resulting phage lysate is diluted and titered on appropriate bacteria to obtain single plaques (for more details, see Thomason et al., 2003).

Oligonucleotides. The oligonucleotides were purchased from Invitrogen without additional purification. The purified oligonuclotide was subjected to electrophoresis in a 15% PAGE-Urea gel, excised from the gel without direct UV irradiation and eluted using the Elutrap electro-separation system (Schleicher and Schuell). The size-purified oligonucleotide was then precipitated with isopropanol, washed with ethanol, dried, and stored at −20jC.

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