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Oligonucleotide Recombination in Gram-Negative Bacteria

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Summary

This report describes several key aspects of a novel form of RecA-independent homologous recombination. We found that synthetic single stranded DNA oligonucleotides (oligos) introduced into bacteria by transformation can site-specifically recombine with bacterial chromosomes in the absence of any additional phage encoded functions. Oligo recombination was tested in four genera of Gram-negative bacteria and in all cases evidence for recombination was apparent. The experiments presented here were designed with an eye towards learning to use oligo recombination in order to bootstrap identification and development of phage encoded recombination systems for recombineering in a wide range of bacteria. The results show that oligo concentration and sequence have the greatest influence on recombination frequency, while oligo length was less important. Apart from the utility of oligo recombination, these findings also provide insights regarding the details of recombination mediated by phage-encoded functions. Establishing that oligos can recombine with bacterial genomes provides a link to similar observations of oligo recombination in archaea and eukaryotes suggesting the possibility that this process is evolutionary conserved.

Keywords

Recombineering; *Pseudomonas*; Homologous Recombination; Oligonucleotide; Gene Conversion

Introduction

Homologous recombination is conserved across all kingdoms of life and serves a fundamental role in promoting genetic exchange between two identical or nearly identical DNA molecules. The primary functions of the endogenous homologous recombination pathways in bacteria are DNA replication repair, DNA damage repair and for genomic plasticity as a mechanism to integrate horizontally acquired DNA into a recipient genome (Kuzminov, 1999; Lusetti and Cox, 2002; Thomas and Nielsen, 2005). These pathways have the potential to result in either reciprocal exchange (crossing over) or gene conversion events, in which there is unidirectional transfer of sequence information from donor to recipient molecule. RecA-mediated homologous recombination reactions involve the formation of an intermediate in which a 3' single stranded DNA (ssDNA) end invades a

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second DNA molecule and anneals with the complementary homologous strand generating a D-loop structure (Amundsen and Smith, 2003). A characteristic feature of the RecA-mediated pathways is that extensive homologies are required for the reaction to be efficient (Lovett *et al.*, 2002).

Bacteriophage encoded homologous recombination systems also have the potential to contribute to genetic exchange in bacteria (Murphy, 1998; Zhang *et al.*, 1998; Yu *et al.*, 2000; Datta *et al.*, 2008; van Kessel *et al.*, 2008). The bacteriophage lambda encodes recombination functions, known as lambda Red, that are expressed during lytic growth and facilitate phage replication as well as the transfer of DNA between phage and/or host bacterium (Poteete, 2001). The lambda Red functions have been adapted for recombineering, an *in vivo* cloning technology that has broad application for engineering recombinant DNA molecules (Ellis *et al.*, 2001; Court *et al.*, 2002; Sawitzke *et al.*, 2007). The Red functions catalyze RecA-independent gene conversion events by facilitating recombination between two DNA molecules, generally a genomic DNA target (either chromosomal or plasmid) and a linear DNA substrate that is introduced into the cell by transformation. The Red functions are encoded by the *bet*, *exo* and *gam* genes and enhance the rates of recombination by generating/stabilizing a single-stranded substrate and facilitating hybridization of the substrate DNA with the target molecule (Little, 1967; Cassuto and Radding, 1971; Kmiec and Holloman, 1981; Karakousis *et al.*, 1998). When ssDNA is supplied as the substrate for recombination only the *bet* gene product, Beta, is required (Ellis *et al.*, 2001). Additionally, the shortest fragments that can recombine are limited by the ability of Beta to bind, and therefore efficient Beta-mediated recombination requires substrate DNA be at least 30 to 40 bases long (Mythili *et al.*, 1996; Ellis *et al.*, 2001; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation).

Recently, Dutra *et al.* 2007 reported using DNA oligonucleotides (oligos) to direct a recombineering-like manipulation of plasmid DNA in *Escherichia coli* without exogenous phage recombinase functions (Dutra *et al.*, 2007). Although the frequency is very low, this mode of recombination resembles lambda Red-mediated recombination particularly with respect to the replication imposed strand bias on gene conversion events directed by oligos and the lack of requirement for RecA. Similar results have also been reported as negative controls used to characterize the background rates of recombination in the absence of lambda Red functions (Datta *et al.*, 2006; Datta *et al.*, 2008).

In the present study, the salient characteristics of this RecA-independent/background mode of recombination were evaluated in terms of its capacity to mediate changes to bacterial chromosomes. Here, we demonstrate that recombination occurs at low but detectable frequencies between oligos and the chromosomes of *E. coli*, *Salmonella typhimurium*, *Shigella flexneri* and *Pseudomonas syringae* suggesting that this mode of oligo recombination is evolutionarily conserved among the gamma subdivision of proteobacteria. This discovery may enable practical recombineering strategies in a wide variety of species.

Results

Single stranded DNA oligos recombine with bacterial chromosomes at specific loci

In the course of attempting to develop a lambda Red-mediated recombineering strategy for use in *P. syringae*, we noticed a significant number of recombinants generated in control reactions in which none of the lambda Red genes were present. These recombinants were generated, simply, by introducing ssDNA oligos directly into *P. syringae* cells by electroporation. The substrate oligos used in these experiments were homologous to regions of the *P. syringae* genome and included changes (point mutations and deletions, see below) centered in these sequences that conferred a selectable phenotype when the change was

incorporated into the genome (Fig. 1). The presence of the directed mutation was then confirmed by sequence analysis, which showed that the chromosomal allele had been converted to match the sequence encoded in the transformed oligo.

