# RecBCD Enzyme "Chi Recognition" Mutants Recognize Chi Recombination Hotspots in the Right DNA Context

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**ABSTRACT** RecBCD enzyme is a complex, three-subunit protein machine essential for the major pathway of DNA double-strand break repair and homologous recombination in *Escherichia coli*. Upon encountering a Chi recombination-hotspot during DNA unwinding, RecBCD nicks DNA to produce a single-stranded DNA end onto which it loads RecA protein. Conformational changes that regulate RecBCD's helicase and nuclease activities are induced upon its interaction with Chi, defined historically as 5′ GCTGGTGG 3′. Chi is thought to be recognized as single-stranded DNA passing through a tunnel in RecC. To define the Chi recognition-domain in RecC and thus the mechanism of the RecBCD-Chi interaction, we altered by random mutagenesis eight RecC amino acids lining the tunnel. We screened for loss of Chi activity with Chi at one site in bacteriophage λ. The 25 *recC* mutants analyzed thoroughly had undetectable or strongly reduced Chi-hotspot activity with previously reported Chi sites. Remarkably, most of these mutants had readily detectable, and some nearly wild-type, activity with Chi at newly generated Chi sites. Like wild-type RecBCD, these mutants had Chi activity that responded dramatically (up to fivefold, equivalent to Chi's hotspot activity) to nucleotide changes flanking 5′ GCTGGTGG 3′. Thus, these and previously published RecC mutants thought to be Chi-recognition mutants are actually Chi context-dependence mutants. Our results fundamentally alter the view that Chi is a simple 8-bp sequence recognized by the RecC tunnel. We propose that Chi hotspots have dual nucleotide sequence interactions, with both the RecC tunnel and the RecB nuclease domain.

**KEYWORDS** chromosomal sites; recombination hotspots; Chi; RecBCD enzyme; DNA context-dependence

HOMOLOGOUS recombination, like other aspects of chromosome metabolism, is controlled by special DNA sites. The sites controlling homologous recombination that are best understood at the molecular level are the Chi hotspots of *Escherichia coli* (Smith 2012). Chi sites control RecBCD enzyme, a complex enzyme with both nuclease and helicase activities essential for the major pathway of DNA double-strand break repair and genetic recombination. These sites, called Chi for **c**rossover **h**otspot **i**nstigator (Lam *et al.* 1974), locally stimulate recombination by altering the multiple activities of RecBCD. Here, we address the question of how RecBCD recognizes Chi, the first step in its stimulation of recombination. Our results force a reanalysis of both the Chi nucleotide sequence and how RecBCD recognizes Chi.

Chi was discovered as a set of mutations that increase the plaque size of phage λ *red gam* mutants, which rely on the *E. coli* RecBCD pathway for growth (Lam *et al.* 1974; McMilin *et al.* 1974; Henderson and Weil 1975). In the absence of its own recombination functions (Red) and of the RecBCD inhibitor (Gam), λ replication forms only monomeric circles. Phage DNA packaging, however, requires dimeric or higher order concatemers, which can be formed by RecBCD-promoted recombination between monomers. Wild-type λ lacks Chi sites; thus, the RecBCD pathway operates on λ *red gam* mutants only at low level, only a few packaged phage are produced, and burst and plaque sizes are small. Mutations creating a Chi hotspot arise at multiple, widely scattered locations in the genome (Henderson and Weil 1975; Stahl *et al.* 1975); increase the frequency of RecBCD-promoted concatemer formation; and result in larger burst and plaque sizes. Analysis of six Chi sites in λ and one in the *E. coli lacZ* gene


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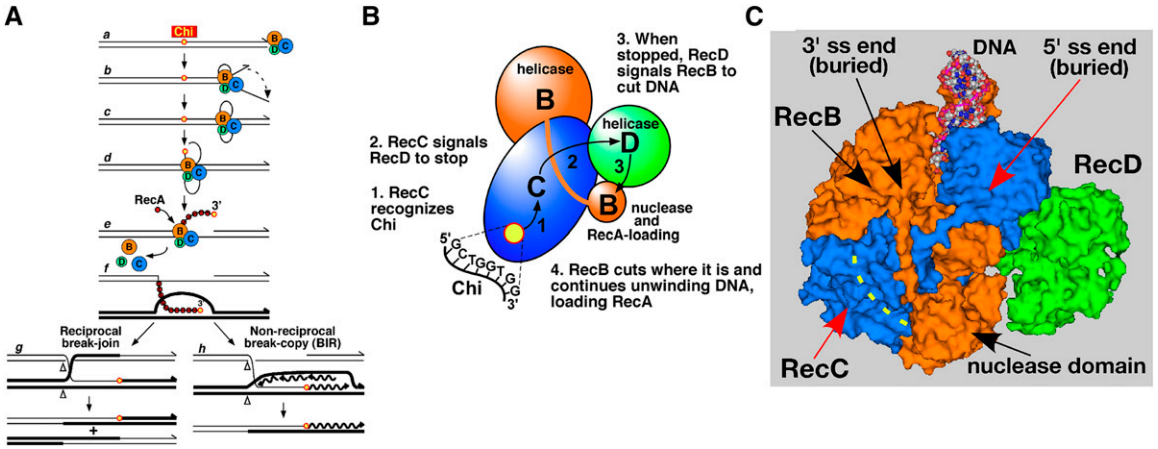


**Figure 1** Models for recombination and regulation of RecBCD enzyme by Chi hotspots, and RecBCD structural features involved in its context-dependent response to Chi. See text for further explanations. (A) Model for RecBCD-promoted recombination (Amundsen *et al.* 2007). Break-induced replication (BIR). (B) Intersubunit signal transduction model for regulation of RecBCD enzyme activities in response to Chi (Amundsen *et al.* 2007). (C) The RecBCD-ds DNA complex shown in a surface representation (PDB 1W36; Singleton *et al.* 2004). The RecB, C, and D subunits are colored as in panels A and B. The bound DNA has four terminal unwound bp; the 3′ terminus extends into the RecB helicase domain and the 5′ terminus extends into RecC headed toward the RecD helicase domain. Dotted yellow line represents the RecC tunnel in which Chi is putatively recognized. See Figure 2 for additional views.

revealed a sequence, 5′ GCTGGTGG 3′, common to all (Smith *et al.* 1984). Mutations creating or inactivating Chi are all in this octamer. The flanking sequences have no discernible similarity, and it was concluded that Chi is 5′ GCTGGTGG 3′, its complement, or the duplex (Smith *et al.* 1981a). Indeed, insertion of synthetic DNA with 5′ GCTGGTGG 3′ results in Chi activity both with purified enzyme and in cells (Dixon and Kowalczykowski 1991; Dabert *et al.* 1992; Dabert and Smith 1997). Analysis of heteroduplex DNA showed that only the strand with 5′ GCTGGTGG 3′ is needed to activate purified RecBCD (Bianco and Kowalczykowski 1997).

Since Chi acts on the host RecBCD pathway but not on the λ Red pathway or on two host pathways activated by suppressors of *recBC* null mutations (Gillen and Clark 1974; Stahl and Stahl 1977), it seemed likely that Chi interacts with RecBCD enzyme, the component unique to the RecBCD pathway. This hypothesis was bolstered by the isolation of intragenic pseudorevertants of a *recC* null mutant that regained at least partial recombination proficiency but lacked detectable Chi activity (Schultz *et al.* 1983).

Direct evidence that RecBCD recognizes Chi came from the demonstration that wild-type RecBCD enzyme, but not that from the pseudorevertants, nicks DNA at Chi during DNA unwinding (Ponticelli *et al.* 1985; Taylor *et al.* 1985). Collectively, these observations supported a model of recombination (Figure 1A) (Smith *et al.* 1981b) in which RecBCD initiates DNA unwinding at a free double-stranded (ds) DNA end, rapidly unwinds the DNA with the production of single-stranded (ss) DNA loops, nicks one strand at a properly oriented Chi site, and continues unwinding. The newly generated 3′ ss end, with Chi near its end, is coated by RecA strand-exchange protein and invades an intact homologous duplex to generate a D-loop. The D-loop is converted into a Holliday junction, which may be resolved into reciprocal recombinants; alternatively, the D-loop may prime DNA replication and generate nonreciprocal recombinants (Smith 1991, 2012). Later reports showed that RecBCD actively loads RecA onto the newly generated 3′ end in a Chi-dependent manner (Anderson and Kowalczykowski 1997).

The multiple activities of RecBCD are altered by Chi, as noted above. The phenotypes of a class of special missense mutations altering the RecB helicase domain led to the "signal transduction" model of Chi's control of RecBCD (Figure 1B) (Amundsen *et al.* 2007). In this model, when Chi is in a tunnel in RecC, this subunit signals the RecD helicase to stop unwinding, which in turn signals the RecB nuclease domain to nick the DNA at Chi and to begin loading RecA onto the newly generated 3′-ended ss DNA as unwinding by RecB continues. Conformational changes in RecBCD upon binding of DNA and again upon encountering Chi have supported this model (Taylor *et al.* 2014), which is consistent with current genetic and biochemical data.

Central to this model is recognition of Chi by RecC. In the crystal structure of RecBCD bound to ds DNA (Figure 1C and Figure 2), the 5′ end of the DNA heads toward the RecD helicase and the 3′ end is in the RecB helicase domain headed toward a tunnel in RecC (Singleton *et al.* 2004; Saikrishnan *et al.* 2008). Amino acids lining this tunnel are altered in the pseudorevertants noted above. A set of RecC mutants, each with 1 of 11 amino acids lining the RecC tunnel changed to alanine, was reported to have reduced or abolished Chi activity (Handa *et al.* 2012), further implicating the RecC tunnel in Chi recognition. That report used Chi at one locus in λ. We report here a different, but overlapping, set of RecC tunnel mutants and that these, as well as some of the previously reported RecC tunnel mutants, in fact have Chi activity; but that this activity depends strongly (up to fivefold) on the

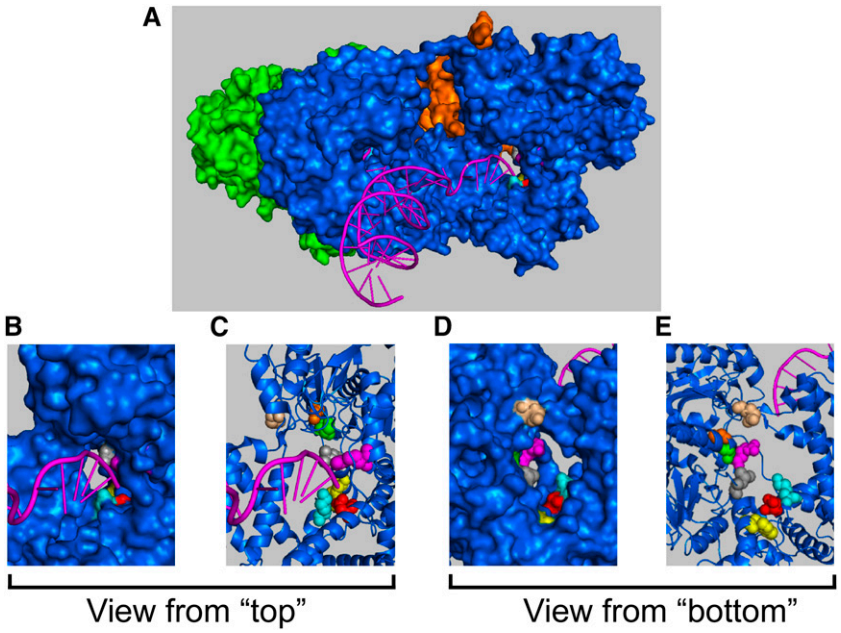


**Figure 2** The RecC tunnel in which Chi is putatively recognized. (A) A portion of the RecBCD-ds DNA complex in surface view (PDB 1K70; Saikrishnan *et al.*, 2008). RecC (blue), the RecB nuclease domain (orange), and RecD (green) are shown. One ss DNA end (magenta) enters a tunnel (right) in RecC leading to the region implicated in Chi recognition. Amino acid residues altered by mutation, seen best in B–E, are colored as follows: S39 (green), G41 (orange), L64 (gray), W70 (yellow), K88 (cyan), D136 (red), Q652 (wheat), R708 (magenta). The other ss DNA end (left) enters a second tunnel in RecC leading to RecD, a helicase. Additional panels show (B and D) enlarged surface and (C and E) ribbon representations of the tunnel. Views B and C are oriented as in A; views D and E are from the opposite side.

nucleotide-sequence context of Chi. Our results force a reanalysis of the essential sequence of Chi and how this sequence interacts with RecBCD enzyme.

## Materials and Methods

### *Bacterial strains, phage, and plasmids*

*E. coli* strains, plasmids, and phage are listed in Supplemental Material, Table S1 with their genotypes and sources. Many plasmids are derivatives of plasmid pSA607, which contains *recB*$^+$, *recD*$^+$, and *recC2773* (*recC* with six histidine codons added to the C terminus) and encodes a fully functional RecBCD enzyme (Taylor *et al.* 2014). Methods to prepare λ phage libraries with 25-bp inserts and various $\chi^+L$ alleles inserted into the λ *gam* gene by recombineering (Thomason *et al.* 2009) are in File S1.

### *Growth media*

Tryptone broth (TB) and agar (TBA), LB broth (LB) and agar (LBA), and suspension medium (SM) have been described (Schultz *et al.* 1983; Cheng and Smith 1989). TB top agar contained 0.75% Bacto-Agar (Becton Dickinson) and TB bottom agar contained 1.0% Bacto-Agar. Tryptone agar plates (BBL YE) for the detection of clear and turbid plaques contained 0.2% yeast extract (Becton Dickinson). Media were supplemented with ampicillin (100 μg/ml) or streptomycin (25 μg/ml) as needed.

### *Mutagenesis and screen for mutants with altered Chi activity*

Mutations altering the tunnel of RecC were produced on plasmid pSA607 using the QuikChange Multi Site-Directed Mutagenesis kit (Agilent Technologies) with the oligonucleotides (Integrated DNA Technologies) listed in Table S2. Oligonucleotides were designed with the desired mutation(s) using the web-based QuikChange Primer Design Program (http://www.genomics.agilent.com/primerDesignProgram.jsp).

For patch mutagenesis, the base composition of the mutagenic oligonucleotide was mixed so that each targeted codon could be efficiently changed to any other. Accordingly, at each position the wild-type base was present at 94% of the total; the remaining 3 bases were present at 2% each (see Table S2). For single-codon random mutagenesis, all 4 bases were mixed (25% each) at each of three contiguous positions in the oligonucleotide. Specific site-directed mutations were made with the QuikChange kit and oligonucleotides designed to introduce the desired point mutation (*e.g.*, W70A or D136A).

