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Probing Cellular Processes with Oligo-Mediated Recombination and Using the Knowledge Gained to Optimize Recombineering ☆

James A. Sawitzke[1], Nina Costantino[1], Xin-tian Li[1], Lynn C. Thomason[2], Mikhail Bubunenko[1,2], Carolyn Court[3] and Donald L. Court[1]*

[1]*Molecular Control and Genetics Section, Gene Regulation and Chromosome Biology Laboratory, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702, USA*
[2]*Gene Regulation and Chromosome Biology Laboratory, Basic Science Program, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD 21702, USA*
[3]*Transcription Control Section, Gene Regulation and Chromosome Biology Laboratory, Center for Cancer Research, National Cancer Institute at Frederick, Frederick, MD 21702, USA*

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Recombination with single-strand DNA oligonucleotides (oligos) in *Escherichia coli* is an efficient and rapid way to modify replicons *in vivo*. The generation of nucleotide alteration by oligo recombination provides novel assays for studying cellular processes. Single-strand exonucleases inhibit oligo recombination, and recombination is increased by mutating all four known exonucleases. Increasing oligo concentration or adding nonspecific 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 6 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 dependent on media and is 10,000-fold reduced for cells grown in minimal *versus* rich medium. Genomewide 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.

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**Corresponding author*. E-mail address: court@ncifcrf.gov.

Abbreviations used: oligo, oligonucleotide; MMR, methyl-directed mismatch repair; MAGE, multiplex automated genome engineering; dsDNA, double-strand DNA; ssDNA, single-strand DNA; MOPS4-morpholinepropanesulfonic acid.

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Introduction

In the past decade, recombineering (*recombi*nation-mediated genetic engi*neering*) has become the method of choice for genetically modifying large DNA molecules. Recombineering is highly efficient,[1,2] is targeted by short (~50 base) DNA homologies,[3–5] is not limited by the availability of restriction sites,[5] and can be automated to rapidly make genomewide alterations.[6] With large DNA molecules such as bacterial artificial chromosomes or bacterial genomes, traditional genetic engineering techniques fail, since finding convenient unique restriction sites proves impossible and 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 in a bacterial artificial chromosome, phage P1 artificial chromosome, virus, or plasmid in *Escherichia coli* before moving back into their organism of choice. Recently, genomewide engineering technologies that utilize recombineering, including multiplex automated genome engineering (MAGE)[6] and trackable multiplex recombineering (TRMR), have been developed.[7] In addition to advantages in genetic engineering, oligonucleotide (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 provide new insights into such processes and 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, with λ Red[3,8–10] or *E. coli* RecET[5] being the most commonly used; however, several new systems are being developed.[11–14] The λ Red system includes three proteins (Exo, Beta, and Gam) and recombines both double-strand DNA (dsDNA)[3,8–10] and single-strand DNA (ssDNA).[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 kb,[6] 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 on a RecA-like function.[18,19] In *E. coli*, numerous recombination functions (Fig. 1) have been categorized as being part of the RecBCD and/or RecF recombination pathway. Both RecBCD and RecF pathways require RecA protein; 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 the Red functions have been briefly expressed 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 ssDNA at the replication fork.[1,2,15,23–25] An annealed oligo containing at least 1 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 Court 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.[1] Mutation of the MMR genes increased oligo-mediated recombination ~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. We have designed[1] recombining oligos 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 change[1] 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 reported[27] 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 a 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 Sharan[17] obtained a high frequency of recombination when 6–20 consecutive