The *rpsL* gene was used to evaluate the ability of this method to direct specific point mutations in the *P. syringae* chromosome and to investigate other characteristics of this mode of recombination (see below). In these experiments, the oligos were designed to match a region of the *rpsL* gene centered on the lysine codon (AAA) at position 43. The oligos contained nucleotide substitutions changing the lysine codon to arginine (K43R), which confers resistance to streptomycin and enables the selection of recombinants. The recombination frequency was calculated by determining the number of colonies that grew on streptomycin containing selective media and standardized to 10^8 viable cells. Using this method, *P. syringae* cells transformed with 5 μg of an 84 nt oligo encoding a 4 bp change (oSWC1255) yielded 2400 recombinants per 10^8 viable cells. The recombination frequency with this oligo was nearly 300,000-fold higher than the background rate of streptomycin resistance ($8.6 \times 10^{-3}/10^8$) in control transformations in which no oligos were added. Sequencing of the streptomycin resistant *rpsL* allele of 6 independent recombinants confirmed that each had acquired the changes directed by the oligo, including a silent marker change in the wobble position of an adjacent codon.

The *rpsL* gene was also used to test whether oligos could recombine with the chromosomes of other species in the absence of the Red functions. To do this, *E. coli*, *Salmonella typhimurium* and *Shigella flexneri* cells were made electrocompetent and transformed with 300 ng of an oligo (str2) that confers streptomycin resistance after recombination with the *rpsL* gene (Table 1). Comparisons of the results of these transformations to negative controls suggest that each of these species is capable of undergoing recombination with the transformed oligos. Similar results were also observed using a different oligo that targeted the *rpoB* gene and conferred rifampicin resistance upon recombination (Table 1).

The capacity of this mode of recombination to delete a segment of the chromosome was also investigated. To produce a deletion, *P. syringae* cells were transformed with an 83 nt oligo (oSWC61) that had sequence identity to the 5' and 3' ends of the *upp* gene (Fig 1B) and recombinants were identified by acquisition of resistance to 5-fluorouracil (5-FU). An average of 192 5-FU resistant clones were obtained per 10^8 viable cells from three independent transformations. Additional screening was necessary to identify the recombinants because the frequency of 5-FU resistance in transformations with the oligo directing the deletion is approximately the same as control transformations in which no oligo was added ($119/10^8$ cells). Eleven representative 5-FU resistant isolates were chosen at random and of these three (3/11) were confirmed by PCR to have the deleted *upp* gene. Sequence analysis of the deleted *upp* allele in these clones showed that the *upp* gene had been deleted precisely as defined by the mutagenic oligo.

Oligo concentration has nonlinear effects on recombination frequency

The amount of oligo used in the transformations was varied in order to assess the influence of oligo concentration on recombination frequency. In this experiment, *P. syringae* cells were transformed in a constant volume containing 0.5 μg , 1 μg , 5 μg , 10 μg or 20 μg of the *rpsLK43R* encoding oligo (oSWC1255) and the frequency of recombination was determined. A 285-fold difference in frequency was observed over the entire range of oligo concentrations tested (Fig. 2A). An 80-fold increase in recombination was noted between 1 μg and 5 μg , which is disproportional to the 5-fold change in oligo concentration. Above 5 μg the concentration of oligo is saturating and no higher recombination is observed. This result suggested that there was an oligo concentration threshold that, if exceeded, would overcome an inhibitory effect on recombination. To test whether the concentration threshold

was sequence dependent, *P. syringae* cells were transformed with 1 μg of the *rpsLK43R* oligo (oSWC1255) in the presence or absence of 4 μg of non-homologous “carrier” oligo (Fig. 2B). Consistent with the threshold hypothesis additional carrier oligo improved the transformation efficiency to a level that was comparable to the full 5 μg of the specific homologous oligo. This result suggests that the barriers inhibiting recombination could be overcome by transformation of excess oligo and that titration of the inhibitor is not sequence dependent.

The effect of oligo concentration on the recombination frequency was also tested in *E. coli*, by transforming cells with two different amounts of oligo 144. This oligo encodes homology to the *galK* gene and directs a single base change that converts the TAG amber codon at position 145 of *galK* in strain HME57 to a TAC tyrosine codon, generating Gal⁺ recombinants (Costantino and Court, 2003). When 0.1 μg of oligo 144 is used the Gal⁺ frequency is $2.3 \times 10^3/10^8$; however, increasing the oligo concentration 100-fold to 10 μg increased the recombination frequency to $3.5 \times 10^4/10^8$. These results show that in *E. coli* the concentration of the oligo does indeed affect the recombination frequency, however, over the range of concentrations tested the degree of the effect was less pronounced than what was observed in *P. syringae*.

Strand annealing dynamics limit oligo lengths that recombine efficiently

Lambda Red-mediated recombination displays a dramatic oligo length dependence (Ellis *et al.*, 2001; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation) that has been attributed to the requirement for the substrate oligos to be long enough to be bound by Beta (Mythili *et al.*, 1996). Oligos shorter than 22 nt recombine at a low rate that is the same in the presence or absence of Beta (data not shown). To investigate the effect of oligo length in the absence of lambda Red functions, *P. syringae* cells were transformed with six oligos of different lengths. The oligos were designed to match the lagging strand with two flanks of various lengths (i.e., 5/5, 10/10, 20/20, 30/30, 40/40 and 60/60) on each side of the four base *rpsLK43R* change (see oSWC1978, oSWC1518, oSWC1251, oSWC1253, oSWC1255 and oSWC1257). There appears to be a threshold between 10 and 20 nt above which recombinants were obtained at equivalent frequencies regardless of the length of the homologies encoded in the oligos (Fig. 3A). Transformation with 10 nt homology oligos resulted in no increase above spontaneous background.