Following mutant-strand synthesis with 100 ng of pSA607 and 125 ng of the appropriate oligonucleotide, DNA was digested with *Dpn*I, and 1.5–5 μl of the reaction mix (50 μl) was used for transformation of XL10 Gold ultracompetent cells (Agilent Technologies). Ampicillin-resistant (Amp$^R$) colonies were selected on LBA plates. For preliminary characterization of the mutagenesis reaction, 5–10 isolated colonies were grown overnight in 5 ml of LB. Plasmid DNA was prepared (Pure Link Miniprep Kit; Invitrogen, Carlsbad, CA), and a portion of *recC* was amplified by a DNA polymerase chain reaction (PCR) using oligonucleotides listed in Table S2. The PCR products were purified (QIAquik PCR purification kit; QIAGEN, Valencia, CA) and sequenced using the appropriate oligonucleotide primers to determine the frequency and distribution of mutations. Sequence data included ~500 bp, centered on the position of the mutation target.

For screening *recC* phenotypes following mutagenesis, ~500 Amp$^R$ colonies were pooled into 5 ml of LB and incubated at 37° for 2 hr. Plasmid DNA was isolated as described

above and used to transform the *hsdR17 hsdM*$^+$ modification-proficient strain DH5α. A total of ~500 Amp$^R$ colonies were pooled; DNA was prepared as before and used to transform strain V2831 (Δ*recBCD2731* < *kan*> *recF143*) to Amp$^R$. The chromosomal *recBCD* deletion allowed us to assess the plasmid-borne RecBCD phenotype, and the *recF* mutation limited recombination to the RecBCD pathway. Unusual Chi-related phenotypes were observed in ~5 and 65% of the colonies screened following patch and single-codon mutagenesis, respectively (see below).

Chi activity in mutant candidates was first estimated by comparing the plaque sizes of λ 872 (χ°; *i.e.*, lacking Chi) and λ 873 (χ$^+$*76*; *i.e.*, containing Chi) phage; both contain *b1453*, which deletes *red* and most of *gam*. For large-scale screening, a 50 μl aliquot of each phage stock (8 × 10$^6$ PFU/ml) was applied as a line (~5 × 85 mm) to a BBL YE plate. Isolated colonies of transformants and *recBCD*$^+$ control strains were picked from LBA plates and cross-streaked first against λ χ° and then against λ χ$^+$*76* phage. Plaque size was examined after overnight incubation at 37°. For isolates producing apparently equal-size plaques, more accurate plaque sizes were determined on lawns of bacteria as described below.

### *Chi alleles*

To assess Chi activity in genetic assays, we used phage with Chi alleles at eight locations in the genome (Figure 3B). χ$^+$*A*, χ$^+$*76*, χ$^+$*C*, χ$^+$*D*, χ$^+$*F*, and χ$^+$*G* have been described previously (see references in Table 2). We created Chi at two new loci, χ*L* in the *gam* gene and χ*J* to its right. χ*J* alleles were identified in a phage library containing insertions of random 25-mers (see File S1). For χ$^+$*L251*, the *gam* gene was inactivated by a 1-bp frameshift near the N terminus of the gene, and the Chi octamer (5′ GCTGGTGG 3′) was created by substitutions at another 3 bp nearby (Figure 3B; Smith *et al.* 1981a). Four nucleotides flanking the Chi octamer on each side were randomized to generate χ$^+$*L252*, *253*, and *254*. The designation χ$^+$ indicates the allele contains the Chi octamer, 5′ GCTGGTGG 3′; χ$^-$ indicates the allele contains a C to T change in the octamer (5′ GTTGGTGG 3′), which inactivates Chi (Schultz *et al.* 1981). The flanking nucleotides are identical for χ$^+$ and χ$^-$ alleles with the same numerical designation.

### *Screen for phage sensitivity and Chi activity by spot test*

Spot tests for phage growth were done as described previously (Schultz *et al.* 1983). Cells were grown in TB supplemented with maltose (0.1%), thiamine (0.5 μg/ml), and ampicillin to ~1 × 10$^8$ CFU/ml. Phage stocks (~3 × 10$^3$ PFU/ml in SM) were applied as 10 μl aliquots to the lawn of bacteria in TB top agar on TBA plates. To eliminate null mutants from further analysis, candidates were tested for the retention of RecBCD nuclease activity using a phage T4 *gene 2* triple-nonsense mutant (Amundsen *et al.* 2012), which forms plaques only on cells lacking RecBCD nuclease activity (Oliver and Goldberg 1977). About 1% of the mutant candidates tested allowed T4 *gene 2* mutant plaque-formation; most of these contained plasmids with *recC* nonsense mutations.

We next assayed λ plaque size by spot test of λ 872 (χ°), λ 873 (χ$^+$*76*), and λ 801 (*red*$^+$ *gam*$^+$). As expected, wild-type (*red*$^+$ *gam*$^+$) λ phage formed large plaques on all strains. Phage containing the χ$^+$*76* allele formed large plaques on *recBCD*$^+$ cells, but not on the RecC tunnel mutants reported here (see *Results*). Phage with other Chi alleles (*i.e.*, χ$^+$*A*, χ$^+$*C*, χ$^+$*D*, χ$^+$*F*, χ$^+$*G*, χ$^+$*J*, or χ$^+$*L*) formed plaques larger than isogenic χ° phage on many tunnel mutants (see *Results*). While the absolute plaque size of a given phage on a given strain varied slightly from day to day, the relative sizes of plaques were highly reproducible.

### *λ recombination and Chi activity assays*

Recombination proficiency and Chi activity in λ crosses were measured as described (Schultz *et al.* 1983). Chi activity was quantified in two types of crosses (Figure 3). (i) Chi hotspot activity was determined from two normalized crosses between λ 1081 and λ 1082 (χ$^+$*D123*; cross 1a) and between λ 1083 and λ 1084 (χ$^+$*76*; cross 1b) (Stahl and Stahl 1977). The frequency of *J*$^+$ *R*$^+$ recombinant phage in both crosses was determined by plating on strain C600 (*supE44*) or strain V3477 (*recD2741 supE44*) for total phage, and on strain 594 (*sup*$^+$) or strain V222 (*recD1013 sup*$^+$) for recombinants. The number of clear and turbid plaques on strain 594 was determined by visual inspection; at least 50, and usually >100, plaques of each type were counted to determine the turbid/clear (t/c) ratio. These crosses are normalized by an interval lacking Chi in each cross; Chi hotspot activity is expressed as $\sqrt{(t/c)_{1a} / (t/c)_{1b}}$, where t/c is the ratio of turbid to clear plaques on strain 594 or V222 from crosses 1a and 1b as indicated (Stahl and Stahl 1977). (ii) Chi activity was determined from a nonnormalized cross between *susJ6 cI857* and *cI*$^+$ *susR5* phage with various homozygous χ*L* alleles. The frequency of *J*$^+$ *R*$^+$ recombinant phage and their plaque morphology were determined as described above. Chi activity is expressed as the ratio of clear to turbid plaques (c/t) on strain 594 or V222. Data are mean ± SEM, or individual values.

### *Hfr recombination assays*

Recombination proficiency was measured by selecting His$^+$ Str$^R$ (streptomycin-resistant) exconjugants following mating between Hfr donor strain V1306 (Hfr PO44 *his*$^+$ *rpsL*$^+$) and plasmid transformants of recipient strain V2831 (Δ*recBCD2731 hisG4 rpsL31*). The ratio of donor to recipient cells was ~1:10.

### *Data availability*

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article and Supplemental Material.

## Experimental design to identify RecBCD mutants having altered interaction with Chi

To rapidly screen RecC tunnel mutant candidates and subsequently to obtain quantitative measures for Chi activity and recombination, we used several phage-based assays to

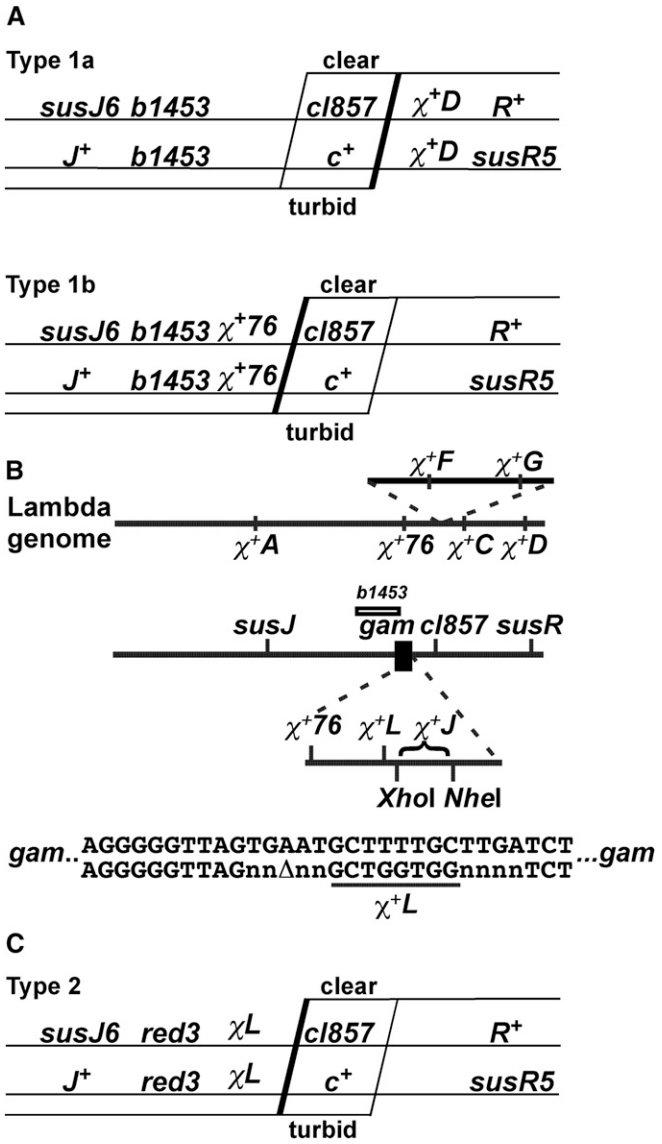


**Figure 3** Physical and genetic map of λ and crosses to assess Chi activity. (A) λ crosses to determine Chi hotspot activity of wild type and RecC tunnel mutants. Single horizontal lines represent the ds DNA of each parent in the cross with the indicated genetic markers. Diagonal lines represent the position of exchanges among $J^+$ $R^+$ recombinants; a thick line indicates exchange stimulated by an active Chi in each cross. The plaque morphology (clear or turbid) generated by the exchange is indicated. Crosses of type 1a and 1b measure Chi hotspot activity (Stahl and Stahl 1977). (B) Physical maps of λ and of the *gam* region. Markers on each line, which represents the λ genome or an insertion in which $\chi^+F$ and $\chi^+G$ arose, are placed to scale. The *b1453* deletion extends from bp 27,728–33,032; *red* (bp 31,348–32,810) with the *red-3* mutation (T to A at bp 31,966, creating *exo* D21E, and G to A at bp 32,382, creating *bet* W143*; unpublished data) is to the left of *gam* (bp 32,816–33,232). $\chi^+76$ is an A to G mutation at bp 33,058 (unpublished data), and the $\chi^+L$ octamer is at bp 33,196–33,203. Restriction enzyme cleavage sites for *Xho*I (bp 33,498) and *Nhe*I (bp 34,679) were used to clone 25-mers creating $\chi^+J$. Part of the nucleotide sequence of *gam* is shown with the 1-bp deletion to create $\chi^+L$ (underlined) flanked in this case by four random nucleotides (n). (C) Type 2 crosses to measure $\chi^+L$ activity and its context-dependence.

measure intracellular nuclease and Chi activity of the RecBCD enzyme. RecBCD influences the growth, and consequently plaque formation, of *E. coli* phages in several ways. RecBCD exonuclease activity blocks the growth and plaque formation of phage T4 mutants lacking the protective *gene 2* protein that binds to the ends of infecting DNA. These protein caps are thought to block RecBCD binding and subsequent exonucleolytic degradation (Oliver and Goldberg 1977), so that T4 $2^+$ but not T4 $2^-$ phage form plaques on RecBCD nuclease-proficient strains. Wild type and all RecC tunnel mutants reported here were RecBCD nuclease-proficient by this assay; indicating that RecBCD enzyme was functionally intact, at least for nuclease activity, which requires the helicase and ATPase activities (Hsieh and Julin 1992; Yu *et al.* 1998).

Chi activity and recombination proficiency were measured using λ *red gam* mutant phage. (Hereafter, "λ" means "λ *red gam* mutant" except where noted.) We used the λ plaque-size assay noted in the Introduction to qualitatively assess Chi activity in wild type and RecC tunnel mutants. We quantified intracellular Chi activity and recombination proficiency in λ vegetative crosses by determining the position of genetic exchange among selected recombinants (Figure 3, A and C). Chi acts primarily to its left as shown here (Faulds *et al.* 1979; Kobayashi *et al.* 1982; Taylor *et al.* 1985), resulting in an excess of exchanges in the Chi-containing interval compared to another without Chi. The first measure of Chi hotspot activity used a pair of crosses, each with a Chi-free interval that acted as a control for the other, thereby normalizing Chi activity (Figure 3A, type 1a and 1b) (Stahl and Stahl 1977). Each parental phage had a mutation (*susJ6* or *susR5*) flanking the region containing a Chi site located to the right ($\chi^+D123$) or left ($\chi^+76$) of $cI^+$ (or the alternative *cI857* allele), a plaque morphology marker. $J^+$ $R^+$ recombinants were selected on nonsuppressing ($sup^+$) *E. coli* host cells, and plaques were scored as turbid ($c^+$) or clear (*cI857*). When Chi is active, there is an excess of turbid plaques in cross 1a and an excess of clear plaques in cross 1b. Chi hotspot activity is expressed as $\sqrt{(t/c)_{1a} / (t/c)_{1b}}$ (Stahl and Stahl 1977), where t/c is the ratio of turbid to clear plaques in cross 1a or 1b, as indicated. In these crosses the Chi hotspot activity of $recBCD^+$ strains is ~5; in *recBCD* null strains, it is essentially 1, as expected from the normalization and from Chi hotspot activity requiring RecBCD.

To determine the role of nucleotide context in the Chi response, as indicated by our results below, we created Chi at a new locus (χ*L*) within the *gam* gene, ~150 bp to the right of the $\chi^+76$ locus (Figure 3B). As a control, we introduced into certain $\chi^+L$ alleles the corresponding $\chi^-L$ mutation, a C to T change in the Chi octamer that inactivates Chi at the well-studied χ*C* locus ($\chi^-C206$; 5′GTTGGTGG 3′; Sprague *et al.* 1978; Schultz *et al.* 1981). We expressed Chi activity as the ratio of clear (*cI857*) to turbid ($cI^+$) plaques (c/t) among $J^+$ $R^+$ phages from such crosses (Figure 3C, type 2). As shown below, alteration of the bp flanking $\chi^+L$ significantly raised or lowered the c/t ratio in wild type and RecC tunnel mutants by as much as fivefold, demonstrating remarkable DNA

context-dependence of Chi activity that is as strong as the stimulation of recombination by Chi itself. [We use "Chi hotspot activity" for normalized crosses and "Chi activity" for the c/t ratio in single (*i.e.*, not normalized) crosses.]

