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Probing cellular processes with oligo-mediated recombination; using knowledge gained to optimize recombineering

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Abstract

Recombination with single-strand DNA oligonucleotides (oligos) in *E. coli* is an efficient and rapid way to modify replicons *in vivo*. The generation of a nucleotide alteration by oligo recombination provides novel assays for studying cellular processes. Single-strand exonucleases inhibit oligo recombination, and by mutating all four known exonucleases recombination is increased. Increasing the oligo concentration or addition of non-specific carrier oligo titrates out the exonucleases. In a model for oligo recombination, λ Beta protein anneals the oligo to complementary single-strand DNA at the replication fork. Mismatches are created and the methyl-directed mismatch repair (MMR) system acts to eliminate the mismatches inhibiting recombination. Three ways to evade MMR through oligo design include, in addition to the desired change 1) a C~C mismatch six bp from that change, 2) four or more adjacent mismatches, or 3) mismatches at four or more consecutive wobble positions. The latter proves useful for making high frequency changes that alter only the target amino-acid sequence and even allows modification of essential genes. Efficient uptake of DNA is important for oligo-mediated recombination. Uptake of oligos or plasmids is growth media-dependent and is 10,000-fold reduced for cells grown in minimal vs rich medium. Genome-wide engineering technologies utilizing recombineering will benefit from both optimized recombination frequencies and a greater understanding of how biological processes such as DNA replication and cell division impact recombinants formed at multiple chromosomal loci. Recombination events at multiple loci in individual cells are described here.

Keywords

mismatch repair; lambda Red; genome engineering; targeted mutagenesis; DNA transformation

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INTRODUCTION

In the past decade, recombineering, recombination-mediated genetic engineering, has become the method of choice to genetically modify large DNA molecules. Recombineering is highly efficient,^{1; 2} is targeted by short (~50 base) DNA homologies,³⁻⁵ is not limited by availability of restriction sites,⁵ and can be automated to rapidly make genome wide alterations.⁶ With large DNA molecules such as BACs or bacterial genomes, traditional genetic engineering techniques fail since finding convenient, unique restriction sites proves impossible and the physical manipulation of large DNA molecules is difficult. Recombineering has enabled researchers working on a wide range of organisms to precisely modify large genetic constructs contained on a BAC, PAC, virus or plasmid in *E. coli*, before moving back into their organism of choice. Recently, genome-wide engineering technologies that utilize recombineering have been developed, including multiplex automated genome engineering (MAGE)⁶ and trackable multiplex recombineering, or TRMR.⁷ In addition to advantages in genetic engineering, oligo recombination provides novel assays for studying cellular processes such as DNA uptake, methyl-directed mismatch repair (MMR), DNA replication, and chromosome segregation. Lessons learned from these experiments are providing new insights about such processes as well as ways to maximize oligo-mediated recombination frequencies. These methods are useful for functional genomic studies in *E. coli*, for techniques such as MAGE, and as a starting point for developing recombineering in new organisms.

Recombineering utilizes bacteriophage-encoded recombination systems, the most commonly used being λ Red^{3; 8-10} or *E. coli* RecET,⁵ however, several new systems are being developed.¹¹⁻¹⁴ The λ Red system includes three proteins, Exo, Beta and Gam, and recombines both double-stranded (dsDNA)^{3; 8-10} and single-stranded (ssDNA) DNA.^{1; 2; 15} Beta, a single-strand annealing protein, is the only λ function required for efficient recombination with ssDNA oligos.² Oligo recombination can be used to make single or clustered base substitutions, deletions up to ~45 kb⁶ or small (20-30 base) insertions.^{6; 16; 17} Beta-like functions from other bacterial species and phage have been isolated and shown to be functional.^{11; 12}

Most homologous recombination depends on RecA or a RecA-like function.^{18; 19} In *E. coli*, numerous recombination functions (Figure 1) have been categorized as being part of the RecBCD and/or RecF recombination pathways. Both the RecBCD and RecF pathways require RecA protein and thus, mutation of the *recA* gene results in a severe recombination defect as neither pathway is functional.¹⁹ Hence it is significant that Red recombination using either dsDNA^{20; 21} or oligos^{1; 2; 15; 22} does not require the *E. coli* RecA protein. This proves useful, as after briefly expressing the Red functions to allow recombination, expression can be shut-off preventing further homology-dependent rearrangements in *recA* mutant cells.¹⁰

Previous studies have established several parameters that affect oligo-mediated recombination.^{1; 2; 6; 15; 22} One critical factor is the direction of DNA replication through the target sequence. Of the two complementary oligos that can be used at any locus, the one corresponding in sequence to the lagging strand is more efficient at recombination.^{1; 2; 15; 22} This observation supports the model that during DNA replication the Beta protein anneals the oligo to complementary single-stranded DNA at the replication fork.^{1; 2; 15; 23-25} An annealed oligo containing at least one base different from the chromosome creates a mismatch, which targets the oligo-mediated recombination events to be repaired by the MMR system. Mismatch repair reduces recombination between diverged sequences.²⁶ Costantino and Court¹ found that avoidance of the MMR system is key for high frequency recombination, as MMR can eliminate more than 99% of the recombinants from an oligo-

mediated recombination. Mutation of the MMR genes increased oligo-mediated recombination by ~100-fold with most mismatches, and nearly 400-fold for the well-repaired G-G mismatch.¹ Temporary inhibition of the MMR system by addition of 2-aminopurine increases oligo recombination 10-fold.¹ Unfortunately, both of these techniques to reduce MMR cause uncontrolled generalized DNA mutagenesis.

Evading mismatch repair in cells with a functional MMR system is desirable for oligo recombination as it avoids generalized mutagenesis. A method we devised¹ is to design the recombining oligo such that the mismatch created during recombination is not recognized by the MutS protein, which binds mismatched base pairs and initiates the repair process. An oligo that creates a C~C mismatch at the targeted change¹ generates high numbers of recombinants since MutS binds poorly to the C~C mismatch.²⁷ This method is highly effective but limited in general applicability. It has been reported²⁷ that the MMR system efficiently repairs the other seven single base mismatches as well as mismatches with up to three adjacent unpaired bases caused by either an insertion or deletion on one strand of the DNA.²⁸ Less is known, however, about the correction of multiple nearby or consecutive mismatches and context effects on individual mismatches.²⁹ *In vitro*, MutS protein has been shown to recognize multiple nearby mismatches, although at a reduced affinity as compared to a similar single mismatch.³⁰ *In vivo*, Yang and Sharan¹⁷ obtained a high frequency of recombination when 6-20 consecutive nucleotides were altered around the desired change; probably because the MMR system is unable to bind and repair these multiple alterations.¹⁶ Here we determine the effect of several types of multiple mismatches on recombination frequencies.

