A set of recombineering plasmids for gram-negative bacteria

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Abstract

We have constructed a set of plasmids that can be used to express recombineering functions in some gram-negative bacteria, thereby facilitating in vivo genetic manipulations. These plasmids include an origin of replication and a segment of the bacteriophage λ genome comprising the red genes (exo, bet and gam) under their native control. These constructs do not require the anti-termination event normally required for Red expression, making their application more likely in divergent species. Some of the plasmids have temperature-sensitive replicons to simplify curing. In creating these vectors we developed two useful recombineering applications. Any gene linked to a drug marker can be retrieved by gap-repair using only a plasmid origin and target homologies. A plasmid origin of replication can be changed to a different origin by targeted replacement, to potentially alter its copy number and host range. Both these techniques will prove useful for manipulation of plasmids in vivo. Most of the Red plasmid constructs catalyzed efficient recombination in E. coli with a low level of uninduced background recombination. These Red plasmids have been successfully tested in Salmonella, and we anticipate that that they will provide efficient recombination in other related gram-negative bacteria.

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1. Introduction

Recombineering is a new means of in vivo genetic engineering that allows DNA modifications to be made easily and efficiently. It is a highly effective method for functional genomic analysis, engineering a wide variety of DNA rearrangements and combining genes with special genetic elements or tags (Copeland et al., 2001; Court et al., 2002). Recombineering allows a researcher to carry out DNA modifications and cloning without restriction enzymes or DNA ligases (Yu et al., 2000; Copeland et al., 2001; Court et al., 2002). The modifying DNA for recombineering is either a double-stranded (ds) PCR product (Murphy et al., 2000; Yu et al., 2000; Lee et al., 2001; Court et al., 2002) or a single-stranded oligonucleotide (oligo) (Ellis et al., 2001; Swaminathan et al., 2001) carrying short regions of target homology at the ends which can be precisely recombined in vivo with its substrate sequences onto any episome within the cell. Recombination between the short homologies is catalyzed by the λ Red functions, Exo, Beta and Gam. The λ Gam protein prevents degradation of transformed linear dsDNA by the host RecBCD and SbcCD nucleases (Unger and Clark, 1972; Kulkarni and Stahl, 1989) while Exo resects the 5’ ends of the dsDNA (Little, 1967) to generate 3’ ssDNA overhangs. Beta binds to these ssDNA overhangs, as well as to oligos, ultimately pairing them with a complementary ssDNA target (Karakousis et al., 1998; Li et al., 1998).

Our laboratory uses a defective λ prophage for optimal expression of the Red functions in E. coli (Yu et al., 2000; Ellis et al., 2001). This defective prophage contains the phage immunity region and the main leftward operon under control of the μI promoter (Fig. 1). The rightward operon encoding the DNA replication genes, lysis cassette, and the structural genes has been
removed by a deletion that extends from cro through the right attachment site, attR and into the bacterial biotin gene bioA. The exo, bet and gam genes are expressed from the pL operon under the control of the temperature-sensitive (ts) repressor, CI857. At low temperature (30–34 °C) the repressor remains active and blocks the pL promoter, shutting off transcription of the red genes. A brief temperature shift to 42 °C results in a transient denaturation of the repressor, allowing Red expression. On shifting back to low temperature the repressor renatures, binds to pL, and again turns off the Red system. Following heat inactivation of the repressor, the expression of gam, bet and exo are initially prevented by the transcription terminators present between pL and the red genes unless the N anti-termination function modifies RNA polymerase to prevent transcription termination (Gottesman et al., 1980).

Here we report a modification of the prophage strain and derive from it a set of plasmids carrying a minimal Red expression cassette under endogenous λ repressor control. These new vectors can be introduced by transformation to different bacterial backgrounds, and in some of them, a ts replication defect provides a means for easy curing from the host. We also report two recombinering applications that are useful for plasmid engineering in general: 1) a technique to clone any gene with a linked drug marker to a multicopy vector by gap-repair and 2) a means to alter the origin of replication (ori) of a plasmid, hence changing its copy number and host specificities.