The influence of oligo length was also evaluated in *E. coli* using eleven oligos that covered a range of lengths between 12 to 70 nt. Each of these oligos matched the lagging strand and encoded the same C to G change that corrects the nonsense mutation in the *galK* gene, conferring galactose prototrophy as described above. Overall, only small differences in recombination frequency were observed among oligos longer than 20 bases (Fig. 3B). In both *P. syringae* and *E. coli*, the absence of increases in efficiencies proportional to increases in oligo length suggests that sequences longer than 20 nt exceed the length at which binding energies impact the binding/dissociation rates. However, for oligos shorter than 20 nt there appears to be a continuous decrease in recombination frequency that is correlated with decreasing oligo length until recombination can no longer be detected at 12 nt, suggesting that for oligos shorter than 20 nt, the recombination frequency is affected by the annealing characteristics of the substrate oligo.

To help determine whether the length threshold was a function of annealing thermodynamics, the recombination frequency was measured in two strains that have different GC contents in the 20 bp region flanking the *galK145* amber codon. A strain was constructed by substituting A or T nucleotides at selected positions in nearby codons, creating silent mutations which reduced the GC content of this region from 52% to 24%. The recombination frequency was then assessed in the parental (52% GC) and reduced GC

(24% GC) strains using oligos that correct the amber codon (TAG to TAC) but otherwise match the sequence in the respective strains. We found that reducing the GC content reduced the recombination frequency for short oligos (21 nt or 23 nt), as would be expected if the strength of annealing is limiting the recombination efficiency (Table 2). Longer oligos were unaffected by the GC changes. These results are consistent with the idea that the length threshold is governed primarily by the thermodynamics of strand annealing and for this particular region, oligos of 21 nt are close to the length where changing the GC content affects the annealing characteristics of the oligo and the recombination frequency.

Oligo sequence influences recombination frequency

The specific sequence mismatches encoded by the oligos used to direct point mutations have been shown to influence the frequency of recombination for lambda Red-mediated recombineering (Costantino and Court, 2003; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation). For example, during Red-mediated recombination, oligos generating a C-C mismatch recombine ~100-fold more efficiently than those generating a T-C mismatch at the same position. Genetic and biochemical evidence supports the idea that this is due to a hierarchy for recognition of specific combinations of mispaired bases by the bacterial methyl directed mismatch repair (MMR) system (Kramer *et al.*, 1984; Costantino and Court, 2003). The effect of MMR on the frequency of Red-independent oligo recombination was tested in *E. coli*. To do this, cells were transformed with lagging strand oligos that encoded either a C-C mismatch (which is not detected by MMR) or a T-C mismatch (which is detected by MMR) and the frequency of recombination was assessed using the galactose prototrophy assay. The data show that the oligo encoding the C-C mismatch is 365 times more efficient than the oligo encoding the T-C mismatch. Additionally, transformation of an isogenic *mmr*- *E. coli* strain with the lagging strand oligo encoding a T-C mismatch produced recombinants at a frequency that was indistinguishable from the C-C mismatch. These results support the idea that MMR influences the frequency of Red-independent recombination events in a manner that is similar to what has been observed for lambda Red recombination.

The inherent asymmetry of DNA replication establishes intermediate states that differ between the two replicating strands, which influences a variety of recombination events. Previous work has shown that the strand used to mediate recombination affects the frequency of recombination in both phage-mediated (Ellis *et al.*, 2001; Li *et al.*, 2003; van Kessel and Hatfull, 2008) and RecA-independent oligo recombination with plasmids in *E. coli* (Dutra *et al.*, 2007). To investigate whether this strand bias also influences recombination of oligos with the *E. coli* and *P. syringae* genomes, cells were transformed with complementary oligos that annealed to the leading or lagging strand of the chromosome. The recombination frequencies were then determined using either the galactose prototrophy or streptomycin resistance assays for *E. coli* or *P. syringae* respectively. In wild-type *E. coli* cells, oligos matching the lagging strand were 3-fold more efficient than oligos matching the leading strand at generating recombinants (Table 3), whereas in the *mutS* strain the lagging strand oligo was nearly 10-fold more efficient. The difference in the degree of the observed strand bias between the wild-type and *mutS* strains is due to the specific mismatches encoded by oligo 100 and 101 and differences in the efficiency with which those mismatches are repaired. Accordingly, the effect of the strand bias is more accurately represented in the *mutS* strain because the confounding effects of MMR are eliminated.

In *P. syringae* the lagging strand oligo was on average 6-fold more efficient than the oligo matching the leading strand (Table 3). The three-nucleotide changes used in these oligos to generate drug resistance may be targeted for MMR. Comparison of the frequency of the lagging strand oligo used in this experiment (oSWC57) to transformations with similar

oligos encoding a 4 base change (Figs. 2a, 2b and 3a), indicates that oligos encoding the 4 base change are 10-fold more efficient. This result resembles observations in *E. coli* where the number of mismatches influences the degree to which MMR affects recombination frequency (Parker and Marinus, 1992; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation), but further analysis will be required to fully characterize the effect of mismatch repair in *P. syringae*. Results found here confirm that this mode of recombination is similar to lambda Red-mediated ssDNA recombination in that it also displays a strand bias that corresponds to the orientation of DNA replication such that the lagging strand oligo generates recombinants more efficiently than the oligo matching the leading strand. This suggests that the strand bias observed in Red mediated recombination is not an inherent property of the enzymes that catalyze the reactions, but rather, is likely due to features that are shared between both types of recombination.