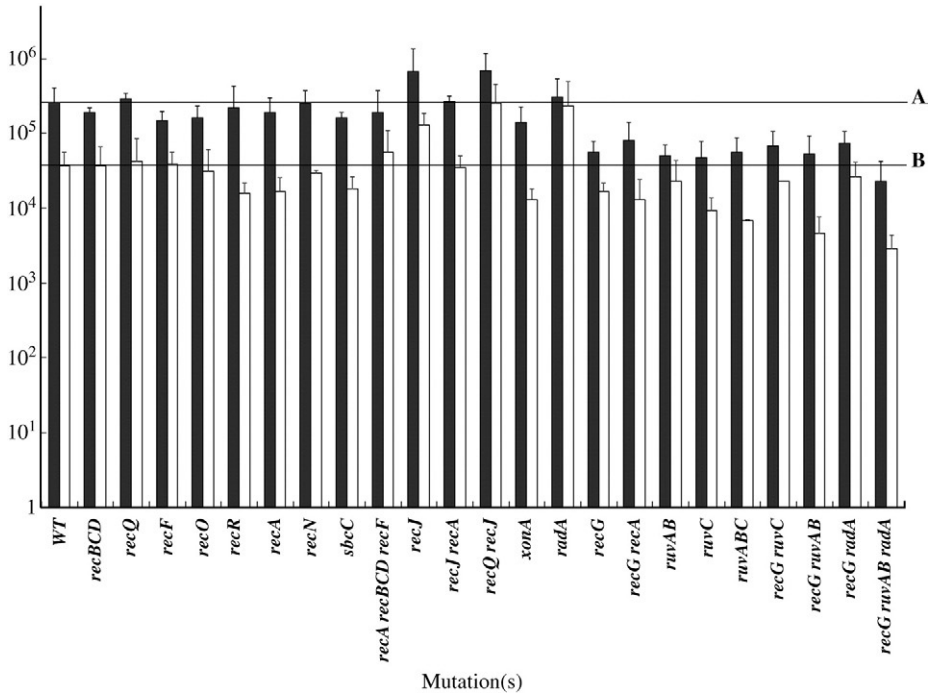

Fig. 1. Oligo recombination in *E. coli* recombination mutant strains. All strains are derivatives of HME6 and contain a knockout of the indicated genes. Recombination frequencies in this figure and in all tables and figures are normalized per 10^8 viable cells, which is approximately the number of survivors 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," and the recombination frequency in wild-type cells with the leading-strand is shown by line "B." Note that the bias between recombinants obtained with the lagging-strand *versus* 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 mismatches on the lagging-strand and the leading-strand are different and are thus repaired at different efficiencies.

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 MAGE[6] 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. In order to better understand this process, we analyzed the recombinants generated when two loci were simultaneously targeted. Furthermore, 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 (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 (Fig. 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 6-fold, but only with the leading-strand oligo. The *recJ* and *recQ recJ* mutant strains showed increased recombination for both leading-strand and lagging-strand. We have determined that

Table 1. The effects of oligo concentration, nonspecific carrier oligo, and single-strand exonucleases

Number of Gal$^+$ oligos/cell	Gal$^+$/10^8 Diluted in H_2O		Gal$^+$/10^8 Diluted in oligo[a]	
	Exo^{+b}	Exo^{-c}	Exo^{+b}	Exo^{-c}
30,000	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

[a] Carrier oligo (LT217) was included at 3000 oligos/cell.
[b] Recombination with oligo 144 in HME6 cells.
[c] Recombination with oligo 144 in XTL74, which has been deleted for ExoI, ExoVII, ExoX, and RecJ exonucleases. All data throughout the table are the average of three or more experiments (3000 oligos/cell = 5 pmol of oligo per reaction). Experiments in XTL74 cells used an oligo concentration 2-fold higher than that indicated in column 1.

these effects are due to RecJ's role in MMR (data not shown).[33]

Concentration of oligo used in recombination

We routinely use 5 picomoles of oligo for recombination,[1,34,35] which is equal to approximately 3000 oligos/bacterial cell electroporated under our conditions. Increasing the number of oligos per cell 10-fold or 100-fold (Table 1; data not shown) does not increase the number of recombinants, indicative of saturation. Table 1 also compares the results obtained when the oligo was diluted either in sterile dH_2O or with a "carrier" oligo. When present, the carrier was added at 3000 oligos/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 ssDNA 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 dH_2O (3000 → 300) decreased recombination only ~2-fold, a further 10-fold dilution (300 → 30 oligos/cell) decreased recombination more than 80-fold. However, by dilution into carrier oligo, the dilution from 300 to 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 host single-strand exonucleases affect recombination?