To measure overall recombination proficiency, we used standard assays of Hfr conjugational and λ vegetative recombination (Stahl and Stahl 1977; Schultz *et al.* 1983). In these assays, the recombinant frequencies in *recBC* null mutants are lower than those in wild type (*recBCD*$^+$) by factors of ~500 and ~10, respectively.

## Results

### *RecC tunnel mutants that lack Chi activity but retain nuclease and recombination-promoting activities*

To identify amino acids involved in detecting or signaling the presence of Chi on ss DNA, we mutagenized four patches of codons encoding amino acids that line the RecC tunnel, as indicated by the crystal structure (Table S3) (Singleton *et al.* 2004). In the crystal structure these patches cover a significant portion of the tunnel surface (Figure 2) and are largely composed of amino acids that extend into the tunnel interior, possibly allowing interaction with ss DNA. Although there is no direct assay for Chi recognition, such as stable binding of RecBCD enzyme to Chi-containing DNA (see *Discussion*), we quickly assessed the consequence of Chi recognition by comparing the plaque sizes of λ with a Chi site ($\chi^+76$) and without ($\chi^\circ$) as noted above.

Mutation of one to three codons within codon patches 37–44, 58–67, 643–652, and 687–691 of *recC* resulted in many mutants (~5% of the isolates tested) on which λ $\chi^\circ$ and $\chi^+76$ made plaques equal in size but ranging from tiny to large, depending on the *recC* mutant (Table S3; wild-type λ *red*$^+$ *gam*$^+$ formed large plaques on all the mutants); in some cases no plaques were observed on the *recC* mutant but were observed on the wild-type control strain. This spectrum of plaque sizes is expected, as mutation of *recC* could lead to loss of Chi activity (no or small-plaque formation by both phage) or to the ability to respond to a sequence present in both $\chi^\circ$ and $\chi^+76$ (large-plaque formation by both phage). Most mutants retained nearly full recombination proficiency as measured following Hfr conjugation (Table S3). Chi hotspot activity in the mutants, using the normalized crosses type 1a and 1b with $\chi^+76$ and $\chi^+D123$ (Figure 3A), ranged from 1.0 to 3.9 compared to 5.0 in the wild-type control (Table S3). These results confirm the loss or reduction of Chi activity initially detected by the plaque-size assay using $\chi^+76$.

We used two criteria for selecting individual codons for further analysis. First, codon changes that arose frequently in the "patch" mutants were identified. For example, 7 of 14 isolates from mutagenesis of codon patch 37–44 in *recC* contained a change in codon G41. Chi activity was reduced whether this mutation was present alone or in combination with additional mutations. Second, we identified additional codons for mutagenesis following alignment of the RecC polypeptides from bacterial species with or without known or suspected Chi activity (Schultz and Smith 1986; Smith *et al.* 1986; McKittrick and Smith 1989). We chose amino acids with 100% identity within the RecC tunnel of species with Chi activity but not in species without suspected Chi activity. Based on these criteria, we mutagenized the eight codons noted below.

### *Single amino acid changes in the RecC tunnel apparently abolishing Chi activity*

To test the requirement for individual amino acids in Chi activity, we mutagenized single codons of plasmid-borne *recC* using DNA oligonucleotides with all three randomized nucleotides at codon Q38, S39, G41, L64, K88, R644, Q652, or R708, whose encoded amino acids protrude into the tunnel (Figure 2). This randomization allowed us to screen, at each position, for any amino acid change that alters Chi activity. From each mutagenesis, we transformed a *recBCD* deletion strain and analyzed ~300 transformants that were T4 *2*$^-$ resistant and therefore RecBCD nuclease-proficient; ~50–65% of these isolates produced equal plaque sizes of λ $\chi^\circ$ and $\chi^+76$ phage and were characterized further (Table 1 and Table S4). Both phage made equally large plaques on some mutants (Q38D, G, and Y; S39A, R, L, P, and W; L64I and Y; K88D; and R644D, Q, E, G, I, S, W, and V), suggesting the presence of a DNA sequence in both $\chi^\circ$ and $\chi^+76$ phage that stimulates recombination in these RecC tunnel mutants. All of these mutants were recombination-proficient, and many had hotspot activity in normalized crosses (unpublished data; see *Discussion*). Other mutants, including the 25 thoroughly analyzed below, had reduced λ plaque size in the presence or absence of a Chi site, suggesting the loss of Chi activity (S39, G41, L64, K88, Q652, and R708; Table 1). This conclusion was supported by strongly reduced hotspot activity in normalized crosses (Table 1 and Table S4; Figure 3A). Recombination proficiency of these mutants in Hfr and λ crosses was only partially reduced (Table S5).

### *RecC tunnel mutants reveal context-dependence of Chi hotspot activity*

The results above defined several RecC tunnel amino acids involved in Chi recognition or signal transduction. We tested the hypothesis that those RecC tunnel mutants on which both $\chi^\circ$ and $\chi^+76$ phage made no visible plaques or only tiny plaques recognize a different "Chi-like" DNA sequence not found in λ. A hotspot sequence active in these mutants could stimulate recombination and thus formation of large λ plaques. We prepared a λ library with insertions of random 25-mers at the newly-defined *χJ* locus in the *gam* gene (Figure 3B). We expected any octamer (like Chi) to occur once per ~4000 phage, given $4^8$ (65,536) octamers and 18 possible positions of an octamer within the insert.

A total of ~10,000 phage from the library were plated on *recBCD*$^+$ cells or four RecC tunnel mutants (L64A, Q652L,

**Table 1 Recombination proficiency and Chi activity of RecC tunnel mutants**

| | | Phage λ recombination | | | |
|---|---|---|---|---|---|
| | | $\chi^+76$, $\chi^+D$ hotspot crosses | $\chi^+L$ crosses | | |
| | | | Chi activity (c/t ratio)[d] | | |
| *recC* codon change[a] | $\chi^\circ/\chi^+76/\ \chi^+L252$ plaque size[b] | Chi hotspot activity[c] | $\chi^+L251$ | $\chi^+L252$ | $\chi^-L252$ |
| + | S/L/L | 5.1 ± 0.2 | 3.9 ± 0.3 | 4.9 ± 0.2 | 1.9 ± 0.1 |
| *recBCD* null | L/L/L | 1.1 ± 0.1 | 1.4 ± 0.2 | 1.3 ± 0.3 | 1.1 ± 0.1 |
| S39E | T/T/T | 1.0 ± 0.1 | 1.5 ± 0.2 | 1.9 ± 0.2 | 1.3 ± 0.1 |
| S39V | T/T/M | 1.5 ± 0.1 | 2.3 ± 0.4 | 4.6 ± 0.4 | 1.6, 1.5 |
| G41Q | N/N/S | 0.9, 1.4 | 2.1 ± 0.3 | 2.3 ± 0.1 | 1.2, 1.4 |
| L64A | T/T/M | 1.5 ± 0.1 | 2.1 ± 0.2 | 3.3 ± 0.3 | 1.4 ± 0.2 |
| L64S | N/N/S | 1.0 ± 0.1 | 2.5 ± 0.2 | 2.9 ± 0.2 | 1.5 ± 0.3 |
| L64V | N/N/S | 1.3 ± 0.1 | 2.7 ± 0.3 | 3.4 ± 0.1 | ND |
| K88I | N/N/M | 1.6 ± 0.3 | 3.3 ± 0.1 | 4.3 ± 0.2 | 1.5 ± 0.2 |
| Q652L | T/T/T | 1.2 ± 0.1 | 2.4 ± 0.2 | 3.7 ± 0.9 | 1.3, 1.2 |
| R708D | N/N/M | 1.4 ± 0.1 | 2.9 ± 0.2 | 3.8 ± 0.2 | 1.3 ± 0.3 |
| R708V | T/T/M | 1.4 ± 0.2 | 2.8 ± 0.2 | 3.2 ± 0.2 | 1.5, 1.3 |

S, small; L, large; T, tiny; M, medium; N, no visible plaques.

[a] Strains are transformants of strain V2831 (Δ*recBCD2371*) with the indicated *recC* codon change on derivatives of plasmid pSA607 (*recBC*$^{2773}$*D*). *recBCD* null is *recB21* (IS*186* insertion).

[b] Relative size of isolated plaques on the indicated strain.

[c] Chi hotspot activity for each set of crosses (λ 1081 × 1082 and λ 1083 × 1084) was determined as described (Stahl and Stahl 1977). Chi hotspot activity = $\sqrt{(t/c_{1a})} / (t/c_{1b})$ where $t/c_{1a}$ is the ratio of turbid ($c^+$) to clear (*cI857*) plaques from the λ 1081 × 1082 cross and $t/c_{1b}$ is the ratio of turbid to clear plaques from the λ 1083 × 1084 cross among $J^+$ $R^+$ recombinants in type 1 crosses (Figure 3A). Data are from two independent experiments (listed separately) or the mean ± SEM from 4–10 experiments.

[d] Chi activity for crosses with λ $\chi^+L$ phage is the ratio of clear (*cI857*) to turbid ($c^+$) recombinant plaques (c/t) among $J^+$ $R^+$ recombinants (determined as described above) in type 2 crosses (Figure 3C).

R708D, and R708V), and a few plaques (1 per ~3000) larger than the vast majority were observed. Seven phage of independent origin found on *recBCD*$^+$ cells contained Chi, as expected. Remarkably, however, all of the six phage of independent origin found on the tunnel mutants also contained Chi (Table 2). (Because of limited diversity in the library, these six were apparently sisters of the first seven found on *recBCD*$^+$ cells.) This unexpected finding indicates that Chi activity is possible in some of the tunnel mutants if the Chi sequence is present at a different position in λ, possibly because of local sequence context. We did not observe larger plaques on nine additional RecC mutants tested (S39E and F; G41E, Q, and W; L64S, T, and V; Q652E), perhaps because of the rarity of such large-plaque phage and the limited number of phage plated. In addition, although we rarely observed a plaque-size increase by Chi in any context with these mutants, most do have detectable hotspot activity (Table S6), indicating that even these mutants recognize Chi (see *Discussion*).

To test the inference that Chi at a different position is active in certain RecC tunnel mutants, we determined plaque-formation by λ containing Chi at five other loci ($\chi^+A$, $\chi^+C$, $\chi^+D$, $\chi^+F$, and $\chi^+G$) on wild type and 20 RecC tunnel mutants. $\chi^+C$, $\chi^+D$, and $\chi^+F$ phage formed significantly larger plaques on 7 of 20 tunnel mutants that allowed no or only tiny plaque-formation by $\chi^+76$ phage; as expected, all of these $\chi^+$ phage formed large plaques on wild-type *recBCD*$^+$ cells. This test of Chi in additional contexts demonstrated that Chi activity in the tunnel mutants was dictated by more than the Chi octamer (5′ GCTGGTGG 3′), which was present in all phage tested.

### *Context-dependence of $\chi^+L$ activity in wild type and RecC tunnel mutants*

In order to assess more thoroughly the role of context on Chi activity in the mutant strains, we created Chi at another newly-defined locus, designated χ*L* in *gam* (Figure 3B). As we reported recently (Taylor *et al.* 2016), the new Chi site, $\chi^+L251$, was active in *recBCD*$^+$ cells with a c/t ratio of 3.9 in type 2 crosses (Table 1; Figure 3C), indicating an excess of exchanges in the interval containing $\chi^+L$. In the standard *recBCD* null mutant *recB21*, the c/t ratio was 1.4 ± 0.2, reflecting low-level RecBCD-independent recombinants about equally frequent in the two intervals left and right of χ*L*. With $\chi^-L251$ (5′ GTTGGTGG 3′), the c/t ratio was 1.3 ± 0.1 in *recBCD*$^+$ cells and 1.1 ± 0.1 in *recB21*, indicating little if any activity of this Chi-minus allele. Remarkably, most of the RecC tunnel mutants had c/t ratios with $\chi^+L251$ that were noticeably higher (2.1–3.3) than that of *recB21* (1.4; Table 1 and Table S6), indicating that in these RecC tunnel mutants $\chi^+L251$ was active, albeit less than in *recBCD*$^+$ cells, and RecBCD dependent.

We tested the idea that the immediately surrounding nucleotide context affects Chi hotspot activity of $\chi^+L251$ by randomizing 4 bp on each side of the Chi octamer. For this analysis, we chose three representative χ*L* alleles with different nucleotide sequences flanking the Chi octamer ($\chi^+L252$, $\chi^+L253$, and $\chi^+L254$; Table 2). On wild type and on the *recC* tunnel mutants tested (L64A, K88I, and R708D), $\chi^+L252$ formed the largest plaques, $\chi^+L253$ formed intermediate-sized plaques, and $\chi^+L254$ formed the smallest plaques. All three phages made plaques much larger than those of $\chi^-L252$. These results indicate that plaque size and

**Table 2 Chi alleles and their nucleotide sequence contexts**

| Chi allele | Sequence (5′ → 3′)[a] | Description (refs)[b] |
|---|---|---|
| $\chi^+76$[c] | GGCGAGCTGCTGGTGGTGACGCGCCC | Chi$^+$ mutant of λ (Stahl and Stahl 1977; Kobayashi *et al.* 1982) |
| $\chi^+D123$ | TCGTGAAAGCTGGTGGCAGGAGGTCG | Chi$^+$ mutant of λ (Smith *et al.* 1981a) |
| $\chi^+C157$ | GCAGATCAGCTGGTGGAAGAGGGACT | Chi$^+$ mutant of λ (Sprague *et al.* 1978) |
| $\chi^-C206$ | GCAGATCAG**T**TGGTGGAAGAGGGACT | Octamer mutation of $\chi^+C157$ (Schultz *et al.* 1981) |
| $\chi^+F225$ | CCGGCTAGGCTGGTGGGGTTGCCTTA | Chi$^+$ mutant of λ (Smith *et al.* 1981b) |
| $\chi^+G218$ | AAACCACCGCTGGTGGCGGTGGTTTT | Chi$^+$ mutant of λ (Smith *et al.* 1981b) |
| $\chi^+J270$ | TGGGTGTGCTCTGCTGGTGGGCGGG | Random 25-bp insertion in *gam* |
| $\chi^+J271$ | TGGTTGTTTTTGGCTGGTGGGTGGT | " |
| $\chi^+J272$ | TGGGGTGTGCTCTGCTGGTGGGCGG | " |
| $\chi^+J273$ | GATCGGGTCAGGGGAGAGCTGGTGG | " |
| $\chi^+J274$ | GGAGGTGCTGGTGGCCTTTGGTACG | " |
| $\chi^+J275$ | CACCAGCTGGTGGTCCTAGGTGTTC | " |
| $\chi^+J276$ | GCTGGTGGAGGCAGGTAATGCCATT | " |
| $\chi^+L251$ | TTAGTGATGCTGGTGGTTGATCTCA | Chi octamer in *gam*; wt flanks |
| $\chi^-L251$ | TTAGTGATG**T**TGGTGGTTGATCTCA | Octamer mutation of $\chi^+L251$ |
| $\chi^+L252$ | TTAGCCATGCTGGTGGACGGTCTCA | Chi octamer in *gam*; mutant flanks |
| $\chi^-L252$ | TTAGCCATG**T**TGGTGGACGGTCTCA | Octamer mutation of $\chi^+L252$ |
| $\chi^+L253$ | TTAGCAAAGCTGGTGGCGACTCTCA | Chi octamer in *gam*; mutant flanks |
| $\chi^+L254$ | TTAGCATAGCTGGTGGAGACTCTCA | Chi octamer in *gam*; mutant flanks |
| $\chi^+L255$ (2-3) | TTAGCCATGCTGGTGGCGACTCTCA | Swap of 5′ and 3′ flanks |
| $\chi^+L256$ (2-4) | TTAGCCATGCTGGTGGAGACTCTCA | " |
| $\chi^+L257$ (3-2) | TTAGCAAAGCTGGTGGACGGTCTCA | " |
| $\chi^+L258$ (4-2) | TTAGCATAGCTGGTGGACGGTCTCA | " |
| $\chi^+L259$ | TTAGTGATGCTGGTGGTCGAAAACA | "Hottest" flank cut by RecBCD |
| $\chi^-L259$ | TTAGTGATG**T**TGGTGGTCGAAAACA | Octamer mutation of $\chi^+L259$ |
| $\chi^+L262$ | TTAGTGATGCTGGTGGGGGCTTCCA | "Colder" flank cut by RecBCD |

refs, references; wt, wild type.