Host factors enhancing recombination activity

A question that remains to be answered is whether or not this recombination phenomenon is an endogenous bacterial trait or whether it is due to recombinases expressed from resident prophage. As an attempt to address this, we used BLAST to search the *P. syringae* and *E. coli* genomes for evidence of open reading frames with homology to the phage encoded Beta or RecT proteins. After 8 iterations of Psi-Blast using the default parameters with the lambda Beta protein or RecT sequence as the query, no matches to any proteins with significant similarity were found in *P. syringae* pv. *tomato* DC3000. We note that a gene encoding a RecT ortholog was identified using PsiBlast in another *P. syringae* strain (i.e., *P. syringae* pv. *syringae* B728a). The *E. coli* genome sequence was queried using BLASTP to identify any genes encoding proteins with similarity to other phage encoded ssDNA-annealing proteins. The only similarities identified were that of the bacteriophage lambda Beta protein and RecT from the *E. coli* Rac prophage, which are members of the same super family of ssDNA annealing proteins (Iyer *et al.*, 2002). Even though it is unlikely that the *recT* gene is expressed in *E. coli* under the conditions used in our assay (Hall *et al.*, 1993), it is a formal possibility that RecT is assisting recombination given that this class of protein has previously been shown to facilitate oligo-mediated recombination in *E. coli* (Zhang *et al.*, 2003; Datta *et al.*, 2008) and *Mycobacteria* (van Kessel and Hatfull, 2007, 2008). To test whether RecT plays a role in *E. coli*, a *recET* deletion was constructed and the frequency of recombination was compared to the parental strain using the galactose prototrophy assay. The results of these experiments confirmed that RecT is not responsible for this recombination in *E. coli* as we have reported (Datta *et al.*, 2008). Other host-encoded proteins that might logically be thought to be involved in this type of recombination have also been tested. These include: RecA, RecBCD, RecF, O,R, Rep as well as RecA and RecET in combination. In all cases, the frequency of recombination of these mutant strains was similar to wild-type (data not shown), which is consistent with what was reported for oligo-plasmid recombination in a *recA* mutant *E. coli* strain (Dutra *et al.*, 2007).

Discussion

Homologous recombination is recognized as serving critical roles across all biological kingdoms. There is interest in homologous recombination both because of its role in genomic homeostasis and for its utility as a tool for experimental alteration of DNA sequences. Here we present the results of a study that was initiated for the purpose of learning to manipulate bacterial genomes in order to support functional genomic and systems level investigations of *P. syringae* genetic regulatory networks. Serendipitously, these investigations also revealed a novel form of bacterial homologous recombination that is conserved in other bacteria and possibly among other kingdoms (see below).

The results of this investigation show that bacteria are capable of using synthetic single stranded DNA oligos as a substrate for homologous recombination to produce recombinant chromosomal DNA molecules. These recombination events are site specific and can be used to introduce different types of mutations directly in the genomes of *P. syringae*, *E. coli*, *S. typhimurium* and *S. flexneri*. We found that these recombination reactions are affected by the concentration of oligo used in the transformation and by the sequence of the oligo encoding the change. The data also show that oligos ranging in lengths between 20 nt and 120 nt recombine with equivalent efficiencies in *P. syringae* and *E. coli*. Finally, no evidence for the involvement of any recombinases encoded by endogenous prophage could be found. Blast analysis failed to identify any proteins encoded by *P. syringae* with similarity to known phage recombinases and in *E. coli*, deletion of the RecET functions did not change the rate of oligo recombination.

Oligo recombination is a general phenomenon

A feature of all homologous recombination reactions is the requirement for a ssDNA intermediate to base pair with the complementary strand in the process. In oligo recombination the donor molecule is introduced in the single stranded form and obviates the requirement for initial processing to expose single-stranded regions. The involvement of DNA replication is suggested by the result that oligos matching the lagging strand of replicated DNA recombine at a higher frequency than oligos matching the leading strand. This strand bias was observed in both *P. syringae* and *E. coli* (also in *S. typhimurium* and *S. flexneri*, data not shown). One hypothesis is that the transformed oligos are able to hybridize with single stranded regions made accessible during DNA replication. Because of the conservation of the DNA replication process it would not be surprising if oligos could find access to ssDNA using the same mechanism in other organisms. Consistent with this idea, we found that oligo recombination could be detected in all four of the species tested here.

There is evidence that oligo recombination exists in organisms beyond bacteria. In the late 1980's, Fred Sherman and coworkers found that recombinants could be generated in *Saccharomyces cerevisiae* transformed with synthetic oligos encoding point mutations that confer a selectable phenotype (Moerschell *et al.*, 1988). This discovery prompted the authors to consider that it would be interesting to investigate whether prokaryotes also had the capability to undergo recombination with synthetic oligos introduced directly by transformation. There are several striking similarities in the details of yeast and bacterial oligo recombination. First, in yeast, carrier DNA also increased the frequency of recombination (Yamamoto *et al.*, 1992a). The authors speculated that carrier enhanced recombination rates by saturating endogenous nucleases. This explanation is also plausible in bacteria, where deletion of genes encoding ssDNA nucleases *xonA* and *recJ* boost oligo recombination rates in *E. coli* (Dutra *et al.*, 2007). Furthermore, the addition of carrier DNA can also enhance the frequency of lambda Red-catalyzed oligo recombination in *E. coli* (Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation). Deletion of exonuclease genes also increases recombination and, consistent with its role in titrating exonucleases, carrier does not further increase the recombination frequency in exonuclease deficient strains (Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation). Second, in yeast, the number of mismatches and the specific mismatches in oligos encoding di-nucleotide changes affected the recombination rates (Yamamoto *et al.*, 1992a). This is reminiscent of our observations of oligo recombination in bacteria where the ability of the MMR system to recognize specific mismatches influences the recombination rates of oligos with different sequences (Table 3). Third, the strand bias was also present in yeast, with one strand consistently generating more recombinants than its complement. At the time, the authors proposed that this was likely due to differences in the preference for leading or lagging strand oligos to incorporate during DNA replication (Yamamoto *et al.*, 1992b).