2. Materials and methods

2.1. Bacterial strains

Unless otherwise specified, strain construction was done using recombinering technology (Yu et al., 2000; Ellis et al., 2001). Strain DY378 is W3110 {λ, CI857Δ(cro-bioA)} (Yu et al., 2000). DY406 was constructed by replacing kil to sieB of λ nucleotide (nt) 33246–35015 (Daniels et al., 1983) in DY378 with a cassette containing both the chloramphenicol (cat) and sacB genes (Lee et al., 2001). DY432 was constructed from DY406 by replacing the cat-sacB cassette plus adjacent λ DNA sequence using oligo recombinering to generate a deletion from N tokil and fusing nt33169 to 35446 of λ. The loss of the catsacB cassette makes DY432 resistant to 5% sucrose and sensitive to chloramphenicol.

The rex genes downstream of CI857 in DY432 were replaced by a drug cassette, either cat or amp, to generate chloramphenicol resistant (CmR) SIMD3 or ampicillin resistant (ApR) SIMD4. Both the cat and amp cassettes contain their own promoter region as described by Yu et al. (2000) and Thomason et al. (2005). The cassettes replace the DNA segment from the ATG of rexA to the stop codon of rexB, deleting λ nt 35828–37114 (Daniels et al., 1983). These drug cassettes are in single copy on the chro-
metrosome, thus lowered levels of antibiotic are used for selection (30 μg/ml ampicillin and 10 μg/ml chloramphenicol). These prophage-carrying strains are deleted for bioA and require biotin for growth on minimal media.

HME6 is W3110 Δ(argF-lac)U169 galKTYR145UAG {XcI857Δ(cro-bioA)} (Ellis et al., 2001) while HME57 is Δ(argF-lac)U169 galKTYR145UAG. P1 transduction was used to create HME57 from HME6 by bringing in the wild-type λ attachment site attB and the bioA region from W3110, selecting for Bio ³ recombinants. HME57 thus lacks the λ prophage but retains the galK amber mutation.

Salmonella enterica serovar Typhimurium LT2 (S. enterica) and its derivative TS616 (his616 tly452 metA22 metE551 mal616::Tn10 galE496 xyl404 rpsL120 fla66 hsdL6 hsdSA29) were obtained from Dr. L. Thomason. The E. coli bacterial strain BR825 (pola468 Tn10) having the rexA1 mutation with the Gln codon at position 298 converted to an amber (Kelley and Joyce, 1983) was obtained from the laboratory of Dr. S. Austin.

2.2. Amplification of the origin of replication of plasmids

Standard PCR conditions were used to amplify the DNA replication origins of different plasmids using a high fidelity Taq DNA Polymerase. Each primer used for the PCR is a hybrid where the 5’ end contains the target homology and the 3’ end primes the origin fragment. The ori of pBR322, from nt 2348–3296 (GenBank accession no. 208958), was amplified as a 949 bp fragment using primers SD1 and SD2 (Table 1) with pPCR-Script Amp (Stratagene, La Jolla, CA) as template. The 5′ end of SD1 has 54 bases of homology to DNA downstream of the stop codon of exo including the transcription terminator tL3, corresponding to nt 31232–31285 of λ (Daniels et al., 1983). SD2 has 41 bases of homology including the pR promoter and part of cro corresponding to nt 38011–38051 of λ (Daniels et al., 1983).

Likewise, the ori of plasmids pSC101 repA⁴, which corresponds to nt 4524–6736 of pSC101 (GenBank accession no. 47833) and pBBR1, nt 800–2517 of pBBR1 (GenBank accession no. X66730) were amplified by PCR using primer pairs SD3–SD4.

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>PCR product</th>
</tr>
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<tbody>
<tr>
<td>SD1–SD2</td>
<td>pBR322 ori flanked by λ homology</td>
</tr>
<tr>
<td>SD3–SD4</td>
<td>pSC101 ori flanked by λ homology</td>
</tr>
<tr>
<td>SD5–SD6</td>
<td>pBBR1 ori flanked by λ homology</td>
</tr>
<tr>
<td>SD7–SD8</td>
<td>RK2 ori flanked by λ homology</td>
</tr>
<tr>
<td>SD9–SD10</td>
<td>amp cassette targeting galK of E. coli</td>
</tr>
<tr>
<td>SD11–SD12</td>
<td>amp cassette targeting galK of S. enterica</td>
</tr>
</tbody>
</table>

Sequences are available on request. The primers were obtained from Integrated DNA Technologies as salt-free but otherwise unpurified.
and SD5–SD6, respectively (Table 1). The RK2 replication origin, oriV, and a trfA\(^{59}\) gene were amplified by PCR with primer pair SD7 and SD8 using pRR10-ts97 as a template, which corresponds to nt 7478–9962 of prRR54, a derivative of pRR10 (GenBank accession no. AY297462, (Roberts et al., 1990). For all PCR amplifications of plasmid origins the 5′ ends of each primer contained the same λ sequences. All PCR products were purified with a PCR clean-up kit (Qiagen, Valencia, CA) and introduced into the recombinogenic bacterial cells by electroporation.