[a] Sequence surrounding the Chi octamer (underlined) in the indicated allele in λ. The $\chi^-$ octamer mutation (C → T) is in boldface type.

[b] See *Materials and Methods* for sources of nonreferenced alleles.

[c] $\chi^+76$ is A → G mutation at nucleotide 33,058 in the *gam* gene, changing gctggtAg to gctggtGg (unpublished data).

presumably Chi activity are influenced by the nucleotides surrounding Chi, since all phage had Chi at the same position in λ but differed only in the 4 bp flanking each side.

To quantify these results, we determined the Chi activity (c/t ratio) from type 2 crosses (Figure 3C) with these four Chi alleles in *recBCD*$^+$ cells and the RecC tunnel mutants (Figure 4; Table 1 and Table S6). In *recBCD*$^+$ cells the c/t ratio of $\chi^+L252$ was 4.9, higher than that for $\chi^+L251$ (3.9); for $\chi^+L253$ and $\chi^+L254$ the c/t ratios were 3.5 and 3.0, respectively, corresponding to the order of their plaque sizes. $\chi^+L252$ gave the highest Chi activity in most of the RecC tunnel mutants; the c/t ratios decreased for the other Chi alleles in the same order as in *recBCD*$^+$ cells ($\chi^+L252 > \chi^+L253 > \chi^+L254$), again corresponding to the order of plaque sizes. High Chi activities (c/t ratios ≥ 3) were frequent and observed in 16 of the 25 mutants tested with codon changes in S39, G41, L64, W70, K88, D136, Q652, and R708 (Table S6 and Figure 4). The Chi activity of three tunnel mutants (S39V, K88I, and R708D) was similar to that in wild type for $\chi^+L252$ despite the very low Chi hotspot activity in normalized crosses with $\chi^+76$ and $\chi^+D123$ in these mutants (Table 1), confirming the locus (context) dependence of Chi activity in these RecC tunnel mutants. Of the tunnel mutants analyzed, S39E had the weakest Chi activity: the c/t ratios from crosses in this mutant with seven of eight Chi alleles were not significantly different from those of two alleles with a mutation in the Chi octamer ($\chi^-L252$ and $\chi^-L259$; Figure 4; Table 1, Table S6, and Table S7; see below).

### *Nucleotide context 3′ of χ$^+$L influences Chi activity in wild type and RecC tunnel mutants*

Our results above suggest that some or all of the 8 bp flanking the Chi octamer strongly influence Chi activity. We therefore randomized the 4 bp adjacent to either the 5′ side or the 3′ side of $\chi^+L252$ (largest plaques on many mutants) and $\chi^+L254$ (smallest plaques on many mutants) and tested the resultant phage for Chi activity by plaque size. A total of 10–25 phage for each of the four randomizations were sequenced, and their plaque sizes, with parental $\chi^+L252$ and $\chi^+L254$ controls, were determined on wild type and RecC tunnel mutants (unpublished data). Alteration of the 5′ context of $\chi^+L252$ or $\chi^+L254$ had no significant effect on plaque size on *recBCD*$^+$ and three *recC* tunnel mutants (L64A, K88I, and R708D). Alteration of the 3′ context of $\chi^+L252$ and $\chi^+L254$, however, had a dramatic effect on plaque size. For example, the plaques of $\chi^+L254$ phage were tiny to small on R708D and K88I, but one-third (4 of 12) of the nucleotide changes 3′ of the Chi octamer resulted in formation of larger plaques. Conversely, $\chi^+L252$ phage formed larger plaques on L64A, K88I, and R708D, and about one-third (9 of 25) of the

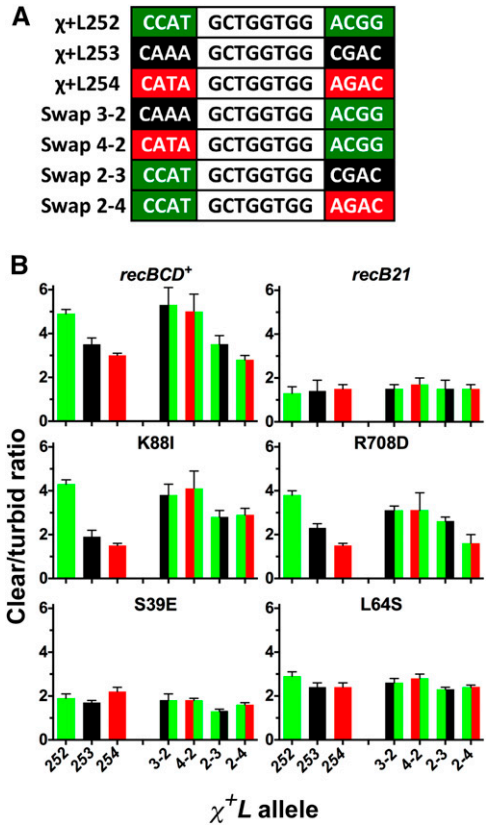


**Figure 4** Context-dependence of Chi activity in wild type (*recBCD*$^+$), RecBCD null (*recB21*), and RecC tunnel mutants. (A) Diagrammatic representation of $\chi^+L$ alleles and swaps of flanking bp tested for context-dependence. The Chi octamer, common to all alleles, is shown in black font. The origin of the 4 bp 5′ and 3′ of the octamer is indicated by the last digit of the allele number separated by a hyphen and the bar colors. For example, $\chi^+L252$ (green) with the 3′ context of $\chi^+L254$ (red) is indicated as 2-4 (green/red). (B) Chi activity of the indicated strains. Strains are transformants of V2831 (Δ*recBCD*::*kan*) with the indicated *rec* alleles or amino acid changes in RecC carried on derivatives of plasmid pSA607 (RecBC$^{2773}$D). Chi activity, measured as the ratio of clear to turbid plaques from type 2 crosses (Figure 3C), is shown for the indicated $\chi^+L$ alleles. Bar colors represent the source of the 4 bp flanking Chi as described above. Data represent the mean ± SEM for four to eight crosses. Data for *recBCD*$^+$ and *recB21* are from Taylor *et al.* (2016).

3′ bp changes resulted in formation of much smaller plaques. These data indicate that sequences flanking Chi on the 3′ side influence the activity of $\chi^+L$, although no correlations between nucleotide sequences and plaque size were evident from this limited analysis (see below).

We extended this analysis in a recent report (Taylor *et al.* 2016) and found that swapping the 3′, but not 5′, flanking 4 bp of the three $\chi^+L$ alleles studied above changed Chi activity in wild-type *recBCD*$^+$ cells. For example, putting the 3′ flank of $\chi^+L252$ (more active) at $\chi^+L254$ (less active) increased the activity of $\chi^+L254$ and, conversely, putting the 3′ flank of $\chi^+L254$ (less active) at $\chi^+L252$ (more active) reduced the activity of $\chi^+L252$. These results were found in both *recBCD*$^+$ cells (Figure 4) and most tunnel mutants (Figure 4; Table S6). For example, in K88I the c/t ratio increased from 1.9 in $\chi^+L253$ to 3.8 in $\chi^+L253$ with the 3′ flank of $\chi^+L252$; similarly, the c/t ratio increased from 1.5 in $\chi^+L254$ to 4.1 in $\chi^+L254$ with the 3′ flank of $\chi^+L252$. Conversely, in K88I the c/t ratio was reduced from 4.3 in $\chi^+L252$ to 2.8 in $\chi^+L252$ with the 3′ flank of $\chi^+L253$ and to 2.9 in $\chi^+L252$ with the 3′ flank of $\chi^+L254$. For all of these comparisons, $P < 0.05$ (unpaired *t*-test; Table S7). Thus, K88I behaves similarly to wild type for this set of Chi sites, even though K88I has little if any activity with $\chi^+76$ and $\chi^+D153$ (Table 1). Similar results were seen with tunnel mutants altered at S39V, L64V, and R708D (Figure 4; Table S6).

### *A continuous spectrum of Chi activity and Chi context-dependence in RecC tunnel mutants*

To determine if the RecC tunnel mutants fell into distinct classes, perhaps from alteration of distinct, separable ways of interrupting the RecBCD-Chi interaction, we evaluated two parameters for each mutant and wild-type RecBCD. First is Chi activity (the ability of each enzyme to respond to Chi), assayed here as the c/t ratio of eight $\chi^+L$ alleles in crosses of type 2, and as the hotspot activity from normalized crosses of types 1a and 1b with $\chi^+76$ and $\chi^+D123$ (Figure 3A). For normalized crosses (type 1), 1 indicates no Chi hotspot activity (Stahl and Stahl 1977); for nonnormalized (type 2) crosses, the c/t ratios in the *recBCD* null mutant *recB21* were ~1.3, indicating no Chi activity. In both types of crosses the highest values were ~5–5.5, indicating strong Chi activity (Table 1 and Table S6). Second is Chi context-dependence (the effect of flanking sequence context on Chi activity), assayed here as the ratio of the activities (c/t) of two $\chi^+L$ alleles in crosses of type 2; values ranged from ~3, indicating strong context-dependence, to 1, indicating no context-dependence (Table S8).

When we ranked the 25 *recC* mutants according to their activity with the most active $\chi^+L$ allele used above, $\chi^+L252$, we found that the Chi activities fell into a continuum with no apparent break points that would suggest classes of mutants (Figure 5A). The highest Chi activity (4.9) was observed with *recBCD*$^+$ cells, not significantly different from that of the *recC* S39V mutant (4.6) ($P = 0.23$; unpaired *t*-test). The lowest Chi activity (1.9) was observed with the S39E mutant, not significantly different from that of the *recB21* null mutant (1.3) ($P = 0.22$), as noted above. Between these two extremes there were *recC* mutants with nearly every activity. The Chi hotspot activity (in normalized crosses) of these mutants also formed a continuum (Figure 5B), although the hotspot activity of the most active mutant G41T (2.0) was much less than that of wild type (5.1). This behavior likely reflects the low activity of $\chi^+76$ in the RecC tunnel mutants, which was used to isolate the mutants, and perhaps of $\chi^+D123$ as well (see *Discussion*). The hotspot activities of several mutants, such as S39E, were not significantly different from that of the *recB21* null mutant, which has no Chi activity (Stahl and Stahl 1977). Ranked according to activity of other $\chi^+L$ alleles, the mutants also formed a continuum (Table S6).

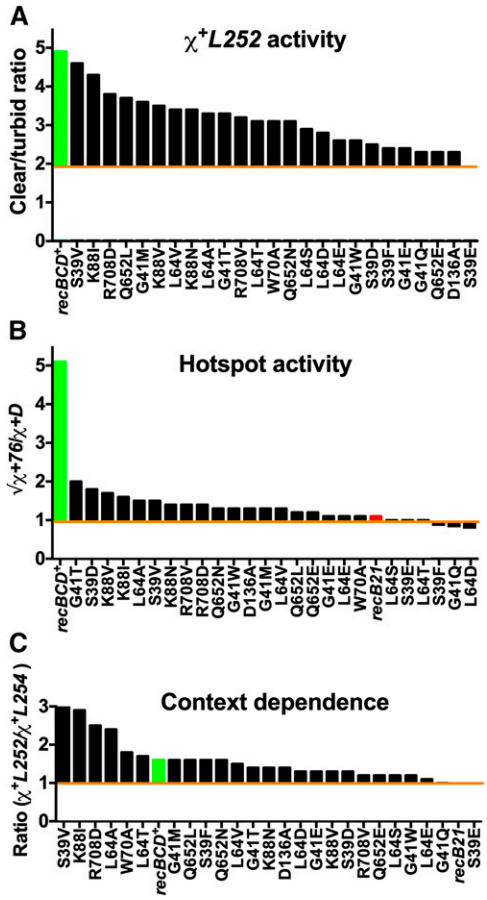


**Figure 5** Chi activity and its context dependence in RecC tunnel mutants vary along a continuum. Data are from Table 1, Table S4, and Table S6. (A) The c/t ratios (Chi activity) from type 2 crosses (Figure 3C) with $\chi^+$*L252* are shown for the indicated RecC tunnel mutants. The c/t ratios in *recBCD*$^+$ for $\chi^+$*L252* (4.9; green bar) and for $\chi^-$*L252* (1.9; orange line) are shown for comparison. (B) Weak Chi hotspot activity of the RecC tunnel mutants in normalized $\chi^+$*D123 and* $\chi^+$*76* hotspot crosses (Figure 3A, type 1 crosses; Table 1). Orange line at 1.0 indicates no Chi hotspot activity. (C) Context dependence (ratio of the c/t ratios from crosses with $\chi^+$*L252 vs.* $\chi^+$*L254*) varies from strong (~3 in some tunnel mutants) to medium (~1.5) in *recBCD*$^+$ and other tunnel mutants, to low (~1) in *recBCD* null and other tunnel mutants. Orange line at 1.0 indicates no Chi context-dependence. Data for *recBCD*$^+$ and *recB21* are from Taylor *et al.* (2016).