Oligos 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 oligo length resulted in decreased recombinant formation; however, those experiments were performed with an oligo that was subjected to mismatch repair.[1] Here we expand on previous experiments by examining a wider range of oligo lengths and by 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 a 70-mer generates the highest level. Reducing the

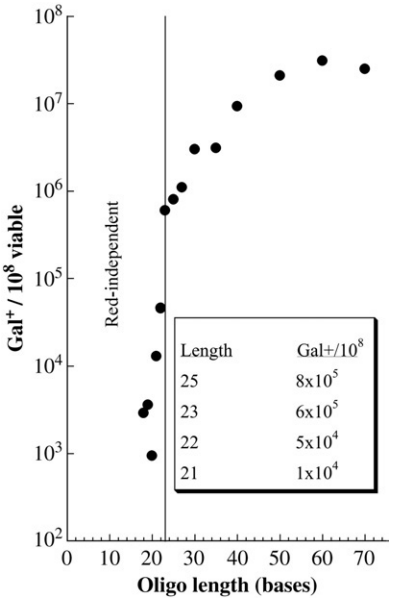

Fig. 2. Effect of oligo length on oligo recombination. The oligo will create a C·C mispair (avoiding the MMR system) when annealed to the target. The oligos are of variable lengths, with the correcting "C" always centered. The host cells were HME6, and the selection was for Gal$^+$. All values represent the average of three or more experiments and are normalized per 10^8 viable cells.

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 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 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,

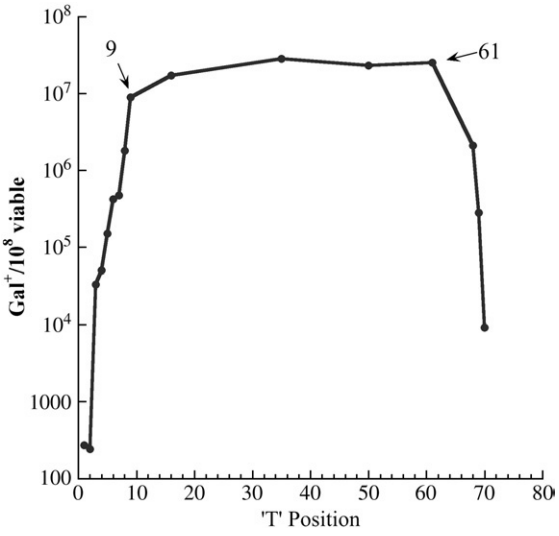

Fig. 3. Effect of the position of the correcting base within an oligo. Each data point shows the position of the correcting base within that oligo. All oligos are 70 bases in length and create a T·C mispair when annealed to the target. To avoid MMR, we performed recombination in the *mutS* mutant strain HME63. All values represent the average of two or more experiments and are normalized per 10^8 viable cells. Control oligos that did not overlap $galK_{am}$ were used, but either ended 3 bases upstream or started 1 base downstream. The Gal^+ frequency for these oligos was $\sim 3\times10^1/10^8$ viable cells, the same as that seen with no oligo added.

yielding approximately 1×10^7 to 3×10^7 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 5 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 (Fig. 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 amber mutation yields Gal^+ recombinants at an average frequency of $3.5\times10^5/10^8$ viable cells (oligo 100). However, if the oligo creates a C·C mismatch (oligo 144), MMR is avoided, and high recombination levels ($2\times10^7/10^8$) 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 (Fig. 4). The level of recombination increased 30-fold if an additional alteration that results in a C·C mismatch is made 6 bases from the correcting "T" (oligos 254 and 176), while a C·C mismatch farther away than 6 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