Technologies such as MAGE⁶ rely on the fact that oligo recombination can be targeted to several loci simultaneously with the goal of achieving multiple changes on a single chromosome. With MAGE, however, it is difficult to tell precisely when and how each recombination event occurred. We analyzed the recombinants generated when two loci were simultaneously targeted in order to better understand this process. Further, we examined oligo recombination in cells grown in minimal medium, which contain fewer sister chromosomes.³¹

RESULTS

Oligo-mediated recombination does not require any known *E. coli* recombination function

Red-mediated oligo recombination has been shown to occur in the absence of RecA.^{1; 2; 15; 22} We asked whether other known *E. coli* recombination functions are involved in oligo recombination. Recombination frequencies were determined by enumerating the number of Gal⁺ recombinants obtained when a 70 base, leading-strand (oligo 101) or lagging-strand oligo (oligo 100) was used to correct the *galK_{tyr145am}* mutation.^{1; 2} In this experiment, the MMR system was functional, thus limiting recombination levels in order to allow detection of any increase or decrease in frequencies.

Recombination frequencies were determined for strains containing single or multiple defects in *E. coli* recombination functions. Our results are consistent with and extend previously published data.³² We saw no major effect on the number of oligo-mediated recombinants in any of the mutant backgrounds (Figure 1). The largest effect seen, about a 10-fold decrease, occurred when four genes involved in the resolution of Holliday junctions, *radA*, *recG*, *ruvA*, and *ruvB* were simultaneously mutated; smaller effects were seen with single mutants. Paradoxically, a *radA* single mutation increased recombination six-fold but only with the leading strand oligo. The *recJ* and *recQ* mutant strains showed increased recombination for both leading and lagging strands. We have determined that these effects are due to RecJ's role in MMR (data not shown).³³

Concentration of oligo used in recombination

We routinely use 5 pmoles of oligo for recombination,^{1; 34; 35} which is approximately 3000 oligos per bacterial cell electroporated under our conditions. Increasing the number of oligos per cell 10- or 100-fold (Table 1 and data not shown) does not increase the number of recombinants, indicative of saturation. Table 1 also compares results obtained when the oligo was diluted in either sterile dH₂O or with a “carrier” oligo. When present, carrier was added at 3000 oligos per cell. The carrier oligo contains no homology to any sequence within the recombining cells, however, like the *galK* oligo, it can be bound by Beta and other single-strand DNA binding proteins including single-strand exonucleases. Reducing the number of *galK* oligos per cell results in fewer recombinants as expected (Table 1). Although the first 10-fold dilution of *galK* oligo in dH₂O (3000→300) decreased recombination only ~2-fold, a further 10-fold dilution (300→30 oligos/cell) decreased recombination more than 80-fold. However, by diluting into carrier oligo, the dilution from 300→30 *galK* oligos/cell results in only a 6-fold drop in recombination frequency. Thus, adding carrier oligo can have a positive effect on recombinant frequencies when the recombining oligo becomes limiting.

Do the host single-strand exonucleases affect recombination?

Oligonucleotides may be sensitive to degradation by single-strand exonucleases within the cell.³⁶ *E. coli* encodes four such exonucleases: ExoI, ExoVII, ExoX and RecJ. We deleted all four of these exonucleases in order to determine how their absence affected Red-mediated recombination with an oligo. Table 1 shows that in the absence of the single-strand exonucleases, up to 40-fold more recombinants are recovered at low oligo concentrations (3 or 30 oligos/cell). Addition of carrier oligo did not result in an increase in recombination frequency for exonuclease mutant cells; in other words, the presence of carrier oligo has the same effect as mutating the four exonucleases. At our standard oligo concentration, the absence of these exonucleases does not affect recombination frequencies, again indicating that recombination is saturated at an oligo concentration of 3000 molecules/cell.

Varying oligo length affects recombinant yield

Ellis *et al.*² found that decreasing the oligo length resulted in decreased recombinant formation, however, those experiments were performed with an oligo that was subject to mismatch repair.¹ Here we expand on previous experiments by examining a wider range of oligo lengths and using oligos that create a C~C mispair and thus, are immune to the MMR system. Figure 2 shows the frequency of Gal⁺ recombinants obtained with oligos of various lengths. Oligos of 40-70 bases yield nearly the same number of recombinants, although a 60-mer or 70-mer generates the highest level. Reducing the length of the oligo from 40 to 23 bases decreases recombinant yield about 20-fold in an exponential fashion. There is a dramatic, 10-fold drop in recombinant yield when an oligo is reduced from 23 to 22 bases in length. The low level of residual recombination with oligos 15-20 bases in length is independent of the λ Red functions (data not shown).³⁷

In a similar experiment where the MMR system was functional and the oligo-generated recombinant was susceptible to it, recombination was reduced approximately 100-fold for each oligo length tested (data not shown). Thus, the general effect of oligo length on recombination frequencies is independent of the MMR system.

Altering the position of the mismatch within the oligo affects recombinant yield

Figure 3 shows the yield of Gal⁺ recombinants obtained with a series of 70 base oligos that contain the same correcting “T” base but the position of the correcting base within the oligo varies. The correcting base is denoted as 1 through 70 along the x-axis, assigned in a 5'→3'

direction. Assays were performed in strain HME63 in which the MMR system is inactive. If the correcting base was from positions 9 to 61, the recombinant yield was similarly high, yielding approximately $1\text{--}3 \times 10^7$ Gal⁺ recombinants/ 10^8 viable cells. If the correcting base was outside of this interval, recombinant yield dropped off precipitously. An oligo with the correcting base at the extreme 5' end, position 1, yielded recombinants well above background but five orders of magnitude lower than when the correcting base was in the middle. Recombination with the correcting base at the extreme 3' end, position 70, generated 10^4 recombinants/ 10^8 viable cells, about 50-fold higher than the 5' end. The low level of recombination observed when the correcting base was on either end was completely dependent on the λ Red functions (data not shown). Experiments using control oligos located such that they end just 5' or 3' of the base to be corrected, i.e. they do not contain the correcting base, and experiments in which no oligo was added had the same very low level of spontaneous Gal⁺ colonies (Figure 3 legend). These results indicate that oligo recombination in the immediate vicinity of the *galK* mutation does not affect the natural reversion frequency found in the absence of oligos.