We observed similar continua when we ranked the mutants according to their context-dependence. For example, the c/t ratio for the most active $\chi^+L$ allele ($\chi^+$*L252*) divided by that of the least active ($\chi^+$*L254*) ranged from 3.1 for S39V (strong context-dependence) to 0.9 for S39E, not significantly different from that of the *recB21* null mutant (no apparent context-dependence) (Figure 5C). (We note that there can be no context-dependence of an inactive Chi site; see *Discussion*.) There was a suggestion of two classes in Figure 5C defined by the difference between L64A (2.4) and W70A (1.8), but this difference was not statistically significant ($P = 0.28$). Ranked according to the ratios of other pairs of $\chi^+L$ alleles, the mutants also formed continua, with maximal context-dependence as high as or higher than that of wild-type RecBCD (~3) and minimal values near that of *recB21* (~1; Table S6).

We noted that several mutants ranked high by both Chi hotspot activity and context-dependence and some low by both criteria. For example, S39V, K88I, and R708D had the highest Chi activity assayed by the c/t ratio with $\chi^+$*L252*, and S39E and G41Q had about the lowest (Figure 5A). These two sets of RecC tunnel mutants also had the highest and lowest context-dependence assayed by the ratio of activities of $\chi^+$*L252* and $\chi^+$*L254* (Figure 5C). Some mutants, however, had moderately strong Chi activity but little context-dependence. For example, L64S, L64D, and G41W were in the middle of the c/t ratio rankings from crosses with $\chi^+$*L252* and $\chi^+$*L253* but had low context-dependence ranking (Chi activity ratios ranging from ~1 to ~1.4).

### *RecC tunnel mutants manifest a range of activities with exceptionally strong Chi alleles*

We recently reported that the Chi octamer flanked by certain 3′ flanking sequences has Chi activity in *recBCD*$^+$ cells even greater than that of the Chi alleles used above (Taylor *et al.* 2016). We tested two of these Chi alleles, $\chi^+$*L259* and $\chi^+$*L262*, in five RecC tunnel mutants that manifested high or low Chi activity with the $\chi^+$*L252* allele used above and found qualitatively similar responses. For example, with $\chi^+$*L259*, tunnel mutants K88I and R708D had c/t ratios in type 2 crosses of 5.4 and 4.8, respectively (Figure 6; Table S9), even higher than they had with $\chi^+$*L252* (4.3 and 3.8, respectively; Table 1 and Table S6; Figure 4). L64A behaved similarly. These high Chi activities depend on the Chi octamer, because the $\chi^-$*L259* mutation gave a low c/t ratio in all cases. Similarly, two RecC tunnel mutants, S39E and L64S, which had low activity with $\chi^+$*L252*, had negligible activity with $\chi^+$*L259* (1.7 and 1.3, respectively), only slightly greater than with the $\chi^-$*L259* Chi octamer mutant (1.3 and 1.0, respectively). Another Chi allele, $\chi^+$*L262*, with high activity in *recBCD*$^+$ cells also had strong activity in the K88I, R708D, and L64A mutants but essentially no activity in S39E and L64S. Thus, these last two mutants had little or no Chi activity in any case tested, whereas the others had activity in all cases tested except with $\chi^+$*76*, used to isolate the mutants, or (for L64A) with the translocated $\chi^+$*C* locus (Handa *et al.* 2012).

## Discussion

To test the hypothesis that the recombination hotspot Chi is recognized by a tunnel in the RecC subunit (Figure 2), we sought *recC* tunnel missense mutants that do not respond to Chi. Unexpectedly, during this study we found that the activity of Chi strongly depends on the nucleotide sequences flanking the Chi octamer 5′ GCTGGTGG 3′. Changing these flanking nucleotides changed Chi activity by factors as great as the stimulation of recombination by Chi itself, about a factor of five. Our study of *recC* tunnel mutants and of Chi's flanking sequences demonstrates a complex interaction

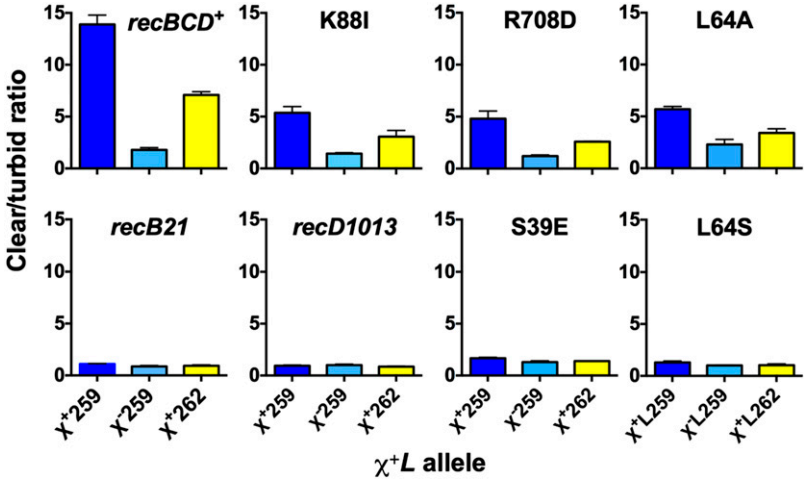


**Figure 6** Activity of strong Chi alleles in RecC tunnel mutants. Shown are results of type 2 crosses (Figure 3C) with two $\chi^{+}L$ alleles that have the same 5′ flanking sequence as $\chi^{+}L251$ but different 3′ flanks (Table 2), which confer high Chi activity in *recBCD*$^{+}$ cells (Taylor *et al.* 2016). The Chi octamer mutant $\chi^{-}L259$ was used as a control, as were the *recBCD* null mutant *recB21* and the *recD1013* mutant, in both of which Chi is inactive (Stahl and Stahl 1977; Amundsen *et al.* 1986). Data for *recBCD*$^{+}$ and *recB21* are from Taylor *et al.* (2016).

between these two parts of Chi and, we propose, two domains of RecBCD (the RecC tunnel and the RecB nuclease domain) that are critical for proper DNA break repair and genetic recombination. Here, we discuss the implications of our results for understanding how RecBCD enzyme is regulated by Chi hotspots.

### *Analysis of RecBCD mutants reveals an additional feature of Chi recombination hotspots: context-dependence of the Chi octamer*

As noted in the Introduction, Chi was equated with 5′ GCTGGTGG 3′ based on seven thoroughly analyzed Chi sites having only this octamer in common and mutations creating or inactivating Chi falling exclusively in this octamer (Smith *et al.* 1984). Synthetic octamer inserted into DNA gives a substrate that purified RecBCD enzyme readily cuts at Chi and onto which it loads RecA protein (Dixon and Kowalczykowski 1991; Anderson and Kowalczykowski 1997). We are unaware, however, of such synthetic Chi sites being previously tested for recombination hotspot activity in living cells, although synthetic Chi on a linear DNA fragment stimulates transformation of *E. coli* cells, and on a circular plasmid it stimulates formation of high molecular weight DNA (Dabert *et al.* 1992; Dabert and Smith 1997). It remained possible, however, that Chi in a given context is active with purified proteins but not as a recombination hotspot in cells. For example, a Chi-related sequence (5′ GCTGGTGCTCG 3′), denoted χ*, weakly stimulates cutting of DNA and loading of RecA protein by the purified RecBC$^{1004}$D pseudorevertant mutant and wild-type RecBCD enzymes, but χ* has no detectable recombination hotspot activity in cells, either *recBCD*$^{+}$ or *recC1004*, a frameshift pseudorevertant with eight amino acid changes in the RecC tunnel (Schultz *et al.* 1983; Handa *et al.* 1997; Arnold *et al.* 2000). With this exception, the published data were consistent with both the genetic and biochemical activities of Chi being determined simply by 5′ GCTGGTGG 3′, but our observations reported here force a revision of this view.

The precise effect of the sequence context on Chi activity remains to be determined, but our data show that the flanking 3′ bp are more influential than the flanking 5′ bp. This conclusion is supported both by our randomization of bp on each side of the octamer and by our swapping of flanking bp between more active $\chi^{+}L$ alleles and less active $\chi^{+}L$ alleles (Figure 4). As predicted, swapping the 3′, but not the 5′, flanks of active alleles increased the activity of less active alleles and vice versa, and randomization of the 3′ flanks revealed more changes affecting Chi activity than randomization of the 5′ flanks. Roughly one-third of the 80 randomizations examined had detectable effects, demonstrating that multiple flanking sequences influence Chi activity, but we were unable to discern a pattern in these sequences indicating the essential features. Our recent analysis of vast numbers of sequences flanking the octamer revealed the importance of bp 4–7 on the 3′ flank for activation of RecBCD's nicking activity at Chi (Taylor *et al.* 2016). At these positions, purines appear to be favored over pyrimidines. We used one of these sequences in $\chi^{+}L259$ (5′ GCTGGTGGTCGAAAA 3′), an exceptionally "hot" Chi allele (Figure 6), to demonstrate that some RecC tunnel mutants, but perhaps not others, retain Chi recognition, as discussed below. The complete rules of how the sequences flanking Chi determine Chi's activity, however, remain to be elucidated.

### *Previously reported RecC Chi-recognition mutants also retain Chi activity*

We began this study with the goal of determining the amino acids in the RecC tunnel that are required for Chi recognition. Some of the *recC* mutants we generated allowed large-plaque formation by λ without Chi, and we surmised that these RecC mutant enzymes recognize a new hotspot with a sequence other than 5′ GCTGGTGG 3′ and present in the 48.5 kb of λ DNA. Handa *et al.* (2012) drew the same conclusion from a study of other RecC tunnel mutants with amino acids changed to alanine and their interaction with Chi. The possibility that their and our mutants of this type recognize a new hotspot sequence remains to be tested because of the

lack of an easy assay for novel Chi sites with activity in these *recC* mutants. Some other *recC* mutants, however, allowed only small or no plaque formation, which provided a simple way to screen or select for Chi-like sequences active in these *recC* mutants. To our surprise, all of the six independent sequences isolated contained Chi (5′ GCTGGTGG 3′) but obviously at a locus different from those used previously ($\chi^+$*76* and $\chi^+$*D123*), which were only weakly active in these mutants. This result implied a context-dependence of Chi's activity, which was demonstrated by our subsequent experiments reported here and by Taylor *et al.* (2016).

But these observations also showed that the *recC* mutants we had isolated were not *bona fide* Chi recognition mutants. Instead, they were Chi context-dependence mutants: their phenotype depended on the Chi site used for assay. After we came to this conclusion, Handa *et al.* (2012) reported 11 *recC* mutants with reduced Chi activity, each with alanine in place of the wild-type amino acid, which they concluded lacked Chi recognition or had relaxed Chi specificity. Their study, however, used Chi at only one locus: transplacement of $\chi^+$*C157* and its surrounding DNA into the λ *int* gene. We tested three of the *recC* mutants they reported (L64A, W70A, and D136A) and found that each in fact has Chi activity with $\chi^+$*L252* (Table 1 and Table S6). Although we have not tested all of the mutants they reported, the high frequency of Chi context-dependence mutants isolated in our screen, which is similar to that of Handa *et al.* (2012), suggests that many, and perhaps most or all, of their mutants are Chi context-dependent mutants. Until a putative Chi recognition *recBCD* mutant is tested with a large number of sequences flanking Chi, it will remain unclear if the mutant genuinely lacks Chi activity.

Some of the mutants we isolated had in fact little or no activity with most of the Chi sites in the 25 different contexts that we tested. For example, S39E was not significantly different from the *recBCD* null mutant *recB21* in its response to Chi in seven of eight contexts tested (Table S6). This mutant may in fact lack Chi recognition, but it may also have Chi activity that depends on a context that we did not test. This possibility complicates the interpretation of mutants that are thought to lack Chi recognition, as noted above.

An additional complication is the lack of a direct assay for Chi recognition. The available assays measure the response to Chi, which involves both recognition of Chi and transduction of the Chi-generated signal to the relevant active site in RecBCD enzyme (Figure 1B; Amundsen *et al.* 2007). Because RecBCD responds to Chi only as it is unwinding DNA rapidly (~1 kb/sec) (Ponticelli *et al.* 1985; Taylor *et al.* 1985), no direct assay, such as binding to Chi, is available. It might, however, be possible to incorporate a moiety at Chi that would emit a signal, such as fluorescence, when productively encountered by RecBCD enzyme without the necessity of further changes to RecBCD.

Several other RecC tunnel mutants were particularly illuminating. The R708D mutant blocked plaque-formation by λ *red gam* $\chi^+$*76* phage but allowed plaque-formation by λ *red gam* $\chi^+$*L252* phage (Table 1). Consistent with these observations, R708D had moderately high Chi activity with $\chi^+$*L252* (c/t = 3.8 compared to 4.9 with wild-type *recBCD*$^+$) but low hotspot activity in normalized hotspot crosses using $\chi^+$*76* and $\chi^+$*D123* (1.4 compared to 5.1 with wild type). R708D also had high context-dependence as assayed by the ratio of activity with $\chi^+$*L252* to that with $\chi^+$*L254* (2.5), even higher than that of wild-type RecBCD (1.6) (Table S4, Table S6, and Table S8; Figure 4 and Figure 5). S39V and K88I behaved similarly. Thus, we might not have detected the R708D and R708V mutants if we had used $\chi^+$*L252* rather than $\chi^+$*76* in our screen. This outcome emphasizes the need to expand the spectrum of Chi sites used to deduce the parts of RecBCD required for Chi recognition.

The L64V and K88V mutants had moderately high Chi activity with $\chi^+$*L252* (3.4 and 3.5, respectively) and with $\chi^+$*L254* (2.2 and 2.8, respectively), and consequently low context-dependences by the ratio of these two activities (1.5 and 1.3, respectively) (Table S6 and Table S8; Figure 5). Yet in normalized crosses with $\chi^+$*76* and $\chi^+$*D123* these mutants had much lower hotspot activities (1.3 and 1.7, respectively) than that in *recBCD*$^+$ (5.1) (Table 1 and Table S4; Figure 5B). These mutants also illustrate differential activity of Chi in different contexts, *i.e.*, Chi context-dependence.