Oligo	TTG CAG CAG CTT TA**G** CAT CTG CCG CTG AAC GTC GTC GAA ATC GTA GAC GGC GAC	$Gal^+/10^8$
100	--- --- --- --- --T --- --- --- ---	$3.5x10^5$
252	--C --- --- --- --T --- --- --- ---	$1.2x10^5$
253	--- --C --- --- --T --- --- --- ---	$4.3x10^5$
230	--- --- --- --- --T --- --- --- --C	$3.6x10^5$
229	--- --- --- --- --T --- --- --C ---	$4.1x10^5$
254	--- --- --**C** --- --T --- --- --- ---	**$1.1x10^7$**
176	--- --- --- --- --T --- --**C** --- ---	**$1.0x10^7$**
144	--- --- --- --- --**C** --- --- --- ---	**$2.0x10^7$**

Fig. 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 boldface. 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 relevant changes are shown. Recombination values obtained from these oligos are an average of three or more experiments and are normalized per 10^8 viable cells. HME6 has a functional MMR system.

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 3 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 (Fig. 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 (Fig. 5b), again demonstrating the importance of sequence context. Such contextual effects have been previously demonstrated.[29]

(a)

Oligo	CAG CTT **TAG** CAT CTG GTC GAA ATC GTA GAC	$Gal^+/10^8$
100	--- --- --T --- ---	$2.1x10^5$
278	--- --- -G- --- ---	$5.1x10^4$
282	--- --- -T- --- ---	$1.3x10^7$
283	--- --- CT- --- ---	$3.9x10^5$
280	--- --- CTA --- ---	$7.7x10^6$
378	--- --- CTT --- ---	$1.0x10^7$
281	--- --A CTA --- ---	$1.3x10^7$
379	--- --A CT- --- ---	$1.4X10^7$
382		

(b)

CAG CTT **TAA** CAT CTG GTC GAA ATT GTA GAC	$Gal^+/10^8$
--- --- --T --- ---	$2.0x10^5$
--- --- -GG --- ---	$5.2x10^4$
--- --- -TG --- ---	$6.2x10^4$
--- --- CTG --- ---	$5.2x10^5$
--- --- CT- --- ---	$5.3x10^6$
--- --- CTT --- ---	$1.5x10^7$
--- --A CT- --- ---	$9.3X10^6$
--- --A CTG --- ---	$9.4X10^6$
--- --A CTC --- ---	$1.7X10^7$

Fig. 5. The effect of multiple changes on oligo recombination frequencies. The double-strand sequence of the region around the *galK* mutations in HME6 (a) and HME58 (b) is shown at the top, where the upper sequence denotes the lagging-strand, and the stop codon is shown in boldface. 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 relevant changes are shown. Recombination values obtained from these oligos are an average of three or more experiments and are normalized per 10^8 viable cells. Both HME6 and HME58 have a functional MMR system.

Evasion of the MMR system by consecutive wobble base alterations

We examined whether changing only the wobble (third) position in several nearby codons while retaining wild-type amino acid sequence could also result in high levels of recombination. As shown in Fig. 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 an 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 MAGE[6] 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 (Fig. 7). In this experiment, immediately after electroporation, cells were plated on L agar, incubated overnight, and independent colonies were 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) were both Mal$^+$ and Gal$^+$. The remaining 240 colonies were white on both indicator plates and thus nonrecombinant. 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, thus allowing for the 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 Discussion. Consistent with Wang *et al.*, we conclude that oligo recombination can occur at more than one locus in the same cell.[6] 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 of 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 been 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

(a)

CAG CTT **TAG** CAT CTG
GTC GAA ATC GTA GAC

Oligo		Gal$^+$/10^8
473	--- --A --T --- ---	4.8x10^5
474	--- --- --T --C ---	5.2x10^5
475	--- --A --T --C ---	1.2x10^7
476	--A --A --T --C ---	1.9x10^7
477	--- --A --T --C --A	2.0x10^7
478	--A --A --T --C --A	1.7x10^7

(b)

CAG CTT **TAA** CAT CTG
GTC GAA ATT GTA GAC

		Gal$^+$/10^8
	--- --A --T --- ---	2.7x10^5
	--- --- --T --C ---	2.9x10^5
	--- --A --T --C ---	5.2x10^6
	--A --A --T --C ---	1.0x10^7
	--- --A --T --C --A	1.0x10^7
	--A --A --T --C --A	2.0x10^7

Fig. 6. (a and b) Changing the wobble position of multiple codons to increase oligo recombination frequencies. See Fig. 5 legend for details.