A C~C mismatch protects a region from mismatch repair

Figure 4 shows the sequence flanking the *galK*_{TYR145UAG} mutation. Recombination with an oligo that substitutes a "T" for the boldface "G" in the stop codon of the amber mutation yields Gal⁺ recombinants at an average frequency of $3.5 \times 10^5/10^8$ viable cells (oligo 100). However, if the oligo creates a C~C mismatch (oligo 144), MMR is avoided and high recombination levels, $2 \times 10^7/10^8$, are achieved.¹ Since it is impossible to make all alterations using a C~C mismatch, we asked whether a second, unselected C~C mismatch near a *galK* T~C correcting mismatch, which is sensitive to the MMR system, can confer high frequency recombination (Figure 4). The level of recombination increased 30-fold if an additional alteration that results in a C~C mismatch is made six bases from the correcting "T" (Oligos 254 and 176), while a C~C mismatch further away than six bases from the correcting base had little or no effect. Inhibition of correction of a mismatch by a nearby C~C mispair has been seen previously in *Streptococcus pneumoniae*.³⁸

MMR cannot repair multiple adjacent mismatches

As large heterologies are not recognized by the MMR system,¹ we reasoned that if we extended the mismatch length base by base, at some point the mismatch distortion would prevent MMR because MutS would be unable to bind. At this length, high level recombination should result. Figure 5 shows experiments with multiple consecutive changes in an oligo, none of which creates a C~C mispair. In all cases when four consecutive bases were altered, and in certain cases with only three base changes, up to 20% of the cells became recombinant indicating avoidance of the MMR system. Surprising exceptions exist; for example, oligo 282 creates only a single T~T mispair (Figure 5A) that is resistant to MMR in its native context. Adding an additional alteration next to this T~T mispair reduces recombination (oligo 283). The same oligo 282 gives a lower recombination frequency when two mismatches are created (Figure 5B), again demonstrating the importance of sequence context. Such contextual effects have been previously demonstrated.²⁹

Evasion of the MMR system by consecutive wobble base alterations

We examined whether changing only the wobble (3rd) position in several nearby codons, while retaining wild type amino acid sequence, could also result in high levels of recombination. As shown in Figure 6, changing the wobble position of three consecutive codons increased the frequency of recombination up to 25-fold more than having changes in two consecutive wobble positions. When four or five consecutive codons were altered in their wobble position, the highest levels of recombination were obtained. Thus, the MMR system can be avoided not only by consecutive changes but also by altering multiple wobble

positions of successive codons. The wobble method of MMR-avoidance allows efficient targeted mutagenesis of genes without amino acid changes in an encoded protein except for the desired change.

Recombination with two different oligos

Techniques such as MAGE⁶ use several different oligos to target multiple loci simultaneously with recombineering technology. However, as MAGE involves automated repeating rounds of recombination, the details of what happens in a given round are unclear. Can more than one locus be successfully recombined during one round of recombineering and how often does the recombination occur on the same sister chromosome? We addressed these questions with two 70 base lagging-strand oligos in a *malK_{am} galK_{am} mutS* strain (HME82). The *malK* and *galK* markers are located on the same arm of the replicon approximately 1.2 megabases apart (Figure 7). In this experiment, immediately after electroporation cells were plated on L agar, incubated overnight and independent colonies patched to MacConkey galactose and MacConkey maltose plates to screen for “red patches” indicating recombinants. Of 490 colonies tested, 24% (118) were Mal⁺ Gal⁻, 18% (90) were Mal⁻ Gal⁺ and 9% (42) had both Mal⁺ and Gal⁺ properties. The remaining 240 colonies were white on both indicator plates and thus non-recombinant. The 42 Mal⁺ Gal⁺ patches were further tested by suspending the original L agar colony in buffer, diluting and plating for single colonies on MacConkey maltose or MacConkey galactose plates, allowing determination of the genetic makeup of individual cells within each of the 42 colonies. During this assay, all red colonies, either Mal⁺ or Gal⁺, were tested on the other sugar indicator. Only two of the original 42 colonies contained a single type of recombinant cell, Mal⁺ Gal⁺. Thirty-one of the 42 colonies contained two recombinant cell types: Mal⁺ Gal⁻ or Mal⁻ Gal⁺. The remaining nine colonies each contained three recombinant cell types, Mal⁺ Gal⁻, Mal⁻ Gal⁺, and Mal⁺ Gal⁺. All of these mixed phenotypes are likely the result of multiple recombination events and are elaborated on in the Discussion. Consistent with Wang *et al.*⁶ we conclude that oligo recombination can occur at more than one locus in the same cell. Most of the cells are competent for oligo uptake and recombination as demonstrated by the >50% recombination frequency. However, the generation of two alterations on one chromosome was rare. We saw only 11/490 (2.2%) colonies in which cells had become genetically *malK⁺ galK⁺*.

Can more than one sister chromosome recombine in a cell?

When *E. coli* grows on a rich medium such as LB, its division time is less than the time required to replicate the full genome. In order to grow this fast, *E. coli* initiates new rounds of replication before the first round has completed. Therefore, in these cells *galK* is present in several copies while the terminus is represented by only one copy until just prior to cell division. We examined whether our high oligo concentrations could target more than one copy of *galK* during oligo recombination.