### *Molecular basis of RecBCD's recognition of Chi and its context-dependence*

Current evidence indicates that Chi is recognized as the ss DNA with 5′ GCTGGTGG 3′ during its passage through the RecC tunnel (Figure 2; Smith 2012; Wigley 2013). Amino acids lining the tunnel are thought to contact specific bases of the DNA; the correct fit presumably sends a signal to the other RecBCD subunits to alter RecBCD's activity (Figure 1B). We report here that this signaling requires more than 5′ GCTGGTGG 3′: additional nucleotides on the 3′ side of this octamer strongly influence the activity of Chi. The context-dependence of wild-type RecBCD is as high as a factor of 5.0 in genetic crosses (Figure 4 and Figure 6; Table 1, Table S6, and Table S9) and 7.4 in enzymatic nicking of DNA (Taylor *et al.* 2016), *i.e.*, as high as or higher than Chi hotspot activity itself (Stahl and Stahl 1977). These nucleotides may extend into the RecB nuclease domain, which is at the exit of the RecC tunnel in the crystal structure (Figure 1 and Figure 2; Singleton *et al.* 2004); if so, Chi may be recognized by more than the RecC tunnel. For example, the RecB nuclease domain may recognize the 3′ flank but have a less stringent sequence requirement than the RecC tunnel has for the Chi octamer. Our recent report supports this interpretation (Taylor *et al.* 2016).

It is interesting that a previously reported RecBCD-Chi mutant combination also requires additional nucleotides on the 3′ side for alteration of RecBCD enzyme activities. The first reported mutants that blocked Chi activation, but retained RecBCD enzymatic activities and recombination proficiency, were pseudorevertants of the *recC73* null allele, as noted in the Introduction (Schultz *et al.* 1983). One of these mutants, *recC1004*, has no detectable activity with standard Chi sites in λ ($\chi^+$*76*, $\chi^+$*C157*, and $\chi^+$*D123*) (Schultz *et al.* 1983) but does have partial activity with the $\chi^*$ sequence 5′ GCTGGTGCTCG 3′

noted above (Handa *et al.* 1997; Arnold *et al.* 2000). This sequence confers large-plaque formation and increased burst size of λ *red gam* phage in both *recC1004* mutant and *recBCD*$^+$ cells, but it does not produce recombination hotspot activity detectable in either strain. It alters the nuclease activity of RecBCD in a way that may allow rolling-circle replication and greater phage yield, and it increases the RecA-loading activity of RecBCD, which is thought to be a function of the RecB nuclease domain (Churchill and Kowalczykowski 2000; Spies and Kowalczykowski 2006); the absence of hotspot activity in χ* mutant cells remains a mystery, however. The putative effects on RecB are consistent with our suggestion above that the 3′ flank of Chi is recognized by RecB. In nearly all of the *recC* mutants reported here, Chi in the correct context does have hotspot activity (Figure 4; Table S6).

Previous reports of mutants with an altered RecC tunnel of RecBCD, or the corresponding AddB tunnel in AddAB of *Bacillus subtilis*, have interpreted the alterations as interfering with the recognition of Chi (Handa *et al.* 2012; Saikrishnan *et al.* 2012; Yang *et al.* 2012; Wilkinson and Wigley 2014). For RecBCD, the crystal structure of the enzyme with ss DNA in the presumed Chi recognition tunnel has not been reported, but the RecC tunnel mutants were interpreted in terms of specific contacts between amino acid side chains and bases in the Chi octamer. Our results with three of these RecC tunnel mutants (L64A, W70A, and D136A) show that they still recognize Chi but respond to it only in the right context (Table 1, Table S4, and Table S6). Thus, these mutants warrant reinterpretation. Some or all of the other previously reported mutants may also be Chi context-dependence, not Chi recognition, mutants. As noted above, studies employing Chi in additional contexts are required to distinguish these possibilities.

## Acknowledgments

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GENETICS

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# RecBCD Enzyme "Chi Recognition" Mutants Recognize Chi Recombination Hotspots in the Right DNA Context

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**Table S1. Bacterial strains, plasmids, and phage**

| Reference number | Genotype | Reference or Source[a] |
|---|---|---|
| Strain | | |
| V66 | *argA21 recF143 hisG4 met rpsL31 galK2 xyl-5* $\lambda^-$ $F^-$ | (SCHULTZ *et al.* 1983) |
| V67 | As V66, plus *recB21* | (SCHULTZ *et al.* 1983) |
| V222 | As V66, plus *recD1013* | (AMUNDSEN *et al.* 1990) |
| V1306 | *thi-1 relA1* $\lambda^-$ (HfrPO44) | (SCHULTZ *et al.* 1983) |
| V2831 | As V66, plus Δ*recBCD2731<kan>* | (AMUNDSEN *et al.* 2007) |
| V3477 | *As C600, plus recD2741<kan > thr-1 leuB6 thi-1 lacY1 tonA21 supE44 rfbD1* $\lambda^-$ $F^-$ | (TAYLOR *et al*. 2016) |
| DY378 | *IN(rrnD-rrnE)1 rph-1* (λ*cI857* Δ(*cro-bioA*)) $F^-$ | (THOMASON *et al.* 2009) |
| C600 | *thr-1 leuB6 thi-1 lacY1 tonA21 supE44 rfbD1* $\lambda^-$ $F^-$ | (SCHULTZ *et al.* 1983) |
| 594 | *lac-3350 galK2 galT22 rpsL179* $\lambda^-$ $F^-$ | (SCHULTZ *et al.* 1983) |
| JM1 | *thr-1 leuB6 Δ(gpt-proA)62 lacY1 tyrT5888 hisG4 rpsL31 argE3* $\lambda^-$ $F^-$ | Our collection |
| C600(P2) | As C600, plus P2 | (SCHULTZ *et al.* 1983) |
| JC8679 | *thr-1 leuB6 ara-14 proA2 lacY1 tsx-33 galK2 hisG4 rpsL31 xyl-5 mtl-1 argE3 thi-1 recB21 recC22 sbcA23 supE44* $\lambda^-$ $F^-$ | (GILLEN 1974) |
| JC9387 | As JC8679, but *sbcA*$^+$ *sbcB15 sup*$^+$ | (GILLEN 1974) |
| Plasmid | | |
| pSA21 | *recB*$^{21}$*CD* | (AMUNDSEN *et al.* 2007) |
| pSA607 | *recBC*$^{2773}$*D* | (TAYLOR *et al.* 2014) |
| pAC5 | *recBCD*$^{1013}$ | (AMUNDSEN *et al.* 1986) |
| Phage | | |
| 801 | wild type | Our collection |
| 816 | *red-3 cI857* | Our collection |
| 872 | *b1453 cI857* | Our collection |
| 873 | *b1453* $\chi^+$*76 cI857* | Our collection |
| 1081 | *susJ6 b1453 cI857* $\chi^+$*D123* | (STAHL AND STAHL 1977) |
| 1082 | *b1453 cI*$^+$ $\chi^+$*D123 susR5* | (STAHL AND STAHL 1977) |
| 1083 | *susJ6 b1453* $\chi^+$*76 cI857* | (STAHL AND STAHL 1977) |
| 1084 | *b1453* $\chi^+$*76 cI*$^+$ *susR5* | (STAHL AND STAHL 1977) |
| 1099 | *b1453 cI* $\chi^+$*A131* | Our collection |
| 1180 | *b221 red-3 gam-210 rex173::IS10* pBR322 *IS10 cI857* $\chi^\circ$ | (SMITH *et al.* 1981) |
| 1188 | *red-3 cI*$^+$ | Our collection |

| | | |
|---|---|---|
| 1209 | *b221 red-3 gam-210 rex173::IS10* pBR322 *IS10 cI857 χ⁺F225* | (SMITH *et al.* 1981) |
| 1211 | *b221 red-3 gam-210 rex173::IS10* pBR322 *IS10 cI857 χ⁺G218* | (SMITH *et al.* 1981) |
| 1321 | *b2 red-3 gam-210 χ⁺C151* | Our collection |
| 1490 | *b1453 nin-5 χ⁺D123* | Our collection |
| 1557 | *b1453 χ⁺C151* | Our collection |
| 1560 | *b1453 cI857 χ⁺C157* | Our collection |
| 1570 | *b1453 cI60* | Our collection |
| 1779 | *b1453 χ⁺J270 cI60* | Materials and methods |
| 1780 | *b1453 χ⁺J271 cI60* | Materials and methods |
| 1781 | *b1453 χ⁺J272 cI60* | Materials and methods |
| 1782 | *b1453 χ⁺J273 cI60* | Materials and methods |
| 1783 | *b1453 χ⁺J274 cI60* | Materials and methods |
| 1784 | *b1453 χ⁺J275 cI60* | Materials and methods |
| 1785 | *b1453 χ⁺J276 cI60* | Materials and methods |
| 1832 | *red-3 χ⁺L251 cI857* | (TAYLOR *et al*. 2016) |
| 1841 | *red-3 χ⁺L252 cI857* | (TAYLOR *et al*. 2016) |
| 1847 | *red-3 χ⁺L253 cI857* | (TAYLOR *et al*. 2016) |
| 1848 | *red-3 χ⁺L254 cI857* | (TAYLOR *et al*. 2016) |
| 1900 | *red-3 χ⁺L256 cI857* | (TAYLOR *et al*. 2016) |
| 1901 | *red-3 χ⁺L258 cI857* | (TAYLOR *et al*. 2016) |
| 1910 | *red-3 χ⁺L257 cI857* | (TAYLOR *et al*. 2016) |
| 1911 | *red-3 χ⁺L255 cI857* | (TAYLOR *et al*. 2016) |
| 1998 | *susJ6 red-3 χ⁺L251 cI857* | (TAYLOR *et al*. 2016) |
| 1999 | *susJ6 red-3 χ⁺L252 cI857* | (TAYLOR *et al*. 2016) |
| 2000 | *susJ6 red-3 χ⁺L253 cI857* | (TAYLOR *et al*. 2016) |
| 2001 | *susJ6 red-3 χ⁺L254 cI857* | (TAYLOR *et al*. 2016) |
| 2002 | *susJ6 red-3 χ⁺L256 cI857* | (TAYLOR *et al*. 2016) |
| 2003 | *susJ6 red-3 χ⁺L258 cI857* | (TAYLOR *et al*. 2016) |
| 2004 | *susJ6 red-3 χ⁺L257 cI857* | (TAYLOR *et al*. 2016) |
| 2005 | *susJ6 red-3 χ⁺L255 cI857* | (TAYLOR *et al*. 2016) |
| 2006 | *red-3 χ⁺L251 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2007 | *red-3 χ⁺L252 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2008 | *red-3 χ⁺L253 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2009 | *red-3 χ⁺L254 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2010 | *red-3 χ⁺L256 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2011 | *red-3 χ⁺L258 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2012 | *red-3 χ⁺L257 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2013 | *red-3 χ⁺L255 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2014 | *susJ6 red-3 χ⁻L251 cI857* | R3434 → 1998 |
| 2015 | *red-3 χ⁻L251 cI⁺ susR5* | R3434 → 2006 |
| 2016 | *susJ6* | (TAYLOR *et al*. 2016) |
| 2017 | *susR5* | (TAYLOR *et al*. 2016) |
| 2025 | *susJ6 red-3 int4 cI857* | (TAYLOR *et al*. 2016) |
| 2026 | *susJ6 int-4 red-3 χ⁺L259 cI857* | (TAYLOR *et al*. 2016) |
| 2029 | *susJ6 int-4 red-3 χ⁺L262 cI857* | (TAYLOR *et al*. 2016) |
| 2030 | *int-4 red-3 χ⁺L259 cI⁺ susR5* | (TAYLOR *et al*. 2016) |

| | | |
|---|---|---|
| 2033 | *int-4 red-3 χ⁺L262 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2034 | *susJ6 int-4 red-3 χ⁻L259 cI857* | (TAYLOR *et al*. 2016) |
| 2035 | *int-4 red-3 χ⁻L259 cI⁺ susR5* | (TAYLOR *et al*. 2016) |
| 2038 | *susJ6 int-4 red-3 χ⁺L252 cI857* | R3159 → 2025 |
| MMS540 | *red-3 int4 cI⁺ susR5* | Lynn Thomason |
| 2039 | *int-4 red-3 χ⁺L252 cI⁺ susR5* | R3159 → MMS540 |
| 2040 | *susJ6 int-4 red-3 χ⁻L252 cI857* | R3499 → 2038 |
| 2041 | *int-4 red-3 χ⁻L252 cI⁺ susR5* | R3499 → 2039 |

[a] R indicates recombineering in which the indicated oligonucleotide is the donor and the indicated phage is the recipient.

**Table S2. Oligonucleotides**

| OL number | Use | Sequence (5' → 3')[a] |
|---|---|---|
| 856 | *recC* sequencing | CAGGAACAGATAGCGGTCG |
| 954 | *recC* sequencing | GCTGGCACAGGAGCGTCCG |
| 989 | *recC* sequencing | GGTAACGCGGAAATACCG |
| 1473 | *recC* sequencing | GCCAGCAGTTCAGTGCTCG |
| 1805 | *recC* sequencing | GAATCGTTGTAGCGTTTCCAG |
| 2893 | Patch mutagenesis A58-S67 | GGT ATT GCG N3N2N1 N1N1N2 N1N4N4 N3N1N4 N4N4N4 N2N2N3 N2N4N3 N2N2N1 N3N2N3 N1N3N2 TTT ATC TGG G |
| 2894 | Patch mutagenesis R687-P691 | GGC GTT TAT CCA N2N3N4 N2N1N3 N2N4N4 N3N2N3 N2N2N1 N4N4N3 GGC TTT GAC C |
| 2895 | Patch mutagenesis Q643-Q652 | GAA CTG GCA N2N1N3 N2N3N4 N2N4N3 N3N1N4 N2N1N1 N3N1N1 N2N3N4 N1N4N2 N1N3N2 N2N1N3 CGT TTT CTC GCC |
| 2896 | Patch mutagenesis V37-Q44 | GAT GAT TCT GN3N4 N3N2N1 N1N1N3 N4N1N2 N2N3N3 N4N1N4 N3N3N2 N1N2N1 N3TG GCT GCA AAT GAC C |
| 2897 | Random mut. *recC* Q38 | GAA CCA GAG ATG ATT CTG GTG NNN AGT ACC GGT ATG GCA CAG TGG |
| 2898 | Random mut. *recC* S39 | CT TTC GAA CCA GAG ATG ATT CTG GTG CAA NNN ACC GGT ATG GCA CAG |
| 2899 | Random mut. *recC* G41 | ATG ATT CTG GTG CAA AGT ACC NNN ATG GCA CAG TGG CTG CAA ATG |
| 2900 | Random mut. *recC* L64 | GCG GCA AAC ATT GAT TTT CCG NNN CCA GCG AGC TTT ATC TGG GAT |
| 2901 | Random mut. *recC* K88 | CCC AAA GAG AGC GCC TTT AAC NNN CAG AGC ATG AGC TGG AAA CTG |
| 2902 | Random mut. *recC* R644 | TTG CGT GAT GAA CTG GCA CAG NNN CTG GAT CAA GAA CGT ATC AGC |
| 2903 | Random mut. *recC* Q652 | G GAT CAA GAA CGT ATC AGC NNN CGT TTT CTC GCC GGA CCG G |
| 2904 | Random mut. *recC* R708 | CGT GGC GAC CGT AGC NNN CGC GAT GAC GAC CGC |
| 2905 | Random mut. R644 | GCT GAT ACG TTC TTG ATC CAG NNN CTG TGC CAG TTC ATC ACG CAA |
| 2906 | Random mut. R708 | GCG GTC GTC ATC GCG NNN GCT ACG GTC GCC ACG |
| 2907 | *recC* sequencing | CGC TTG TCG CTA TCG TCA G |
| 2908 | *recC* sequencing | CCG AAT GCA GAC ATC GCA G |
| 2931 | $\chi^+J$ primer extension | CCTGTGAGACACTAGTCG |
| 2932 | $\chi^+J$ *Xho*I/*Nhe*I, | CTCGCGAATGGTCGACATTCTTCTCGAGNNNNNNNNNNNNNNNNNNNNNNNNNNGCTAGCTGACCGACTAGTGTCTCACAGG |
| 2936 | Lambda 33433f | CAACAGGCCACCCTGCAATG |
| 2937 | Lambda 34715r | ATCGCTTATATCTGGCGCTG |
| 2938 | Lambda 33004f | TTTCCATGTCGTCTGCCAGTTC |
| 2951 | Lambda 33264r | GATGTTTCGTGAAGCCGTCG |
| 3008 | Lambda 33098 | GTAATAAGCGTTCATGGCTG |
| 3009 | Lambda 33430r | GCCATTGCAGGGTGGCCTGTTGCTG |