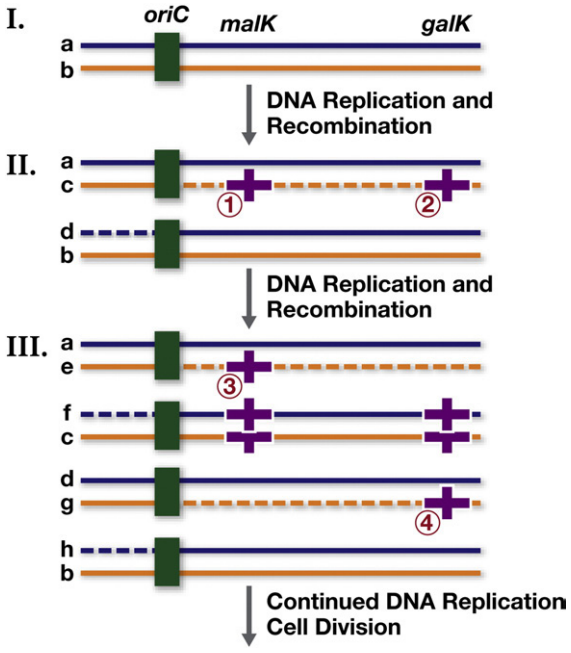

Fig. 7. Diagram of potential routes to create multiple recombinant cell types in an individual colony. (I) One copy of the double-strand *E. coli* chromosome is denoted as linear for simplicity. The DNA replication origin *oriC* is shown as a green box. The relative positions of the *malK* and *galK* genes are shown. This diagram is not drawn 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 represented by a broken line when on the lagging-strand and by a solid line when on the leading-strand, but both are represented by a solid line when they replicate again. The purple "+" signs indicate an oligo recombination event on that DNA strand. From (I) to (II), DNA replication and oligo recombination with both *malK* (①) and *galK* (②) oligos occur. (III) The replication origins in (II) have initiated another round of DNA replication, and there are two more recombination events: one at *malK* (③) and one at *galK* (④) on different strands. It is important to note that, under our growth conditions, about 66% of cells will have four origins, with 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 Mal^+ Gal^+, Mal^+ Gal^-, and Mal^- Gal^+ recombinant cell types, in addition to the Mal^- Gal^- parental type.

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 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 being dependent on the chromosomal position of the targeted gene, the number of targets recombined, and how early in the cell cycle the recombination occurs (i.e., in a newly divided "baby" cell *versus* 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 under 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 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–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, creating a C·C mismatch; this should thus give the highest possible recombination frequency.[1] When cells were grown in M63 glucose medium, however, recombination levels decreased nearly 1000-fold from the 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). Adding a small amount of yeast extract (0.2%) to M63 glucose medium or using MOPS defined medium[40] to grow cells increased recombination to similar levels, approximately 100-fold over the levels seen in M63 glucose medium but still 10-fold lower than the levels seen in LB. Recombination frequencies were normal if cells were grown in LB even when the recovery medium was M63 glucose.

Table 2. Percentage of recombinant cells within a colony

Group	Expected results when eight copies of *galK* are present upon recombination[a]		Observed percentage of Gal$^+$ cells found in individual colonies[b]
	Number of *galK* recombined	Percentage 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

[a] This population of cells is not synchronized; thus, when recombination occurs, there are a variable number of sister chromosomes present. However, under our growth conditions, cells have four or eight sister chromosomes.[39]
[b] Analysis of variance of these data—assuming that 1/16, 2/16, 3/16, and so on of the DNA strands have recombined with the oligo—confirms that the model is supported by the data. The *p*-value was highly significant ($p<0.001$ for all groups except 37.5 and 43.75 where the number of data points in the group was limiting).