Finally in both yeast and bacteria an endogenous recombinase that directly facilitates oligo recombination has not been identified. *S. cerevisiae* encodes a Beta-like protein, Rad52, but it was found not to affect oligo recombination (Yamamoto *et al.*, 1992a). Since the initial discovery of oligo recombination in yeast, similar evidence for oligo recombination in archaea (Grogan and Stengel, 2008) and mammalian cells (Campbell *et al.*, 1989) has also been obtained. Even though the mechanistic details of oligo recombination in eukaryotes, archaea and bacteria have not been elucidated these similarities prompt us to speculate that this process is evolutionarily conserved across biological kingdoms.

Insights relevant to bacteriophage-mediated recombination

Oligo recombination can help us understand how aspects of the lambda Red-mediated recombination reaction are affected by the phage-encoded recombinases. Lambda Red is able to catalyze recombination of a transformed ssDNA, presumably because oligos resemble a 5' resected dsDNA substrate generated by lambda exonuclease. Unlike the dsDNA substrates where both lambda Exo and Beta are required, lambda Beta is the only protein required when oligos are used as a substrate for recombination (Ellis *et al.*, 2001) and functions by binding to ssDNA forming a protein-DNA filament to protect and facilitate the interaction of the DNA at the target site (Karakousis *et al.*, 1998). In Red-independent oligo recombination, the disproportionate effect of oligo concentration or the addition of carrier DNA on the frequency of oligo recombination is consistent with this hypothesis. Excess DNA (homologous or carrier) apparently promotes recombination by reducing the influence of inhibitors, so that recombination takes place before the donor molecule can be eliminated by nucleases.

Oligo recombination and phage-mediated recombination also display comparable sensitivities to the effects of DNA replication (Ellis *et al.*, 2001; van Kessel and Hatfull, 2008) and MMR (Costantino and Court, 2003; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation). The results from oligo recombination suggest that DNA replication and MMR are not directly affecting the phage-encoded enzymes, but rather, are likely to be influencing the interaction between the oligo substrate and the target DNA molecule. These results raise the possibility that the bacteriophage-encoded recombinases have adapted to enzymatically enhance the endogenous oligo recombination process.

Length dependence provides a point of contrast between Red-independent and Beta-mediated oligo recombination

Red-mediated and Red-independent oligo recombination each have a different basis for oligo length dependence. Beta mediated recombination requires that oligos be at least 22 nt long, and the recombination frequency of oligos longer than 22 nt increases proportionally with length (Ellis *et al.*, 2001; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation). These length dynamics are dictated by the ability of Beta to bind the oligo. In contrast, without Beta, the length of the oligo affects the recombination frequency through its influence on the annealing properties of the oligo. We observed that oligos between 15 nt and 19 nt have incremental effects on recombination frequency that are proportional to changes in length, and that reducing the GC content of the oligo-target interaction reduces recombination frequency. Furthermore, using oligos longer than 21 nt provided no significant increase in recombination frequency. Together these data support the idea that there is a thermodynamic threshold for annealing, and that oligos that exceed this threshold can facilitate maximal levels of recombination.

Biological engineering and recombineering considerations

Experimental engineering of genomic sequences in *Pseudomonas* species and many other bacteria is generally performed using plasmid replicons containing homologies to the target

locus. This is a time consuming process because it requires cloning steps and a selection strategy to identify recombinants. There has already been limited success adapting bacteriophage recombination systems to different species (Datta *et al.*, 2006; Ranallo *et al.*, 2006; van Kessel and Hatfull, 2007, 2008; Katashkina *et al.*, 2009); development of such systems in other bacteria would greatly increase the variety and rapidity of manipulations that could be engineered directly in the native genomic context. Some progress has been made with recombineering in *Pseudomonas aeruginosa*, where transformation of long homology PCR products in the presence of plasmids encoding the lambda Red genes produced recombinant alleles of target genes (Lesic and Rahme, 2008).

Here we have described the initial characterization of an endogenous oligo recombination process in *P. syringae* and show that this activity is present in several different bacteria and likely to be even more widely conserved. Oligo recombination could provide strategies to develop more sophisticated recombineering systems in the pseudomonads and in other bacteria. The approaches described in this report establish a framework for identifying and optimizing ssDNA recombination (phage-mediated or otherwise) in organisms where no such methods currently exist, such as in many important bacterial pathogens.

The maximum frequency of Red-independent oligo recombination is currently in the range of 1×10^{-4} recombinants per viable cell, which makes selection necessary to identify individual bacteria that have undergone recombination. In *Mycobacteria*, the frequency of Che9c gp60/61 mediated recombination is likely to be limited by the cells' ability to take up DNA (van Kessel and Hatfull, 2008). We were interested in determining whether something similar might also be limiting the recombinase independent events and causing the low frequency of recombination. We tested this by co-transforming *E. coli* with both the *rpsL* and *rpoB* oligos (conferring resistance to streptomycin or rifampicin respectively). After transformation, cells were grown with selection for a single antibiotic, and then analyzed to determine the fraction of cells that were resistant to both antibiotics. The frequency for acquiring each change separately was $\sim 1 \times 10^{-4}$, while the number of doubly resistant clones was less than 1×10^{-8} , indicating that recombination of each oligo is independent of the other. This suggests that the dynamics of the recombination process are limiting the frequency rather than something like genetic competence as has been observed in *Mycobacteria* (van Kessel and Hatfull, 2008).

It appears that genetic transformation is the only prerequisite for oligo recombination, and does not require that plasmids or other extrachromosomal replicons exist. This system has the potential to be used in many ways. For example, many pathogens develop drug resistance and genome analysis often reveals multiple single base polymorphism in their genes. Recombineering could be used to determine which polymorphisms are responsible for resistance. Additionally, because the recombination of oligos can confer a selectable phenotype, this system could be used to develop transformation protocols in bacteria for which plasmid replicons are not available.