2.3. Preparation of cells for recombineering

The cultures to be used in recombineering were prepared as described previously (Yu et al., 2003; Thomason et al., 2005). For DNA retrieval by gap-repair, the PCR-amplified linear ori fragment of pBR322 was electroporated into SIMD3 or SIMD4 that had been induced 15 min at 42 °C for Red expression and subsequently made electro-competent. Following electroporation the culture was diluted with 10 ml LB and grown non-selectively overnight at 32 °C. Plasmid DNA was then purified and used to transform strain W3110, selecting for Cm\(^{R}\) or Ap\(^{R}\) as appropriate. Plasmids (pSIM2, Cm\(^{R}\) or pSIM4, Ap\(^{R}\)) from these purified transformants were isolated and checked by restriction analysis to show the presence of the λ genes and pBR322 origin.

To replace the pBR322 ori with another origin, W3110 carrying pSIM2 or pSIM4 was induced for Red expression at 42 °C followed by electroporation of PCR product containing the desired plasmid origin and target homologies. After overnight growth of the electroporated culture in 10 ml LB at 32 °C, plasmid DNA was extracted and used to transform the polA mutant strain, BR825, selecting for the appropriate drug resistance. Plasmid DNA was again isolated from BR825 and checked by restriction analysis to confirm the loss of the pBR322 origin and the gain of the new origin.

2.4. Recombination assays with an oligo and dsDNA

The oligo #144 (Costantino and Court, 2003) used for recombineering corrects the amber TAG stop codon of the E. coli galK gene to a TAC tyrosine codon. HME57 was transformed with the different Red plasmids from this study. A saturating level of oligo (5 pmol) was electroporated into these plasmid-containing strains that had been either induced for Red expression by growth at 42 °C for 15 min or that had remained uninduced. After electroporation 1 ml LB was added and cells were incubated at 32 °C for 2 h with shaking. Gal+ recombinant colonies were selected on M63 minimal galactose plates with biotin, and total viable cells were counted on LB agar (Costantino and Court, 2003).

For recombineering in S. enterica, oligo #337 (5′AAGTGGCGGCTGGCACCCGTCTTTCCAGCAGCTTTAC-CACCTGCGCTGGACGGCGGCAAAATTGCCTCTCAA) was used to generate a mutation in the galK gene. In this oligo the 145th codon of the Salmonella galK (nt 19679–20840, GenBank accession no. AE006468) was changed from a tyrosine to a stop codon followed by 4 adjacent base changes, as indicated by nucleotides in lower-case. A correcting oligo #336 (5′ AAGTGGCGGCTGGCACCCGTCTTTCCAGCAGCTTTAC-
SIMD3 carries the cat cassette while the prophage in SIMD4 contains the amp cassette.

3.2. Creating Red expression plasmids

Two different in vivo techniques, described below, were used to create a set of Red expression plasmids. These Red expression vectors were constructed by combining the minimal defective λ prophage from either SIMD3 or SIMD4 with various plasmid origins.

3.2.1. Gap-repair method

The replication origin of pBR322 was amplified by PCR to generate a linear ori DNA flanked by homology to the prophage. This 949 bp linear fragment contains the minimal plasmid ori and lacks both the copy number control region and a drug marker. The fragment was used to retrieve the prophage from SIMD3 and SIMD4 in a gap-repair reaction catalyzed by Red (Fig. 2B). Recombination of the linearized ori vector with the prophage target restores a circular plasmid that is able to replicate. Only those plasmid clones that have undergone successful recombin- neering will also inherit the antibiotic resistance marker from the prophage, allowing selection in the presence of appropriate drug.