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 among 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 of 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 *versus* 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 within a period of 15–30 min. Recombinant colonies formed after various recovery times were assayed by plating on MacConkey galactose plates, where both recombinants and nonrecombinants can grow. We scored red sectored colonies as Gal$^+$ (Fig. 8). Table 4 shows that recombination levels, like survival, are inhibited by plating immediately after electroporation. Optimal recombination is seen with a recovery of 15–30 min.

Discussion

Parameters affecting recombination

We find that host recombination functions have little or no effect on oligo recombination (Fig. 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 that is identical in sequence to the lagging-strand is always more efficient;[1,2,15,22] however, the

Table 3. Transformation and recombination efficiencies with cells grown in either minimal or LB medium

Growth medium	Gal$^+$/10^8 viable cells[a]	Plasmid transformants/10^8 viable cells[b]	Gal$^+$ recombinants/plasmid transformant (%)
M63 glucose	1.1×10^4	3.0×10^3	4.1
LB	8.7×10^6	1.4×10^7	34

[a] Values are the average of three or more experiments throughout the table. Recombination was performed with oligo 144 in HME6.
[b] Transformation was performed with 20 ng of plasmid pLT60.

Table 4. Effect of recovery/outgrowth time on oligo recombination

32° outgrowth (min)	Viability[a]	% Recombinants[b]
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

[a] Titers determined on L plates at the time shown after electroporation.
[b] Percentage of recombinant colonies on MacConkey galactose plates. Sectored red/white colonies were scored as recombinants. Recombination was performed with oligo 144 in HME6 cells. Values are an average of three or more experiments throughout the table.

level of bias varies.[1,2,13,15] At least some of this variability may be due to the concentration of oligo used. Under our standard conditions, the lagging-strand/leading-strand bias is approximately 15-fold 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-strand region on the leading-strand is limiting and thus more restrictive when oligo concentration is also limiting.

With either a leading-strand oligo or a 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/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 inhibitory exonuclease activities.

Oligos from 40 to 70 bases in length showed similar recombinant frequencies (Fig. 2). Others have reported maximum recombination with oligos of 90 or ~120 bases, respectively;[6,22] however, these frequencies were less than 2-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-base 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 15–20 bases in length is independent of the Red proteins, as described by Swingle *et al.*, possibly because Beta cannot anneal such short oligos.[37] 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′

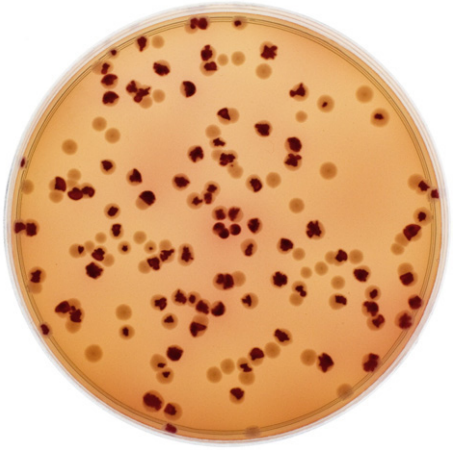

Fig. 8. Photograph of an optimized oligo recombination of $galK_{am}$. Strain HME6 was recombined with the lagging-strand 70-base oligo 144, which creates a C·C mispair when annealed to the target and thus avoids the MMR system. After a 30-min recovery, the cells were diluted and plated on a MacConkey galactose plate. Approximately 70% of the colonies contain red (i.e., Gal^+) cells. Most of the Gal^+ colonies are sectored, consistent with multiple copies of the *galK* locus being present under these growth conditions.