Experimental Procedures

Oligonucleotides

The sequences of all oligos used are shown in Table S1. Oligos were purchased from Integrated DNA Technologies (IDT), Inc., Coralville, IA. Oligos used in experiments with *P. syringae* were PAGE purified to enrich for full-length product except for oSWC1257, which was synthesized as an Ultramer™. PAGE purification of oligos is optional for recombination in *P. syringae* (data not shown) but was done to maintain consistent methods between experiments. Oligos used in other bacteria were desalted but not further purified. Oligos were then diluted in sterile diH₂O or TE and the indicated amount of oligo was added

to electrocompetent cells. The sequence of oSWC1447 and oSWC1448 were composed of 84 nt where each position was randomly chosen and did not contain any matches to the *P. syringae* genome of greater than 15 bp.

Oligo recombination in *P. syringae* pv. tomato DC3000

Electrocompetent *P. syringae* pv. *tomato* DC3000 (Table S2) was prepared using the method described in (Choi *et al.*, 2006). Briefly, *P. syringae* overnight cultures inoculated from a single colony were diluted 25-fold in 125 ml of KB medium (King *et al.*, 1954) and grown to an OD₆₀₀ of 0.8–1.0. Cells were harvested by centrifugation at 20°C, washed twice with equal volume of room temperature 300 mM sucrose and finally resuspended in 1/60 volume of 300 mM sucrose. In each experiment, 5 µl of the indicated amount of oligo (0.5–20 µg) was added to 100 µl of electrocompetent cells and transformed by electroporation at 2.5kV, 25 µF, 200 Ω in a 0.2 cm cuvette using a Gene-pulser (Bio-Rad Laboratories, Hercules CA). SOC medium (5.0 ml) was then added and the cells were incubated with shaking at 30°C overnight to allow the wild-type *rpsL* and recombinant *rpsLK43R* alleles to segregate. To determine the frequency of oligo-directed mutagenesis, dilutions of the transformation outgrowth culture were spread on selective (100 µg/ml streptomycin) or non-selective KB-agar plates and the number of streptomycin resistant transformants were normalized to 10⁸ viable cells. Recombination frequencies are the average of at least three independent experiments and the error bars indicate the standard deviation.

The *P. syringae upp* gene was used as a target to study the capacity of oligo recombination to direct a deletion. The presence of *upp* confers sensitivity to the nucleotide analog 5-fluorouracil. The product of *upp*, uracil phosphoribosyl transferase (Andersen *et al.*, 1992), converts 5-fluorouracil to 5-FdUMP, which exerts its toxic effects by inhibiting thymidylate synthase (Pritchard and Ahmad, 1971). *P. syringae* mutants acquiring the deletion of *upp* were identified by selection for resistance to 50 µg/ml 5-fluorouracil. Transformation of *P. syringae* with oSWC61 resulted in a 556 bp deletion of the *P. syringae* chromosome (from 1242582..1243138 in the *P. syringae* genome sequence, NC_004578).

Oligo recombination in *E. coli*, *S. typhimurium* and *S. flexneri*

Oligo recombination in *E. coli*, *S. typhimurium* and *S. flexneri* (see Table S2 for specific strain information) were done under standard conditions as for Red-dependent recombination (Sawitzke *et al.*, 2007; Sharan *et al.*, 2009) except where noted. For experiments testing recombination with the *rpsL* and *rpoB* genes of *E. coli*, *S. typhimurium* and *S. flexneri*, cells were grown at 32°C, harvested at OD₆₀₀ ~ 0.4, washed twice with ice cold sterile water, electroporated with 300 ng of the indicated oligo and then outgrown in 0.9 ml LB for 4 hours at 32°C and spread on LB agar plates supplemented with 100µg/ml streptomycin or 50µg/ml rifampicin respectively. The selection of *galK* gene recombinants in *E. coli* was done using the galactose prototrophy assay as has been described in (Costantino and Court, 2003). In all assays the recombination frequency was calculated as the number of recombinants per 1 × 10⁸ viable cells, which is approximately the number of cells in a standard electroporation.

Acknowledgments

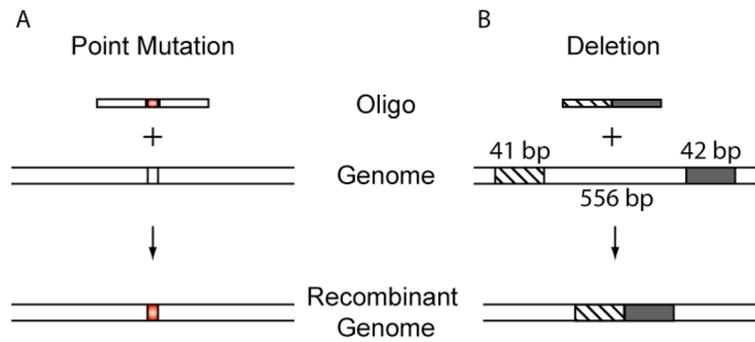
We thank Philip Bronstein, Melanie Filiatrault, Chris Myers, Jim Sawitzke, David Schneider and Lynn Thomason for many useful discussions. This work was supported in part by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research, and in part by a Trans National Institutes of Health/Food and Drug Administration Intramural Biodefense Program Grant of National Institutes of Health and Infectious Disease (to D.L.C.).