3.2.2. Exchanging plasmid origins by recombineering

We created a lower copy number Red expression vector with the DNA replication origin of pSC101 by engineering a precise replacement of the pBR322 ori segment in pSIM2 and pSIM4 with the ori and repA′ gene of pSC101′. After recombineering and overnight growth at 32 °C in non-selective LB medium,
plasmid DNA was isolated and used to transform the polA mutant strain, BR825, selecting for the appropriate drug resistance at 32 °C. Since the pBR322 origin does not replicate in a polA mutant strain (Kingsbury and Helsinki, 1970), only clones in which the pBR322 ori was replaced by the pSC101 ori replic, generating pSIM5 and pSIM6 (Table 2). This same technique of selecting for recombinants in a polA mutant was also used to replace the pBR322 ori segment of pSIM2 and pSIM4 with the broad host range pBBR1 origin, creating pSIM7 and pSIM8, and with the broad host range RK2 ts origin, creating pSIM9.

3.3. Recombineering with an oligo

We tested the recombination efficiency of the various Red expression plasmids by correcting a galK amber mutation in HME57 with oligo #144. Similar experiments were performed with the defective chromosomal prophage strains, HME6 and SIMD3, to compare the recombineering efficiencies of these strains with the Red plasmid systems. All Red plasmids catalyzed recombination efficiently yielding ≥10^9 Gal− recombinants per 10^8 viable cells (Table 3). Upon annealing, oligo #144 creates a C:C pairing that is not repaired by the host mismatch repair system and thus gives this very high recombination frequency (Costantino and Court, 2003).

We also used HME57 carrying pKD119 (Datsenko and Wanner, 2000) to carry out similar recombination assays. Using 1 mM arabinose to induce Red expression, Gal− recombinants were generated at a frequency of 2.8 × 10^7/10^8 viable cells; this is about 100-fold lower than that obtained using the prophage-expression plasmids (Table 3). HME57 is Ara− and will metabolize the arabinose, affecting the induction of ParaBAD. We found that raising the arabinose concentration to 10 mM increased the frequency of Gal− transformants (Table 3) but pKD119 was still less efficient than the prophage regulated systems described here.

3.4. Control of Red expression-background recombination

The expression of Gam, Beta and Exo from multicopy plasmids may not be as tightly controlled as in the chromosomal context.

Table 3
Red-mediated oligo recombineering in E. coli

<table>
<thead>
<tr>
<th>Source of Red</th>
<th>Plasmid origin</th>
<th>Gal− recombinants/10^8 viable cells</th>
<th>E. coli</th>
<th>S. enterica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Red induced</td>
<td>Red uninduced</td>
<td></td>
</tr>
<tr>
<td>HME6^b</td>
<td>NA^c</td>
<td>1.5 × 10^7</td>
<td>1.8 × 10^7</td>
<td></td>
</tr>
<tr>
<td>SIMD3^b</td>
<td>NA^c</td>
<td>9.0 × 10^6</td>
<td>2.7 × 10^6</td>
<td></td>
</tr>
<tr>
<td>pSIM2</td>
<td>pBR322</td>
<td>1.7 × 10^7</td>
<td>1.1 × 10^7</td>
<td></td>
</tr>
<tr>
<td>pSIM5</td>
<td>pSC101</td>
<td>1.8 × 10^7</td>
<td>1.5 × 10^7</td>
<td></td>
</tr>
<tr>
<td>pSIM7</td>
<td>pBBR1</td>
<td>2.7 × 10^7</td>
<td>1.5 × 10^7</td>
<td></td>
</tr>
<tr>
<td>pSIM9</td>
<td>RK2</td>
<td>1.4 × 10^7</td>
<td>1.6 × 10^7</td>
<td></td>
</tr>
<tr>
<td>pKD119</td>
<td>pSC101</td>
<td>2.8 × 10^8</td>
<td>7.3 × 10^8</td>
<td></td>
</tr>
</tbody>
</table>

^a Values indicated are the average of 3 or more experiments.
^b See Section 2.1 for complete genotype.
^c Not applicable.
^d 1 mM arabinose.
^e 10 mM arabinose.