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 the 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 an 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 an 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 (Fig. 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 6 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 9 bases or farther away, recombination is not enhanced (Fig. 4). This indicates that a C·C mismatch may create a region of 6 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 corepair 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 5-base insertion/deletion on one strand and poorly recognizes a 4-base insertion/deletion.[28] Consistent with these results, we found that substituting four or more consecutive bases (Fig. 5; 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 (Fig. 5). These "exceptions" are likely due to sequence context effects.

Methods that use a C·C mismatch or alter four or more consecutive bases, although useful, 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 (Fig. 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 stability[43] 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, we suggest changing 5 bases or more to be safe. 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 (four to eight) of the chromosome,[39] such that recombination might occur on any one strand of any sister chromosome.[1] However, even if recombination occurred at every target on all sister chromosomes, 50% of the DNA strands, at most, 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 (Fig. 8). Pure recombinant colonies are obtained only if the recombined cells are allowed several generations of outgrowth (about 3 h at 30 °C), permitting DNA replication and cell division. During 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-fold 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 they are allowed to recover for less than 15 min. Thus, we recommend that the minimal length of recovery should be 30 min. This allows for full recovery from 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 in a nontoxic medium (e.g., selecting Gal^+) or screening for recombinants nonselectively 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 coelectroporated 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; however, consistent with our findings, it was previously shown that phage DNA transfection by electroporation into *E. coli* is less efficient when cells are grown in minimal medium.[46]

Recombination with multiple oligos

We have shown that two different oligos can be used to simultaneously alter 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 the amount of DNA that 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 coelectrotransformation by two different oligos. Within a single recombinant colony (plated without any recovery period), we found cells that were Mal^+ Gal^-, Mal^- Gal^+, and 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 by 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 (Fig. 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 Fig. 7, during DNA replication, one or more of the target lagging-strands may be recombined. In the four chromosome examples 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 nonoverlapping pattern (i.e., with 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, and 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 the following: (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 under optimal conditions. 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 automated systems such as MAGE[6] and for researchers using standard recombineering procedures.

Materials and Methods

Bacterial strains

The HME6 strain used is W3110 $galK_{TYR145UAG}$ Δ*lacU169* [λ *cI857* Δ*(cro-bioA)*].[2] Strains shown in Fig. 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] The HME63 strain used is HME6 *mutS<>amp*. The XTL74 strain used is HME6 *recJ<>amp xonA<>kan xseA<>tet exoX<>spec*. HME58 is the same as 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.

Oligos and plasmids

Oligos were purchased from IDT salt free, but otherwise unpurified. Oligos 100, 101, and 144, and the *malK* oligo were used as described previously.[1] The "carrier" oligo was LT217, an oligo with homology to a plasmid not present 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 performed as described by Costantino and Court.[1] Cells were prepared for recombination by standard methods.[34,35] Five picomoles of oligo was electrotransformed into the cells, 1 ml of LB was added, and the 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 the total viable cells or to screen for recombinants among the total. For the experiments in minimal medium, the cells were grown to an OD_{600} of 0.4 and prepared for recombineering by the standard protocol.[34] Other relevant methods are described in the table and figure legends.

Proper mixing is important for optimized recombination levels

Pipette electrocompetent cells into chilled microfuge tubes or cuvettes. Add 0.5– 1 μl of salt-free oligo. With a 200 μl pipette tip, pipette up and down several times to mix. At no time should electrocompetent cells be vortexed. Use care to keep the cells in the electrotransformation chamber of the cuvette before electrotransformation. Once electrotransformation has been completed, quickly add 1 ml of LB for recovery, pipetting up and down several times before finally transferring the entire volume immediately to an recovery tube. Never electrotransform without prior mixing of the DNA and the cells.

Media

LB, L agar, M63 galactose with biotin, M63 glucose, TMG, MOPS defined medium,[40] MacConkey galactose, and MacConkey maltose were used as described previously.[1,4,34] For the plasmid transformation experiment, ampicillin was added at 100 μg/ml.

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