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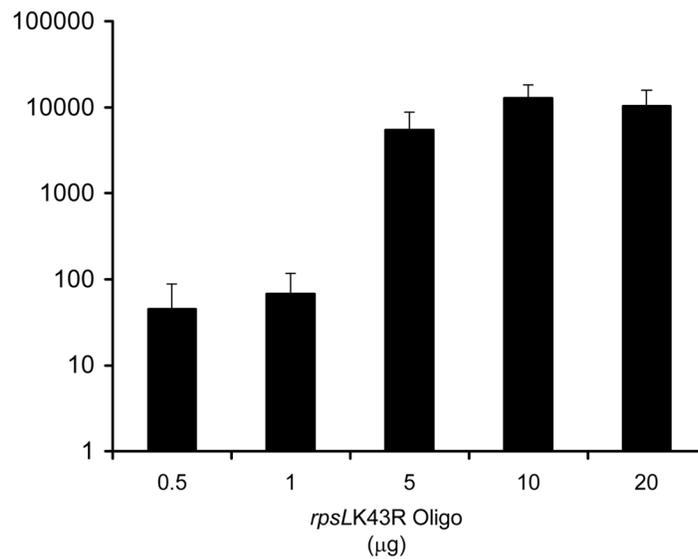
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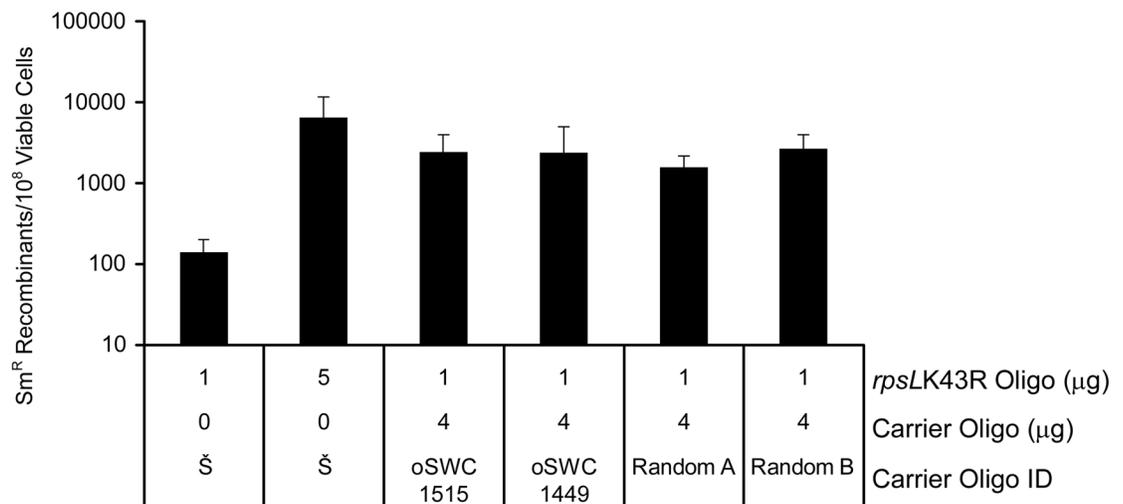
**Fig. 1.**

Two classes of mutations generated using oligo recombination. Changes encoded by synthetic oligos were incorporated at specific genomic loci and recombinants were identified by growth on selective media. (A) Point mutations were incorporated by transforming cells with ssDNA oligos containing nucleotide substitutions at desired locations of the *rpsL*, *rpoB* and *galK* genes. Unless otherwise noted, the nucleotide substitutions used were chosen to minimize any effects of methyl directed mismatch repair on recombination frequency (Parker and Marinus, 1992; Sawitzke, J.A., Costantino, N., Li, X.T., Court, D.L., In Preparation) (B) Deletions were constructed by transforming cells with ssDNA oligos that had segments (41 nt and 42 nt) of sequence identity to the 5' and 3' ends of an open reading frame. A 556 bp internal section of the *P. syringae upp* gene was deleted.

A



B

**Fig. 2.**

Effect of DNA concentration on recombination frequency. Recombination frequencies are the average of at least three independent transformations and are shown with error bars indicating standard deviation. (A) *P. syringae* cells were transformed with different amounts of oligo oSWC1255, which encodes a 4 base change that directs the *rpsLK43R* plus a silent marker, which is used to confirm that the mutation is derived from the oligo. (B) The recombination rate was determined for *P. syringae* cells transformed with the *rpsLK43R* encoding oligo (oSWC1255) in the presence or absence of carrier oligo. Carrier oligos oSWC1515 and oSWC1449 have sequence homology to the leading or lagging strands, respectively, within gene PSPTO_5020 in the *P. syringae* sequence. The PSPTO_5020 gene was chosen because it and *rpsL* are equidistant from the origin of replication on opposite

arms of the chromosome. Carrier oligos random A (oSWC1447) and Random B (oSWC1448) are complementary oligos. They were designed by generating a random sequence of 84 nt for Random A and then creating its complement Random B.