As in the previous experiment, we plated for colonies on L agar immediately after the electroporation, thus before allele segregation. Within a recombinant colony, some cells will be recombinant and some will be parental with the exact percentage of recombinant cells dependent on the chromosomal position of the targeted gene, the number of targets recombined, and how early in the cell cycle the recombination occurs, ie. in a newly divided “baby” cell vs an “old” dividing cell. A newly divided cell has half as many targets as a cell actively undergoing division and a recombinant chromosome in a baby cell will likely replicate before cell division increasing the percentage of recombinant cells in the resulting colony. We expect four or eight replicating copies of the chromosome with our growth conditions.³⁹ As more targets within a cell are modified and then replicate, the recombinant cell frequency within the colony should increase. If recombination occurred at every target

on all sister chromosomes then the maximum percentage would be 50%. By a similar calculation, if eight chromosomes are being generated and only one recombines, then there should be ~6% (1/16 of the DNA strands) recombinant cells in the resultant colony. To address these predictions we determined the percentage of Gal⁺ cells in individual recombinant colonies taken from L agar plates. Table 2 demonstrates that the percentage of Gal⁺ recombinant cells within a recombinant colony varies but that these percentages sort into restricted groups or bins, suggesting that various numbers of the targeted gene had recombined in the different groups. The major groups I to V (Table 2) consisted of colonies with ~6%, ~13%, ~19%, ~25%, and ~31% recombinants in accord with our predictions. Other colonies with 37 to ~50% Gal⁺ recombinants were found less frequently.

Effects of growth medium on oligo recombination

Theoretically, the generation of recombinants at multiple loci on the same chromosome should be easier with cells grown in minimal medium as the cells contain fewer sister chromosomes due to the reduced growth rate.³¹ Accordingly, we determined how the choice of growth medium affects oligo recombination. All experiments used oligo 144, which creates a C~C mismatch and thus, should give the highest possible recombination frequency.¹ When cells were grown in M63 glucose medium, however, recombination levels decreased nearly 1000-fold from levels seen with cells grown in LB, yielding only 1×10^4 recombinants per 10^8 viable cells (Table 3). Supplementing the minimal medium with all 20 amino acids did not increase recombination (data not shown). Either adding a small amount of yeast extract (0.2%) to M63 glucose medium, or using MOPS defined medium⁴⁰ to grow cells increased recombination to similar levels, approximately 100-fold over levels seen in M63 glucose medium but still 10-fold lower than LB. Recombination frequencies were normal if cells were grown in LB even when the recovery medium was M63 glucose. Thus, the medium used for cell growth determines recombination level rather than the recovery or plating medium.

What causes poor oligo-mediated recombination in cells grown in minimal medium? We asked whether recombination itself is inhibited or if cells grown in minimal medium are defective for DNA uptake. If the latter is true and recombinants are scored amongst cells that have been successfully transformed with a plasmid, the relative recombination frequency might be improved. Table 3 shows that transformation of a supercoiled plasmid is reduced ~10,000-fold with cells grown in M63 glucose medium. However, if only plasmid transformants are sampled, recombinants on the chromosome are readily found (4% of transformants). Thus, poor uptake of plasmids and presumably oligos via electroporation of cells grown in minimal medium appears to be the main reason that recombination appears defective in these cells. The normalized recombination among plasmid transformants is about 10-fold less efficient in minimal medium vs LB grown cells. This lower level may reflect in part fewer replicating copies of the target gene per cell in the more slowly growing bacteria.³¹

The effect of recovery time on recombinant yield

To find a recombinant generated by oligo recombination, one must either select for or against a function, or screen for the desired mutation. The length of time that elapses between addition of LB after electroporation and plating of the recombinant mix, previously referred to as “outgrowth time”^{16; 34; 35}, impacts cell survival and cell growth thereby affecting recombinant frequency.

Cell survival following electroporation was determined by plating on L agar after various amounts of time (Table 4). If cells are plated immediately after electroporation, they suffer a 10-fold loss in viability but recover with a 15-30 min recovery period. Recombinant

colonies formed after various recovery times were assayed by plating on MacConkey galactose plates, where both recombinants and non-recombinants can grow. We scored red sector colonies as Gal⁺ (Figure 8). Table 4 shows that recombination levels, like survival, are inhibited by plating immediately after electroporation. Optimal recombination is seen with a 15-30 minute recovery.

DISCUSSION

Parameters affecting recombination

We find that host recombination functions have little or no effect on oligo recombination (Figure 1). However, several oligo parameters can greatly affect recombination. At any locus, of the two complementary oligos that can be used for recombination, the oligo identical in sequence to the lagging-strand is always more efficient,^{1; 2; 15; 22} however, the level of bias varies.^{1; 2; 13; 15} At least some of this variability may be due to the concentration of oligo used. With our standard conditions, the lagging/leading-strand bias is approximately 15- to 20-fold,¹ however if less oligo is used, the bias increases dramatically (our unpublished results). We believe that the bias arises because the length of the available single-stranded region on the leading strand is limiting and thus, more restrictive when oligo concentration is also limiting.

With either a leading- or lagging-strand oligo, if the oligo concentration is too low, recombination is adversely affected by single-strand exonucleases. At our standard oligo concentration, mutation of all four single-strand exonucleases present in *E. coli* has little effect (Table 1), however, at 100-fold lower oligo concentrations, the mutations enhance recombination levels indicating that the exonucleases are degrading oligos. Thus, the optimal oligo concentration of 3000 molecules per cell avoids issues with single-strand exonucleases and yields consistently high recombination levels. If one uses less than optimal amounts of targeting oligo, carrier oligo can be added to titrate the inhibitory exonuclease activities.

Oligos from 40 to 70 bases in length showed similar recombinant frequencies (Figure 2). Others have reported maximum recombination with oligos of 90 or ~120 bases, respectively,^{6; 22} however, these frequencies were less than two-fold higher than with 70 base oligos, and since the synthesis of these longer oligos is more expensive and more mistakes are generated, we consider 70 bases to be optimal. Even with 70 base oligos, the recombinants should be sequenced as errors may have occurred during oligo synthesis.⁴¹ Note that recombination with oligos longer than ~120 bases occurs at reduced frequencies (our unpublished results),^{6; 22} possibly due to oligo secondary structure and/or increased mistakes in synthesis. Recombination drops off exponentially as oligo length is decreased from 40 to 23 bases, consistent with *in vitro* studies showing Beta binding poorly to oligos shorter than 36 bases.⁴² For oligos shorter than 23 bases, a very low level of recombination is found. The level of recombination seen with 21 and 22 base oligos appears to be partially Beta-dependent as this frequency of recombination is not reached in the absence of Beta at this oligo concentration.³⁷ Recombination with oligos 15 to 20 bases in length is independent of the Red proteins as described by Swingle *et. al.*³⁷ possibly because Beta cannot anneal such short oligos. Finally, the MMR system reduces recombination frequencies for all oligo lengths.