Constitutive expression of Gam would inactivate RecBCD, which can affect cell growth and interfere with plasmid replication (Sergeev et al., 2001; Court et al., 2002) while leaky expression of recombination functions leads to unwanted recombination events, especially at repetitive sequences (Murphy and Campbellone, 2003). We assayed the oligo recombination in uninduced cells for an indication of background Red expression. In the strains that carry chromosomal prophages, the background recombination was 2 × 10^5 Gal− recombinants per 10^8 viable cells (Table 3). The pBR322-based plasmid (pSIM2) lacking copy number control gave an unacceptably high number of recombinants (1 × 10^5) in the absence of Red induction. The strain carrying pSIM2 grew abnormally slow even in rich media, which is likely the result of unwanted expression of the Red functions. In strains carrying the lower copy number pSC101-derived pSIM5 and RK2-derived pSIM9, the level of unwanted background recombination was as low as that observed with the chromosomal prophages, making them ideal candidates for recombineering. pSIM7, having a pBBR1 origin and intermediate copy number, generated an intermediate 1.5 × 10^4 Gal− recombinants/10^8 viable cells.

3.5. Recombineering with dsDNA

We compared the recombineering efficiency of the various plasmids with linear dsDNA by assaying the frequency of galK gene replacement with an amp cassette. Ap^R recombinants were selected at 32 °C and their Gal− phenotype was confirmed by streaking on Mac-Gal indicator agar. All Ap^R colonies tested gave colorless (Gal−) colonies as expected. All pSIM expression plasmids yielded more than 10^4 Ap^R recombinants/10^8 viable cells, levels similar to the defective prophages (Table 4). Induction of pKD119 with 10 mM arabinose generated recombinants, but again at a 10-fold lower level than the pSIM plasmids.

3.6. Recombineering in Salmonella

S. enterica was transformed with pSIM5 and a Gal− derivative was made by recombineering with oligo #337. This Gal− strain was used to test the efficiency of pSIM5 in catalyzing oligo
recombineering in *Salmonella* using oligo #336 to repair the mutation in *galK*, thereby converting the strain back to Gal⁺. On average, $5 \times 10^6$ Gal⁺ recombinants were observed per $10^9$ viable cells, while the background recombination in uninduced cells was $4 \times 10^2$ Gal⁺ colonies/$10^8$ cells surviving electroporation.

*S. enterica* and its derivative TS616, transformed with various Red expressing plasmids, were used to study dsDNA recombineering using a PCR-generated *amp* cassette targeted to replace the *S. enterica galK* gene. Ap⁺ *Salmonella* recombinants were recovered at a frequency of $10^3$ per $10^8$ viable cells (Table 4). *Salmonella* carrying pKD119 again gave about a 10-fold lower frequency of Ap⁺ transformants relative to pSIM5.

### 3.7. Curing of plasmids

The ts-replicons of pSIM5 and pSIM9 permit curing of plasmids by growing the host strains at 37 °C once the desired recombination is confirmed. W3110 carrying either plasmid was first grown at 32 °C then shifted to 37 °C (see Section 2.5). After 2 h growth at 37 °C pSIM9 was lost from 60% of the colonies screened while pSIM5 was lost from only 4% of the colonies. However, when allowed to grow for 4 h at 37 °C, both pSIM5 and pSIM9 were lost from 92% of the colonies. After 8 h of growth at 37 °C all colonies tested had lost the plasmid. For plasmid curing, it is preferable to use 37 °C as the *pL* operon remains mostly repressed by CI857 at this temperature, blocking unwanted Red expression in the host strain.

### 4. Discussion

Here we report the construction of a series of Red expression plasmids, retaining the native λ control, but with a wider host range. Recombineering techniques were used to create these vectors by in vivo plasmid cloning. We utilized the λ Red system to both gap-repair a DNA segment of choice onto a vector and exchange the plasmid origin to alter copy number and/or host range.

The plasmids constructed in this study coordinate express *gam*, *bet* and *exo* of λ in their natural prophage context under tight regulation, thereby achieving high levels of recombination with a low induced background similar to that of the chromosomal prophage system. Modifications ranging from point mutations to large substitutions can be created using these plasmids. These plasmids can readily transform and catalyze recombination in *recA* mutant strains where the defective λ prophage cannot be easily introduced by P1 transduction. They will be particularly useful in engineering BACs or P1 artificial chromosome (PACs) carrying large DNA clones, which are usually propagated in the *recA* mutant strain DH10B (Lee et al., 2001). The plasmids can be easily introduced where a special *E. coli* background is required; plasmids with the ts-replicons can be easily eliminated from such strains allowing the newly created modifications to be retained in a plasmid-free background.