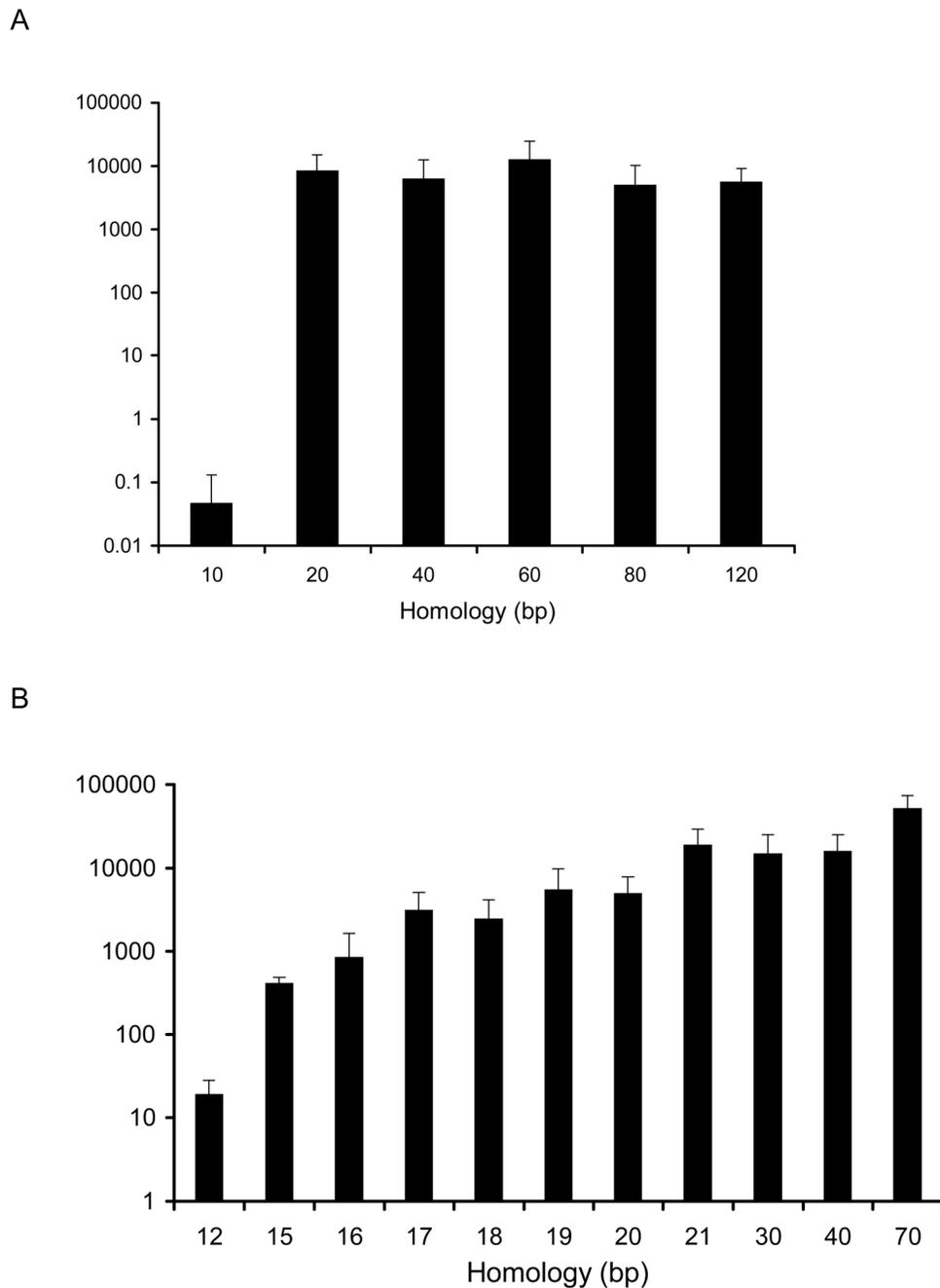


Fig. 3. Effect of oligo length on recombination frequency. Recombination frequencies are the average of at least three independent transformations and are shown with standard deviations. (A) *P. syringae* cells were transformed with 5 μ g of oligos that had different length sequences flanking the 4 base *rpsLK43R* change. Homology length indicates the combined length of each flank, but does not include the 4 base change. (B) The effect of oligo length was tested in *E. coli* by transforming with 10 μ g of each oligo that ranged in length from 12 to 70 nts. The spontaneous rate of Gal+ is 12 per 10^8 viable cells, which was determined from electroporations without oligo added (data not shown).

Table 1

Oligo recombination in enteric bacteria. The number of streptomycin and rifampicin resistant recombinants per 10^8 viable cells produced by transformation with a 76 nt or 75 nt oligo, carrying the *rpsL*K87R or *rpoB*P564L changes, respectively. These data are representative of a typical experiment.

Species	Sm ^R		Rif ^R	
	<i>rpsL</i> K87R oligo		<i>rpoB</i> P564L oligo	
	+	-	+	-
<i>E. coli</i> MG1655	16	0	200	0.7
<i>S. flexneri</i> BS547	330	0	830	4
<i>S. typhimurium</i> LT2	430	6	500	3

Table 2

The effect of GC content on the recombination frequency of short oligos. $T_m = 4(G+C)+2(A+T)$, where G, C, A or T represents the number of the indicated nucleotide in the oligo sequence.

Length/ID	% GC	T _m	Recombination Frequency	Fold- Difference
21 nt/Wt GC	52%	62 C	$1.5(\pm 0.9) \times 10^4$	17.2
21 nt/Low GC	24%	50 C	$8.8(\pm 5.3) \times 10^2$	
23 nt/Wt GC	54%	68 C	$2.8(\pm 2.0) \times 10^4$	9.5
23 nt/Low GC	27%	56 C	$2.9(\pm 2.5) \times 10^3$	
25 nt/Wt GC	52%	74°C	$3.2(\pm 1.8) \times 10^4$	3.8
25 nt/Low GC	28%	62°C	$8.6(\pm 8.3) \times 10^3$	
30 nt/Wt GC	53%	90°C	$1.5(\pm 1.0) \times 10^4$	1.7
30 nt/Low GC	33%	78°C	$8.9(\pm 5.6) \times 10^3$	
70 nt/Wt GC	56%	>90°C	$5.2(\pm 2.1) \times 10^4$	1.6
70 nt/Low GC	47%	>90°C	$3.3(\pm 2.0) \times 10^4$	

Fold-difference indicates the ratio of the average recombination frequencies for the GC variants at each oligo length.

Table 3

Frequency of recombination in *E. coli* and in *P. syringae*. Entries marked ND, have not been determined because a *mutS* deficient *P. syringae* strain has not been created. MMR is methyl directed mismatch repair.

Species	Oligo ID	Strand	Recognized by MMR	WT	<i>mutS</i>
<i>E. coli</i>	100	Lagging	+	2.0×10^2	3.0×10^4
<i>E. coli</i>	101	Leading	+	7.0×10^1	3.8×10^3
<i>E. coli</i>	144	Lagging	-	7.3×10^4	3.5×10^4
<i>P. syringae</i>	57	Lagging	ND	$5.1(\pm 3.8) \times 10^2$	ND
<i>P. syringae</i>	56	Leading	ND	$8.5 (\pm 3.0) \times 10^1$	ND