The position of the correcting base along the length of a 70 base oligo has little effect on recombination frequency as long as it is not less than 9 bases from an end. Once the change is less than 9 bases from an end, recombination frequencies decrease significantly. This effect could be largely due to degradation of the marker by exonucleases when the change is too close to an end (our unpublished results), although oligos with the correcting base at the

extreme 5' or 3' end still generate recombinants. We have determined that the low levels of recombination seen with the correcting bases at the extreme 5' or 3' ends of the oligo are Red-dependent. It was previously reported that if four unpaired changes were on the 5' or 3' end, recombination did not occur,²² however, we find that an oligo with a single alteration at the last base of the 3' end yields 10^4 recombinants per 10^8 viable cells. Thus, we presume that some DNA polymerase(s) is able to utilize, albeit poorly, this unpaired 3' end as a substrate for DNA synthesis and repair. Likewise, an oligo with the correcting base on the 5' end generates recombinants but at an even lower level, $10^2/10^8$ viable cells. Here, to get a recombinant, DNA ligase must join, albeit inefficiently, the mismatched 5' end base to an adjoining upstream DNA molecule.

Evading mismatch repair by oligo design

Although much is known about MMR in *E. coli*, oligo recombination provides a powerful new tool to examine behavior of the system *in vivo*. It was previously shown that eliminating MMR by mutation can increase oligo recombination more than 100-fold.¹ Using a MMR mutant strain to increase recombination can be useful, but a drawback is the accompanying accumulation of mutations throughout the genome.²⁷ Here we have identified three ways to design oligos that increase recombination in a MMR-proficient host and thus avoid generalized mutagenesis. The *galK_{tyr145am}* model system is excellent for studying mismatch repair since amino acid substitutions in this region of the GalK protein are well tolerated without affecting the Gal⁺ phenotype (Figure 5).

Mismatch repair does not recognize C~C mismatches *in vitro*,²⁷ and we have previously shown that an oligo creating a C~C mispair when annealed to its target greatly increases recombination *in vivo*.¹ We show here that creating a C~C mismatch located six bases from a desired change leads to higher levels of recombination, indicating that the MMR system does not recognize this double mispair. In contrast, if the C~C mismatch is nine bases or farther away, recombination is not enhanced (Figure 4). This indicates a C~C mismatch may create a region of six bases to either side that is refractory to mismatch repair. On the other hand, a correcting C~C mismatch can be repaired resulting in lower recombination frequencies if an additional repairable mismatch is 9 bases from the C~C mismatch (data not shown). In this case efficient co-repair of the C~C mismatch with the repairable mismatch has likely occurred.

Previously it has been shown that the MMR system does not recognize a five base insertion/deletion on one strand and poorly recognizes a four base insertion/deletion.²⁸ Consistent with these results, we found that substituting four or more consecutive bases (Figure 5 and data not shown) also escapes MMR. However, we observed exceptional cases where a single T~T mismatch or three alterations in a row resulted in high levels of recombination (Figure 5). These "exceptions" are likely due to sequence context effects. Although useful, methods using a C~C mismatch or altering four or more consecutive bases have limitations. Creation of a C~C mismatch is not always feasible, and changing four consecutive bases of a gene may alter coding sequence and function. A powerful new way to avoid both of these problems is to alter four or more wobble positions of adjacent codons in addition to the desired alteration (Figure 6). This configuration of mismatches evades the MMR system allowing high frequency targeted mutagenesis without additional undesired changes to the encoded protein. This method is of general utility and is particularly useful for mutating essential genes with high efficiency in a single recombination reaction. It has been used to generate over 40 mutations in the *E. coli* RNA polymerase gene, *rpoB* (our unpublished results). Although the amino acid sequence need not be altered due to the wobble changes, it should be kept in mind that infrequently, synonymous substitutions lead to altered protein expression; such altered expression is often due to mRNA stability⁴³ rather than codon bias. If this is a concern, an additional recombination step can be included to revert the wobble

positions to wild type, leaving the desired mutation intact. This altered wobble position procedure should yield high efficiency targeted modification within any protein-coding sequence, allowing high throughput alterations of genomes. For either consecutive or wobble position changes, to be safe we suggest changing five or more bases. Having five changes allows specific detection of mutations by colony PCR. These advances in recombineering technology should be readily applicable to other organisms where MMR exists and oligo recombination has been established such as *Salmonella*, *Shigella*, *Yersinia*, and *Pseudomonas*.

Growth considerations

Bacteria grown in LB contain several replicating copies (4-8) of the chromosome,³⁹ such that recombination might occur on any one strand of any sister chromosomes.¹ However, even if recombination occurred at every target on all sister chromosomes, at most, 50% of the DNA strands can be recombinant. Thus, if cells are plated immediately after electroporation, the colony that forms will always be a mixture of parental and recombinant cells (Figure 8). Pure recombinant colonies are obtained only if the recombined cells are allowed several generations of outgrowth (about three hrs at 30°C) permitting DNA replication and cell division. During the outgrowth, the recombinant and parental chromosomes segregate away from each other and into daughter cells. DNA replication and cell division reduce the frequency of cells containing recombinants as much as 8- to 16-fold for *E. coli* growing in LB.³⁹ If the recombinant cell has a growth disadvantage and the recombination mix is allowed to recover for extended periods, recombinants will be greatly diluted out by cell growth and division.

We note a loss of viability of electroporated cells when allowed to recover less than 15 minutes. Thus, we recommend that the minimal length of recovery should be 30 minutes. This allows for full recovery from the electroporation yet avoids segregation and dilution of the recombinant chromosome, which make it harder to screen for the recombinant. This short recovery is also ideal for selecting for growth on a non-toxic medium (e.g. selecting Gal⁺), or screening for recombinants non-selectively by diluting and plating on L agar plates after the recovery period. Screening techniques such as PCR and colony hybridization^{16; 44} can be used to identify recombinants.