| | | |
|---|---|---|
| 3010 | Lambda 33336r | CATACTTAATCAGCCAGGAGTCCC |
| 3012 | Lambda 33149f | GCGAAATGCCGGGCTGATTAGG |
| 3159 | Construction of *χ+L252* | GCCGGGCTGATTAGGAAAACAGGAAAGGGGGTT AGCCATGCTGGTGGACGGTCTCAGTTTCAGTATT AATATCCATTTTTTATAAGC |
| 3205 | Randomize $\chi^+$*L252* $n_4$ 3’ | GCCGGGCTGATTAGGAAAACAGGAAAGGGGGTT AGCCATGCTGGTGGNNNNTCTCAGTTTCAGTATT AATATCCATTTTTTATAAGC |
| 3210 | Randomize 5’ $n_4$ $\chi^+$*L252* | GCCGGGCTGATTAGGAAAACAGGAAAGGGGGTT AGNNNNGCTGGTGGACGGTCTCAGTTTCAGTATT AATATCCATTTTTTATAAGCG |
| 3216 | Randomize 5’ $n_4$ $\chi^+$*L254* | CGGGCTGATTAGGAAAACAGGAAAGGGGGTTAG NNNNGCTGGTGGAGACTCTCAGTTTCAGTATTAA TATCCAT |
| 3217 | *recC* D136A 1 | GACCTGTTTGCCCAGTATCTGG |
| 3218 | *recC* D136A 2 | CGCCGCTTTTGAGGAAAG |
| 3220 | *recC* W70A 2 | GCTGGCAGCGGAAAATCA |
| 3224 | *recC* W70A 1 | GAGCTTTATCGCGGATATGTTCGTCCGGGTG |
| 3291 | Randomize $\chi^+$*L254* $n_4$ 3’ | CGGGCTGATTAGGAAAACAGGAAAGGGGGTTAG CATAGCTGGTGGNNNNTCTCAGTTTCAGTATTAAT ATCCAT |
| 3434 | Construction of $\chi^-$*L251* | CGGGCTGATTAGGAAAACAGGAAAGGGGGTTAG TGATGTTGGTGGTTGATCTCAGTTTCAGTATTAAT ATCCATTTTTTATAAGCG |

[a] For nucleotides other that A, T, C, and G, the percentage of mixed bases at the indicated position is as follows: N = 25% A, 25% C, 25% G, 25% T; N1 = 94% A, 2% C, 2% G, 2% T; N2 = 2% A, 94% C, 2% G, 2% T; N3 = 2% A, 2% C, 94% G, 2% T; N4 = 2% A, 2% C, 2% G, 94% T.

**Table S3. Characterization of *recC* mutants in patches of the tunnel**

| Isolate[a] | Amino acid changes (codon) | Relative Hfr recombinant frequency[b] | χ°/χ⁺*76* plaque size[c] | χ⁺*76*, χ⁺*D* Chi hotspot activity[d] |
|---|---|---|---|---|
| *recBCD*⁺ | Wild type | 1.0 | S/L | 5.0 |
| *recB21* | IS*186* insertion | 0.003 | L/L | 1.0 |
| VQ2 | Q38K, S39I, A43T | 0.2 | S/S | 1.3 |
| VQ3 | V37G | 0.1 | N/N | 1.5 |
| VQ4 | Q38H, M42I | 0.5 | S/S | 1.8 |
| VQ6 | S39I, T40Y | 0.3 | S/S | 1.3 |
| VQ7 | V37M, S39R, T40A | 0.05 | N/N | 1.0 |
| VQ14 | G41D | 0.1 | T/T | 1.2 |
| VQ59 | S39N | 0.7 | T/T | 3.9 |
| VQ79 | G41V, Q44K | 0.6 | T/T | 1.7 |
| VQ127 | Q38L, G41C | 0.4 | N/N | 1.2 |
| VQ173 | G41V | 0.4 | T/T | 1.1 |
| VQ195 | G41C, Q44K | 0.5 | S/S | 2.4 |
| VQ212 | G41V, A43S | 0.4 | T/T | 1.3 |
| VQ213 | S39I, T40I, M42I | 0.5 | T/T | 1.3 |
| VQ218 | V37A, Q38P, G41S | 0.2 | N/N | 1.2 |
| AS63 | L64V | 0.05 | T/T | 1.2 |
| AS68 | I60S, P63Q, S67T | 0.3 | M/M | ND |
| AS95 | P63L | 0.8 | S/M | ND |
| AS130 | P65Q, S67G | 0.2 | T/T | 1.2 |
| AS181 | P63T, L64V, P65L | 0.3 | L/L | 1.1 |
| AS185 | P63T | 0.9 | M/M | ND |
| QRP184 | Q652L, Q688R | 0.1 | T/T | 1.2 |
| QQ54 | Q643H | 0.2 | T/T | ND |
| QQ75 | R649L, Q652L | 0.3 | S/S | ND |
| QQ83 | L645M | 0.1 | M/M | ND |
| QQ90 | R644L, Q652L | 0.3 | S/S | ND |
| QQ208 | R649C, S651R | 0.2 | T/T | 2.3 |
| QQ291 | E648K, Q652L | 0.3 | S/S | 1.2 |

[a]Strains are transformants of strain V2831 [Δ*recBCD2371*] with the indicated *recC* codon changes on derivatives of plasmid pSA607 (*recBC*$^{2773}$*D*).

[b]Frequency of His⁺ [Str$^R$] recombinants per Hfr donor cell (corrected for viability of the recipient cells) relative to *recBCD*⁺, which was 3.2%.

[c]Relative size of isolated plaques on the indicated strain. N, no visible plaques; T, tiny; S, small; M, medium; L, large.

[d]Chi hotspot activity, determined as described (STAHL AND STAHL 1977) = $\sqrt{}(t/c_1)\div(t/c_2)$, where $t/c_1$ is the ratio of turbid (*c*⁺) to clear (*cI857*) plaques from the λ 1081 x 1082 cross and $t/c_2$ is the ratio of turbid to clear plaques from the λ 1083 x 1084 cross among *J*⁺ *R*⁺ recombinants in type 1 crosses (Fig. 3A).

**Table S4. Sequence of *recC* alleles**

| *recC* allele[a] | Nucleotide sequence[b] | | Codon change | Phage λ recombination $\chi^+76$, $\chi^+D$ hotspot cross |
|---|---|---|---|---|
| | | | | Chi hotspot activity[c] |
| + | | | | 5.1 ± 0.2 |
| *2774* | (AGT) | GTG | S39V | 1.5 ± 0.1 |
| *2775* | | TTT | S39F | 0.9 ± 0.01 |
| *2776* | | GAT | S39D | 1.8 ± 0.2 |
| *2777* | | GAG | S39E | 1.0 ± 0.1 |
| *2778* | (GGT) | ACG | G41T | 1.8, 2.2 |
| *2779* | | ATG | G41M | 1.3 ± 0.3 |
| *2781* | | TGG | G41W | 1.3, 1.5 |
| *2783* | | GAG | G41E | 1.1, 1.4 |
| *2784* | | CAG | G41Q | 0.9, 1.4 |
| *2785* | (CTG) | GCG | L64A | 1.5 ± 0.1 |
| *2786* | | GTG | L64V | 1.3 ± 0.1 |
| *2787* | | TCA | L64S | 1.0 ± 0.1 |
| *2788* | | ACA | L64T | 1.0 ± 0.1 |
| *2789* | | GAG | L64E | 1.1 ± 0.2 |
| *2790* | | GAT | L64D | 0.9 ± 0.01 |
| *2791* | (TGG) | GCG | W70A | 1.1 ± 0.1 |
| *2792* | (AAA) | ATA | K88I | 1.6 ± 0.3 |
| *2793* | | AAT | K88N | 1.4 ± 0.3 |
| *2794* | | GTG | K88V | 1.7 ± 0.4 |
| *2795* | (GAC) | GCG | D136A | 1.3 ± 0.4 |
| *2796* | (CAG) | AAT | Q652N | 1.3 ± 0.1 |
| *2797* | | CTG | Q652L | 1.2 ± 0.1 |
| *2798* | | GAA | Q652E | 1.2 ± 0.1 |
| *2799* | (CGT) | GAC | R708D | 1.4 ± 0.1 |
| *2800* | | GTG | R708V | 1.4 ± 0.2 |

[a]These alleles also have six histidine codons at the C-terminus, as in the parent allele *recC2773*.

[b]The wild-type sequence (5' → 3') at each codon is indicated in parentheses.

[c]Strains are transformants of strain V2831 [Δ*recBCD2371*] with the indicated *recC* codon change on derivatives of plasmid pSA607 (*recBC*$^{2773}$*D*). Chi hotspot activity, determined as described (STAHL AND STAHL 1977), = $\sqrt{(t/c_1)\div(t/c_2)}$, where $t/c_1$ is the ratio of turbid ($c^+$) to clear (*cI857*) plaques from the λ 1081 x 1082 cross and $t/c_2$ is the ratio of turbid to clear plaques from the λ 1083 x 1084 cross among $J^+ R^+$ recombinants in type 1 crosses (Fig. 3A). Data are from two independent experiments (listed separately) or the mean ± SEM from four to ten experiments. Hotspot activity for a *recB21* null mutant is 1.1 ± 0.1 (Table 1).

**Table S5. Recombination-proficiency of RecC tunnel mutants**

| *recC* codon change[a] | Relative Hfr recombinant frequency[b] | %$J^+R^+$ recombinants | |
|---|---|---|---|
| | | $\chi^+76$, $\chi^+D$ hotspot cross[c] | $\chi^+L252$ crosses[d] |
| + | 1 | 13.9 ± 1.8 | 6.1 ± 0.6 |
| *recBCD* null | 0.004 ± 0.001 | 0.4 ± 0.1 | 0.8 ± 0.1 |
| S39D | 0.09 ± 0.01 | 1.5 ± 0.3 | 3.1 ± 0.4 |
| S39E | 0.06 ± 0.02 | 2.7 ± 0.6 | 4.1 ± 0.3 |
| S39F | 0.21 ± 0.06 | 3.5 ± 0.6 | 4.4 ± 0.3 |
| S39V | 0.32 ± 0.06 | 2.3 ± 0.2 | 2.5 ± 0.1 |
| G41E | 0.07 ± 0.01 | 1.5 ± 0.5 | 1.9 ± 0.1 |
| G41M | 0.05 ± 0.01 | 1.4 ± 0.6 | 2.1 ± 0.2 |
| G41Q | 0.08 ± 0.01 | 0.6 ± 0.3 | 1.9 ± 0.1 |
| G41T | 0.21 ± 0.06 | 2.3 ± 0.2 | 4.3 ± 0.2 |
| G41W | 0.09 ± 0.03 | 4.0 ± 0.9 | 2.6, 1.5 |
| L64A | 0.32 ± 0.04 | 2.3 ± 0.5 | 2.4 ± 0.1 |
| L64D | 0.04 ± 0.01 | 1.5 ± 0.2 | 2.0 ± 0.2 |
| L64E | 0.05 ± 0.02 | 1.6 ± 0.3 | 1.9 ± 0.2 |
| L64S | 0.32 ± 0.07 | 3.6 ± 1.1 | 1.5 ± 0.2 |
| L64T | 0.04 ± 0.01 | 1.5 ± 0.1 | 2.2 ± 0.1 |
| L64V | 0.03 ± 0.01 | 2.9 ± 0.1 | 1.9 ± 0.3 |
| W70A | 0.03 ± 0.01 | 2.6 ± 0.5 | 3.9 ± 0.2 |
| K88I | 0.17 ± 0.04 | 3.7 ± 0.2 | 4.1 ± 0.2 |
| K88N | 0.47 ± 0.03 | 3.9 ± 0.5 | 4.1 ± 0.2 |
| K88V | 0.47 ± 0.03 | 3.7 ± 0.4 | 4.3 ± 0.3 |
| D136A | 0.08 ± 0.03 | 2.5 ± 0.2 | 2.6 ± 0.1 |
| Q652E | 0.06 ± 0.01 | 1.7 ± 0.3 | 1.6 ± 0.2 |
| Q652L | 0.17 ± 0.05 | 3.1 ± 0.2 | 2.9 ± 0.2 |
| Q652N | 0.22 ± 0.06 | 3.3 ± 0.3 | 4.2 ± 0.3 |
| R708D | 0.29 ± 0.07 | 2.8 ± 0.1 | 2.6 ± 0.1 |
| R708V | 0.38 ± 0.01 | 3.4 ± 0.2 | 4.1 ± 0.1 |

[a]Strains are transformants of strain V2831 [*ΔrecBCD2371*] with the indicated *recC* codon change on derivatives of plasmid pSA607 (*recBC*$^{2773}$*D*).

[b]Frequency of His$^+$ [Str$^R$] recombinants per Hfr donor cell (corrected for the viability of the recipient cells) relative to that in *recBCD*$^+$ (6.6%). Data are the mean ± SEM from three or four experiments.

[c]The mean frequency of $J^+ R^+$ recombinants in lysates of crosses with λ 1081 x 1082 and λ 1083 x 1084 determined by plating on strain 594 (*sup*$^+$) for recombinants and on strain C600 (*supE*) for total phage.

[d]The mean frequency of $J^+ R^+$ recombinants in lysates of crosses with λ $\chi^+L252$ phage determined as described above.