All the plasmid vectors constructed here contain a minimal λ prophage in which most of the unwanted regions of the *pL* operon have been removed, including the toxic *kil* gene, transcription terminators, and the anti-termination gene *N*. The *pL* promoter on these constructs is regulated by the *ts* CI857 repressor, and the presence of the λ *oX* and *oR* operators ensure the tightest repression control (Dodd et al., 2001). We have retained the native tL₃ transcription terminator downstream of *exo* in all the plasmids. This prevents excessive transcription beyond *exo* into the *ori* region; such transcription interferes with plasmid replication. A plasmid identical to pSIM2 but without the *tL₃* terminator could not be established in a standard strain lacking a λ repressor.

Tight regulation of gene expression by the λ repressor and the low copy number of the plasmid expression system appear to be critical for effective recombineering. The pSIM2 and pSIM4 plasmids have unacceptably leaky expression of Red and should not be used for recombineering. On the other hand, the most promising of the plasmid systems are pSIM5 and pSIM9; these have a ts-replicon and catalyze recombination efficiently with a low level of unwanted constitutive recombination. pSIM7 gave a recombineering efficiency similar to pSIM5 and pSIM9 but generated a 10-fold higher background. Using the native λ control for Red expression in our vectors we observed higher recombination frequencies than we obtained with pKD119, another pSC101-based *ts-ori* plasmid, where Red genes are taken completely out of the context of λ control and are transcribed from the *ParaBAD* promoter (Datsenko and Wanner, 2000).

The pSIM2 plasmid was generated by recombineering technology using a gap-repair method. This method entailed the retrieval the *red* genes from the defective λ prophage onto a linear 949 bp DNA segment carrying the pBR322 *ori* flanked by appropriate homologies for recombination with the prophage. This modified application of gap-repair makes it convenient to clone almost any region linked to a drug marker. This method can replace standard cloning technology and has the advantage that the cloned segment is not replicated in vitro, eliminating the potential for mutations caused by PCR. A second advantage is that the presence of a drug marker at the target DNA rather than on the linear retrieval fragment allows only the desired recombineant plasmids to emerge with the drug marker, minimizing unwanted background of end-joined vector (Zhang et al., 2000).

We changed the plasmid origin of our pBR322-based plasmids by the precise replacement with origins of other plasmids to alter both copy number and confer different host ranges. The ability to “swap out” a pBR322 origin relies on the inability of pBR322 to propagate in a *polA* mutant. This is a very useful trick to get rid of the pBR322 backbone and replace it with another origin of lower copy number, of different host range, or carrying special elements such as expression promoters and gene tags for example. Such an in vivo trade of plasmid DNA is not limited to origins but can also be used to exchange drug markers. In fact two other pSIM5 derivatives, pSIM18 and pSIM19 have been made by changing the Cm⁺ marker in pSIM5 to hygromycin resistant and spectinomycin resistant respectively (Dr. Pentao Liu and Dr. David Friedman, personal communication).

We expect that our Red expression plasmids will be able to catalyze recombineering in Gram-negative bacteria closely related to *E. coli* and *S. enterica*. It is preferable to first attempt oligo recombineering in different organisms as this requires only λ Beta protein (Ellis et al., 2001) and is more likely to be successful. Intelligent design of oligos helps to increase the
number of recombinants, even in strains competent for mismatch repair. For example, if the recombining oligo forms a C:C mismatch when annealed to the target sequence (Costantino and Court, 2003) or has 4–6 adjacent base changes covering the desired mutational site (N. Costantino, unpublished), it evades the methyl-directed mismatch repair system, maximizing recombination. If oligo recombining is successful then dsDNA engineering should be attempted. In dsDNA recombining, λ Gam is able to protect the electropropated linear dsDNA from degradation by inhibiting RecBCD of E. coli and Salmonella, but in other organisms this may not be the case. For more distantly related Gram-negative bacteria and Gram-positive species, development and use of an alternate phage recombination system may be required. Such systems could be modified and adapted for use on plasmid vectors similar to that shown in this study.

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