Cells grown in minimal medium take up DNA poorly, yielding very low recombination levels with oligos and poor transformation with plasmids. However, among cells selected as plasmid transformants when co-electroporated with oligos, the relative frequency of oligo recombination increased 100-fold (Table 3). Similar techniques to find recombinants have proven useful in *Saccharomyces cerevisiae*⁴⁵ and *Mycobacterium tuberculosis*¹³ and should be considered when moving oligo recombination technology to organisms where cells must be grown in minimal medium or DNA uptake may be an issue. To our knowledge, poor uptake of DNA by electrotransformation of cells grown in minimal medium has not been extensively studied but consistent with our findings, it was previously shown that phage DNA transfection by electroporation into *E. coli* is less efficient when cells were grown in minimal medium.⁴⁶

Recombination with multiple oligos

We have shown that two different oligos can be used to alter simultaneously two genes in an individual cell with a single round of recombination. We expect that numerous different oligos can be used to modify single or multiple loci simultaneously, with the limiting factors being how much DNA can be introduced into a cell and having effective screens or selections to find the changes.⁶ The presence of several DNA replication forks in rapidly dividing *E. coli* provides multiple targets for oligo recombination, and explains our results

with co-electrotransformation by two different oligos. Within a single recombinant colony (plated without any recovery period), we found cells that were Mal⁺ Gal⁻, Mal⁻ Gal⁺, Mal⁺ Gal⁺, as well as the parental Mal⁻ Gal⁻. One of the recombinant classes observed, Mal⁺ Gal⁺, can be explained by a recombination event occurring when a replication fork reaches *malK*, and a second recombination event on that same DNA strand after the fork travels another 1.2 megabases to the *galK* locus. In the original recombinant cell, two additional recombination events involving other forks on sister chromosomes are likely to have occurred to generate the Mal⁺ Gal⁻ and Mal⁻ Gal⁺ recombinants found. Thus, in this cell, we speculate that three separate DNA forks and four oligo recombination events were involved in generating the recombinants seen in a single colony (Figure 7). The recombination events could occur concurrently in one cell as described or, if Beta and the oligo persist, additional recombination events might occur even after the cell starts to divide to form the colony. A much more extensive study of recombinants in a single colony is warranted. For example, having the two loci on opposite sides of the origin of DNA replication or one near the origin and one near the terminus should prove fruitful for better understanding multiple oligo recombination events with the process of DNA replication.

For cells grown in LB, four or eight copies of the genome can be present.³⁹ As seen in Figure 7, during DNA replication one or more of the target lagging-strands may be recombined. In the four chromosome example shown, anywhere from 1/8 to 1/2 of the total DNA strands (lagging plus leading) can be recombined for a given marker, resulting in 12-50% of the cells in the colony being recombinant. If there are eight copies of the genome in a targeted cell, then 1/16 to 1/2 of the DNA strands can be modified. Initial analysis of individual cells from recombinant colonies has demonstrated recombinant frequencies from different colonies with this non-overlapping pattern, ie. 1/16, 1/8, 3/16, 1/4, 5/16, 3/8, or 1/2 of the cells being recombinant (Table 2). This pattern would be expected when as many as eight copies of *galK* are present. Some cells, however, would have had four chromosomes when recombination occurred yielding a subset of these groups (1/8, 1/4, 3/8, 1/2). Here again a broader study is required to understand the targeting and frequency of oligo recombination within a single cell.

Key results to consider when using recombineering as a technique

Recombination with an oligo is a precise, rapid and simple way to modify both large and small replicons and can be used to create point mutations as well as deletions and small insertions. General guidelines for optimizing oligo recombination include: 1) avoid the MMR system 2) use a lagging-strand oligo 3) use saturating oligo concentrations 4) use a 70 base oligo and 5) locate the altered bases >9 bases from an end. Figure 8 shows *E. coli* recombinants obtained where conditions are optimal. Avoiding the mismatch repair system through careful oligo design can maximize the frequency of oligo recombination in cells wild type for MMR. These techniques avoid the generalized mutagenesis associated with the absence of mismatch repair and are of general utility for both automated systems such as MAGE⁶ and researchers using standard recombineering procedures.

MATERIALS AND METHODS

Bacterial Strains

HME6 is W3110 *galK_{tyr145UAG} ΔlacU169 [λ cI857 Δ(cro-bioA)]*.² Strains shown in Figure 1 are derivatives of HME6. All mutations in the recombination genes are complete gene replacements where the coding sequence was replaced with a drug-resistance cassette by dsDNA recombineering.¹⁰ HME63 is HME6 *mutS* <> *amp*. Strain XTL74 is HME6 *recJ* <> *amp* *xonA* <> *kan* *xseA* <> *tet* *exoX* <> *spec*. HME58 is HME6 but with *galK_{tyr145UAA}*

instead of an amber mutation. HME82 is HME6 *malK_{tyr84UAG} mutS*<>amp. Details of strain constructions are available upon request.

Oligonucleotides and plasmids

Oligos were purchased from IDT as salt-free but otherwise unpurified. Oligos 100, 101, 144 and the *malK* oligo were as described previously.¹ The “carrier” oligo was LT217, an oligo with homology to a plasmid not in the cells of this experiment.⁴⁷ The DNA sequences of all oligos are available upon request. Plasmid pLT60 is an amp-resistant pUC-derivative with a *kan_{tyr39UAG}* allele.⁴⁷

Recombination assay

Unless otherwise noted, the recombination assay for all experiments was as described in Costantino and Court.¹ Cells were prepared for recombination by standard methods.^{34; 35} Five pmoles of oligo was electroporated into the cells, 1 ml of LB was added and cells were allowed to recover at 30°C with shaking. The length of recovery time varies from experiment to experiment and is indicated in the figures, tables, and text. Cells were diluted in TMG or M9 salts and plated on M63 minimal galactose plates with biotin to select for Gal⁺ recombinants.¹ Cells were also diluted and plated on L plates to determine total viable cells or to screen for recombinants among the total. For the experiments in minimal medium, cells were grown to an OD₆₀₀ of 0.4 and prepared for recombineering by the standard protocol.³⁴ Other relevant methods are described in table and figure legends.

Proper mixing is important for optimized recombination levels—Pipette the electro-competent cells into the chilled microfuge tubes or cuvettes. Add the 0.5-1 µl of salt free oligo. Using a 200 µl pipette tip, pipette up and down several times to mix. At no time should electro-competent cells be vortexed. Use care to keep the cells in the electroporation chamber of the cuvette before electroporation. Once electroporation is complete, quickly add the 1 ml of LB for recovery, pipetting up and down several times before finally transferring the entire volume immediately to a recovery tube. Never electroporate without prior mixing of the DNA and cells.

Media

LB, L agar, M63 galactose with biotin, M63 glucose, TMG, MOPS,⁴⁰ MacConkey galactose, MacConkey maltose are as described previously.^{1; 4; 34} For the plasmid transformation experiment, ampicillin was added at 100 µg/ml.

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Abbreviations

MMR	methyl-directed mismatch repair
MAGE	multiplex automated genome engineering

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Figure 1. Oligo recombination in *E. coli* recombination mutant strains

All strains are derivatives of HME6 and contain a knockout of the indicated genes. Recombination frequencies in this and all tables and figures are normalized per 10⁸ viable cells, which is approximately the number of survivors in each electroporation. (~) Recombination with lagging-strand oligo 100. (~) Recombination with leading-strand oligo 101. The recombination frequency in wild type cells with the lagging strand is shown by line “A”, the leading strand by line “B”. Important note: the bias between recombinants obtained with the lagging vs the leading strand is variable in these experiments as some of the host mutations affect MMR. Additionally, the strand bias is directly affected by MMR because the mismatch on the lagging strand and the leading strand are different and thus repaired at different efficiencies.

Figure 2. Effect of oligo length on oligo recombination

The oligo will create a C~C mispair, avoiding the MMR system, when annealed to the target. The oligos are of variable length with the correcting "C" always centered. The host cells were HME6 and the selection was for Gal⁺. All values represent the average of three or more experiments and are normalized per 10⁸ viable cells.

Figure 3. Effect of the position of the correcting base within an oligo

Each data point shows the position of the correcting base within that oligo. All oligos are 70 bases in length and create a T~C mispair when annealed to the target. To avoid MMR, recombination was done in the *mutS* mutant strain, HME63. All values represent the average of two or more experiments and are normalized per 10^8 viable cells. Control oligos were used that did not overlap the *galK_{am}*, but ended either three bases upstream or started one base downstream. The Gal⁺ frequency for these oligos was $\sim 3 \times 10^1 / 10^8$ viable cells; the same as was seen with no oligo added.

<u>Oligo</u>	TTG	CAG	CAG	CTT	TAG	CAT	CTG	CCG	CTG
	AAC	GTC	GTC	GAA	ATC	GTA	GAC	GGC	GAC
	<u>Gal⁺/10⁸</u>								
100	---	---	---	---	--T	---	---	---	---3.5x10 ⁵
252	--C	---	---	---	--T	---	---	---	---1.2x10 ⁵
253	---	--C	---	---	--T	---	---	---	---4.3x10 ⁵
230	---	---	---	---	--T	---	---	---	--C3.6x10 ⁵
229	---	---	---	---	--T	---	---	--C	---4.1x10 ⁵
254	---	---	-- C	---	--T	---	---	---	---1.1x10 ⁷
176	---	---	---	---	--T	---	-- C	---	---1.0x10 ⁷
144	---	---	---	---	-- C	---	---	---	---2.0x10 ⁷

Figure 4. A nearby c~C mismatch can increase the recombination frequency of a correcting base
 The double-strand sequence of the region around the *galK_{am}* mutation in HME6 is shown at the top where the upper sequence denotes the lagging-strand and the “G” of the amber codon is shown in bold. Oligos listed below are identical in sequence to the lagging-strand except for the indicated bases. All oligos are 70 bases in length and only the relevant changes are shown. Recombination values obtained from these oligos are an average of three or more experiments and are normalized per 10⁸ viable cells. HME6 has a functional MMR system.

A.						B.					
CAG CTT TAG CAT CTG						CAG CTT TAA CAT CTG					
GTC GAA ATC GTA GAC						GTC GAA ATT GTA GAC					
<u>Oligo</u>						<u>Gal⁺/10⁸</u>					
100	---	---	--T	---	---	2.1x10 ⁵	---	---	--T	---	2.0x10 ⁵
278	---	---	-G-	---	---	5.1x10 ⁴	---	---	-GG	---	5.2x10 ⁴
282	---	---	-T-	---	---	1.3x10 ⁷	---	---	-TG	---	6.2x10 ⁴
283	---	---	CT-	---	---	3.9x10 ⁵	---	---	CTG	---	5.2x10 ⁵
280	---	---	CTA	---	---	7.7x10 ⁶	---	---	CT-	---	5.3x10 ⁶
378	---	---	CTT	---	---	1.0x10 ⁷	---	---	CTT	---	1.5x10 ⁷
281	---	--A	CTA	---	---	1.3x10 ⁷	---	--A	CT-	---	9.3x10 ⁶
379	---	--A	CT-	---	---	1.4x10 ⁷	---	--A	CTG	---	9.4x10 ⁶
382							---	--A	CTC	---	1.7x10 ⁷

Figure 5. The effect of multiple changes on oligo recombination frequencies

The double-strand sequence of the region around the *galK* mutations in HME6 (panel “A”) and HME58 (panel “B”) is shown at the top where the upper sequence denotes the lagging-strand and the stop codon is shown in bold. Oligos listed below are identical in sequence to the lagging-strand of the strain above except for the indicated bases. All oligos are 70 bases in length and only the relevant changes are shown. Recombination values obtained from these oligos are an average of three or more experiments and are normalized per 10⁸ viable cells. Both HME6 and HME58 have a functional MMR system.

A.							B.							
		CAG	CTT	TAG	CAT	CTG			CAG	CTT	TAA	CAT	CTG	
		GTC	GAA	ATC	GTA	GAC			GTC	GAA	ATT	GTA	GAC	
Oligo						Gal ⁺ /10 ⁸						Gal ⁺ /10 ⁸		
473		---	--A	--T	---	---	4.8x10 ⁵		---	--A	--T	---	---	2.7x10 ⁵
474		---	---	--T	--C	---	5.2x10 ⁵		---	---	--T	--C	---	2.9x10 ⁵
475		---	--A	--T	--C	---	1.2x10 ⁷		---	--A	--T	--C	---	5.2x10 ⁶
476		--A	--A	--T	--C	---	1.9x10 ⁷		--A	--A	--T	--C	---	1.0x10 ⁷
477		---	--A	--T	--C	--A	2.0x10 ⁷		---	--A	--T	--C	--A	1.0x10 ⁷
478		--A	--A	--T	--C	--A	1.7x10 ⁷		--A	--A	--T	--C	--A	2.0x10 ⁷

Figure 6. Changing the wobble position of multiple codons to increase oligo recombination frequencies
See Figure 5 legend for details.

Figure 7. Diagram of potential routes to create multiple recombinant cell types in an individual colony

I) One copy of the double-stranded *E. coli* chromosome is denoted as linear for simplicity. The DNA replication origin, *oriC*, is shown as a green box. Relative positions of the *malK* and *galK* genes are shown. This diagram is not to scale. Throughout the diagram, DNA strands are labeled on the left end for identification with newly made strands receiving new labels. Newly synthesized DNA is dashed when on the lagging-strand and solid when on the leading strand, but both are solid when they replicate again. The purple “+” signs indicate an oligo recombination event on that DNA strand. From panel “I” to “II”, DNA replication and oligo recombination with both *malK* (R) and *galK* (S) oligos occurs. III) The replication origins in “II” have initiated another round of DNA replication and there are two more recombination events; one at *malK* (R) and one at *galK* (P) on different strands. It is important to note that under our growth conditions, about 66% of cells will have four origins, the rest having eight,³⁹ so the events depicted can potentially happen in a single cell before any cell division. Continued DNA replication and cell division will occur as the cell

generates a colony that will contain $\text{Mal}^+ \text{Gal}^+$, $\text{Mal}^+ \text{Gal}^-$, and $\text{Mal}^- \text{Gal}^+$ recombinant cell types in addition to the $\text{Mal}^- \text{Gal}^-$ parental type.

Figure 8. Photograph of optimized oligo recombination of *galK_{am}*

Strain HME6 was recombined with the lagging-strand, 70 base oligo 144, which creates a C~C mispair when annealed to the target and thus avoid the MMR system. After a 30 minute recovery, cells were diluted and plated on a MacConkey galactose plate. Approximately 70% of the colonies contain red, ie. Gal⁺ cells. Most of the Gal⁺ colonies are sectorized, consistent with multiple copies of the *galK* locus being present under these growth conditions.

Table 1

The effects of oligo concentration, non-specific carrier oligo, and single-strand exonucleases.

# of Gal ⁺ oligos/cell	Gal ⁺ /10 ⁸ : Dilute in H ₂ O		Gal ⁺ /10 ⁸ : Dilute in Oligo ³	
	<i>I</i> _{Exo⁺}	<i>2</i> _{Exo⁻}	<i>I</i> _{Exo⁺}	<i>2</i> _{Exo⁻}
30000	1.4×10 ⁷			
3000	1.6×10 ⁷	3.9×10 ⁷		
300	7.3×10 ⁶	1.3×10 ⁷	1.3×10 ⁷	9.3×10 ⁶
30	8.7×10 ⁴	1.5×10 ⁶	2.2×10 ⁶	1.5×10 ⁶
3	6.4×10 ³	3.9×10 ⁵	2.2×10 ⁵	3.7×10 ⁵

¹ Recombination with oligo 144 in HME6 cells.

² Recombination with oligo 144 in XTL74, which is deleted for the Exo I, Exo VII, Exo X and RecJ exonucleases. All data throughout the table are the average of three or more experiments. 3000 oligos/cell is 5 pmoles of oligo per reaction. Experiments in XTL74 cells used 2-fold higher oligo concentration than indicated in column 1.

³ Carrier oligo (LT217) was included at 3000 oligos/cell.

Table 2

Percentage of recombinant cells within a colony.

Group	Expected results if 8 copies of <i>galK</i> are present when recombination occurs ¹		Observed % Gal ⁺ found in individual colonies ²
	# of <i>galK</i> recombined	% of Gal ⁺ cells expected	
I.	1	6.25	5, 5, 6, 6, 6, 6, 7, 7
II.	2	12.5	10,10,10,11,12,12,13,14
III.	3	18.75	17,17,17,17,18,18,20,20
IV.	4	25	23,24,24,25,27
V.	5	31.25	29,30,30,33
VI.	6	37.5	37
VII.	7	43.75	40,41
VIII.	8	50	52,52

¹This population of cells is not synchronized so when recombination occurs, there is a variable number of sister chromosomes present. However, under our growth conditions cells have four or eight sister chromosomes.³⁸

²Analysis of variance (ANOVA) of these data, assuming 1/16, 2/16, 3/16 etc. of the DNA strands have recombined with the oligo confirm the model is supported by the data. The p-value was a highly significant $p < 0.001$ for all groups but 37.5 and 43.75 where the number of data points in the group was limiting.

Table 3

Transformation and recombination efficiencies with cells grown in either minimal or LB medium:

Growth medium	Gal ⁺ /10 ⁸ viable ¹	Plasmid Transformants/10 ⁸ Viable ²	Gal ⁺ Recombinants/plasmid transformant
M63 glucose LB	1.1×10 ⁴	3.0×10 ³	4.1%
	8.7×10 ⁶	1.4×10 ⁷	34%

¹ Values are the average of three or more experiments throughout the table. Recombination was with oligo 144 in HME6.

² Transformation was with 20 ng of plasmid pLT60.

Table 4

Effect of recovery/outgrowth time on oligo recombination.

32° outgrowth (min.)	Viability ¹	% Recombinants ²
0	9.0×10 ⁵	7.6
15	7.1×10 ⁶	25.2
30	7.8×10 ⁶	17.2
45	9.7×10 ⁶	18.8
60	1.2×10 ⁷	22.2
75	2.0×10 ⁷	22.7
90	1.4×10 ⁷	22.8
120	3.3×10 ⁷	18.2

¹Titers determined on L plates at the time shown after electroporation.

²Percentage of recombinant colonies on MacConkey galactose plates. Sectorred red/white colonies were scored as recombinants. Recombination was with oligo 144 in HME6 cells. Values are an average of three or more experiments throughout the table.