**Table S6. $\chi^{+}L$ activity of RecC tunnel mutants**

| *recC* codon change[a] | $\chi^{+}L$ activity (c/t ratio) in crosses with the indicated Chi allele[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | $\chi^{+}L251$ | $\chi^{+}L252$ | $\chi^{-}L252$ | $\chi^{+}L2$-3 | $\chi^{+}L2$-4 | $\chi^{+}L253$ | $\chi^{+}L3$-2 | $\chi^{+}L254$ | $\chi^{+}L4$-2 |
| + | 3.9 ± 0.3 | 4.9 ± 0.2 | 1.9 ± 0.1 | 3.5 ± 0.4 | 2.8 ± 0.2 | 3.5 ± 0.3 | 5.3 ± 0.8 | 3.0 ± 0.1 | 5.0 ± 0.8 |
| *recB21* | 1.4 ± 0.2 | 1.3 ± 0.3 | 1.1 ± 0.1 | 1.5 ± 0.4 | 1.5 ± 0.2 | 1.4 ± 0.5 | 1.5 ± 0.2 | 1.5 ± 0.2 | 1.7 ± 0.3 |
| S39D | 2.0 ± 0.1 | 2.5 ± 0.1 | ND | 2.0 ± 0.1 | 2.0 ± 0.2 | 2.3 ± 0.1 | 2.9 ± 0.1 | 2.0 ± 0.1 | 2.5 ± 0.1 |
| S39E | 1.5 ± 0.2 | 1.9 ± 0.2 | 1.3 ± 0.1 | 1.3 ± 0.1 | 1.6 ± 0.1 | 1.7 ± 0.1 | 1.8 ± 0.3 | 2.2 ± 0.2 | 1.8 ± 0.1 |
| S39F | 1.7 ± 0.2 | 2.4 ± 0.1 | ND | 1.2 ± 0.1 | 1.7 ± 0.3 | 2.0 ± 0.2 | 2.8 ± 0.2 | 1.5 ± 0.1 | 2.3 ± 0.1 |
| S39V | 2.3 ± 0.4 | 4.6 ± 0.4 | 1.6, 1.5 | 3.0 ± 0.3 | 2.2 ± 0.3 | 2.4 ± 0.2 | 3.0 ± 0.5 | 1.5 ± 0.1 | 4.0 ± 0.8 |
| G41E | 2.2 ± 0.4 | 2.4 ± 0.1 | ND | 1.5 ± 0.1 | 1.8 ± 0.4 | 2.1 ± 0.1 | 2.6 ± 0.3 | 1.8 ± 0.1 | 2.5 ± 0.1 |
| G41M | 1.6 ± 0.5 | 3.6 ± 0.5 | ND | 2.0 ± 0.1 | 2.3 ± 0.2 | 1.7 ± 0.3 | 2.9 ± 0.3 | 2.2 ± 0.1 | 2.7 ± 0.2 |
| G41Q | 2.1 ± 0.3 | 2.3 ± 0.1 | 1.2, 1.4 | 1.9 ± 0.1 | 1.5 ± 0.3 | 2.0 ± 0.1 | 2.0 ± 0.4 | 2.3 ± 0.2 | 2.3 ± 0.3 |
| G41T | 2.4 ± 0.1 | 3.3 ± 0.1 | ND | 2.0 ± 0.1 | 2.2 ± 0.3 | 2.4 ± 0.3 | 3.2 ± 0.1 | 2.3 ± 0.1 | 3.2 ± 0.1 |
| G41W | 2.1 ± 0.2 | 2.6 ± 0.3 | ND | 2.2 ± 0.1 | 2.1 ± 0.1 | 2.3 ± 0.2 | 2.5 ± 0.1 | 2.2 ± 0.1 | 2.6 ± 0.2 |
| L64A | 2.1 ± 0.2 | 3.3 ± 0.3 | 1.4 ± 0.2 | 2.1 ± 0.1 | 2.2 ± 0.6 | 2.1 ± 0.1 | 2.9 ± 0.4 | 1.4 ± 0.1 | 3.2 ± 0.3 |
| L64D | 2.4 ± 0.2 | 2.8 ± 0.3 | ND | 1.7 ± 0.2 | 1.4 ± 0.1 | 2.1 ± 0.1 | 2.0 ± 0.2 | 2.1 ± 0.1 | 2.5 ± 0.1 |
| L64E | 2.3 ± 0.3 | 2.6 ± 0.2 | ND | 1.8 ± 0.1 | 1.1 ± 0.2 | 1.4 ± 0.3 | 2.3 ± 0.2 | 2.3 ± 0.1 | 2.5 ± 0.1 |
| L64S | 2.5 ± 0.2 | 2.9 ± 0.2 | 1.5 ± 0.3 | 2.3 ± 0.1 | 2.4 ± 0.1 | 2.4 ± 0.2 | 2.6 ± 0.2 | 2.4 ± 0.2 | 2.8 ± 0.2 |
| L64T | 2.0 ± 0.2 | 3.1 ± 0.5 | ND | 1.6 ± 0.1 | 1.1 ± 0.3 | 2.3 ± 0.2 | 2.1 ± 0.2 | 1.8 ± 0.2 | 2.4 ± 0.1 |
| L64V | 2.7 ± 0.3 | 3.4 ± 0.1 | ND | 2.4 ± 0.3 | 2.4 ± 0.2 | 2.2 ± 0.1 | 2.8 ± 0.1 | 2.2 ± 0.1 | 3.0 ± 0.1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| W70A | 1.6 ± 0.1 | 3.1 ± 0.4 | ND | 2.4 ± 0.1 | 2.1 ± 0.1 | 2.5 ± 0.2 | 3.2 ± 0.1 | 1.7 ± 0.2 | 2.9 ± 0.1 |
| K88I | 3.3 ± 0.1 | 4.3 ± 0.2 | 1.5 ± 0.2 | 2.8 ± 0.3 | 2.9 ± 0.3 | 1.9 ± 0.3 | 3.8 ± 0.5 | 1.5 ± 0.1 | 4.1 ± 0.8 |
| K88N | 3.1 ± 0.2 | 3.4 ± 0.2 | ND | 2.6 ± 0.1 | 2.5 ± 0.2 | 2.4 ± 0.2 | 3.3 ± 0.2 | 2.4 ± 0.3 | 3.5 ± 0.1 |
| K88V | 2.9 ± 0.2 | 3.5 ± 0.1 | ND | 2.6 ± 0.2 | 2.5 ± 0.2 | 2.5 ± 0.2 | 3.2 ± 0.2 | 2.8 ± 0.1 | 3.2 ± 0.1 |
| D136A | 1.9 ± 0.2 | 2.6 ± 0.1 | ND | 1.2 ± 0.1 | 1.7 ± 0.1 | 2.5 ± 0.1 | 2.7 ± 0.1 | 1.7 ± 0.2 | 2.5 ± 0.1 |
| Q652E | 2.1 ± 0.4 | 2.3 ± 0.1 | 1.1, 1.3 | 1.7 ± 0.3 | 1.5 ± 0.2 | 1.6 ± 0.2 | 2.5 ± 0.2 | 1.9 ± 0.3 | 2.6 ± 0.2 |
| Q652L | 2.4 ± 0.2 | 3.7 ± 0.9 | 1.3, 1.2 | 1.7 ± 0.1 | 2.1 ± 0.6 | 1.9 ± 0.2 | 3.1 ± 0.1 | 2.3 ± 0.1 | 3.4 ± 0.1 |
| Q652N | 2.6 ± 0.5 | 3.1 ± 0.2 | ND | 2.4 ± 0.3 | 1.9 ± 0.4 | 1.8 ± 0.3 | 2.9 ± 0.1 | 2.0 ± 0.1 | 3.2 ± 0.6 |
| R708D | 2.9 ± 0.2 | 3.8 ± 0.2 | 1.3 ± 0.3 | 2.6 ± 0.2 | 1.6 ± 0.4 | 2.3 ± 0.2 | 3.1 ± 0.2 | 1.5 ± 0.1 | 3.1 ± 0.8 |
| R708V | 2.8 ± 0.2 | 3.2 ± 0.2 | 1.5, 1.3 | 2.2 ± 0.1 | 2.3 ± 0.1 | 2.8 ± 0.1 | 3.3 ± 0.1 | 2.6 ± 0.1 | 3.3 ± 0.2 |

[a]Strains are transformants of strain V2831 [Δ*recBCD2371*] with the indicated *recC* codon change on derivatives of plasmid pSA607 (*recBC*$^{2773}$*D*).

[b]Chi alleles are defined in Table 2 and Figure 4. Chi activity is expressed as the ratio of clear (*cI857*) to turbid (*cI*$^{+}$) plaques among selected *J*$^{+}$ *R*$^{+}$ recombinants in type 2 crosses (Fig. 3C). Data are the mean ± SEM from at least four experiments unless experimental results are listed individually. At least 50 plaques were counted for each recombinant type.

**Table S7. Statistical analysis of $\chi^{+}L$ context-dependence in RecC tunnel mutants**

| | Comparison[a] | *recBCD*$^{+}$ | S39E | S39V | K88I | R708D | L64A | G41Q | L64S | L64V | *recB21* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 251 vs $^{-}$252 | 0.0032 | 0.421 | 0.0400 | 0.0008 | 0.0056 | 0.0686 | 0.0960 | 0.0340 | ND | 0.2510 |
| | 252 vs $^{-}$252 | 0.0002 | 0.055 | 0.0060 | 0.0002 | 0.0008 | 0.0062 | 0.0240 | 0.0097 | ND | 0.5610 |
| | 251 vs 252 | 0.0323 | 0.1880 | 0.0020 | 0.0010 | 0.0090 | 0.0070 | 0.5410 | 0.1880 | 0.0512 | 0.7870 |
| | 251 vs 253 | 0.3810 | 0.3920 | 0.8280 | 0.0010 | 0.0599 | 1.0000 | 0.7580 | 0.7310 | 0.1450 | 1.0000 |
| | 251 vs 254 | 0.0292 | 0.0328 | 0.0810 | 0.0010 | 0.0001 | 0.0107 | 0.5910 | 0.7310 | 0.1450 | 0.7310 |
| | 252 vs 253 | 0.0083 | 0.4100 | 0.0030 | 0.0006 | 0.0020 | 0.0688 | 0.0800 | 0.1270 | 0.0001 | 0.8690 |
| | 252 vs 254 | 0.0001 | 0.3300 | 0.0003 | 0.0001 | 0.0001 | 0.0688 | 1.0000 | 0.6700 | 0.0001 | 0.5990 |
| 3' swaps | 2-2 vs 2-3 | 0.0670 | 0.0400 | 0.0180 | 0.0060 | 0.0050 | 0.0090 | 0.0300 | 0.0360 | 0.0190 | 0.7030 |
| | 2-2 vs 2-4 | 0.0139 | 0.2300 | 0.0030 | 0.0080 | 0.0030 | 0.1520 | 0.0450 | 0.0660 | 0.0050 | 0.5990 |
| | 3-3 vs 3-2 | 0.0400 | 0.7600 | 0.3000 | 0.0170 | 0.0300 | 0.1040 | 1.0000 | 0.5060 | 0.0050 | 0.8590 |
| | 4-4 vs 4-2 | 0.0090 | 0.1200 | 0.0200 | 0.0180 | 0.0400 | 0.0010 | 1.0000 | 0.2070 | 0.0010 | 0.5990 |
| 5' swaps | 2-2 vs 3-2 | 1.0000 | 0.7910 | 0.0470 | 0.3890 | 0.0480 | 0.4540 | 0.4940 | 0.3290 | 0.0050 | 0.5910 |
| | 2-2 vs 4-2 | 0.7830 | 0.6700 | 0.5270 | 0.8160 | 0.4250 | 0.8210 | 1.0000 | 0.7360 | 0.0300 | 0.3680 |
| | 3-3 vs 2-3 | 0.4420 | 0.0300 | 0.1470 | 0.0780 | 0.3290 | 1.0000 | 0.5060 | 0.5500 | 0.5540 | 0.8670 |
| | 4-4 vs 2-4 | 0.0117 | 0.0360 | 0.0690 | 0.0040 | 0.8160 | 0.2360 | 0.0680 | 1.0000 | 0.4060 | 1.0000 |

[a] The c/t ratios obtained in crosses with the indicated $\chi^{+}L$ alleles or $\chi^{-}L252$ (designated "$^{-}$252") were compared by unpaired t tests. p values are listed in black ($p > 0.081$), blue ($p = 0.051 – 0.081$), or red ($p < 0.05$).

**Table S8. Context-dependence of $\chi^+L$ activity in RecC tunnel mutants**

| *recC* codon change | Ratio of Chi activity ($\chi^+L1/\chi^+L2$)[a] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *252/251* | *252/253* | *252/254* | *252*/3-2 | *252*/4-2 | *252*/2-3 | *252*/2-4 | 3-2/*253* | 4-2/*254* |
| *recBCD*$^+$ | 1.26 | 1.40 | 1.62 | 0.92 | 0.98 | 1.40 | 1.75 | 1.51 | 1.66 |
| *recB21* | 0.93 | 0.93 | 0.87 | 0.87 | 0.76 | 0.87 | 0.87 | 1.07 | 1.13 |
| S39D | 1.25 | 1.09 | 1.25 | 0.86 | 1.00 | 1.25 | 1.25 | 1.26 | 1.25 |
| S39E | 1.27 | 1.12 | 0.86 | 1.06 | 1.06 | 1.46 | 1.19 | 1.06 | 0.82 |
| S39F | 1.41 | 1.20 | 1.60 | 0.86 | 1.04 | 2.00 | 1.41 | 1.40 | 1.53 |
| S39V | 2.00 | 1.92 | 3.07 | 1.53 | 1.15 | 1.53 | 2.09 | 1.25 | 2.67 |
| G41E | 1.09 | 1.14 | 1.33 | 0.92 | 0.96 | 1.60 | 1.33 | 1.24 | 1.39 |
| G41M | 2.25 | 2.12 | 1.64 | 1.24 | 1.33 | 1.80 | 1.57 | 1.71 | 1.23 |
| G41Q | 1.10 | 1.15 | 1.00 | 1.15 | 0.92 | 1.21 | 1.53 | 1.00 | 1.09 |
| G41T | 1.38 | 1.38 | 1.43 | 1.03 | 1.03 | 1.65 | 1.50 | 1.33 | 1.39 |
| G41W | 1.24 | 1.13 | 1.18 | 1.04 | 1.00 | 1.18 | 1.24 | 1.09 | 1.18 |
| L64A | 1.57 | 1.57 | 2.36 | 1.14 | 1.03 | 1.57 | 1.50 | 1.38 | 2.29 |
| L64D | 1.04 | 1.27 | 1.33 | 1.40 | 1.12 | 1.17 | 1.17 | 0.91 | 1.19 |
| L64E | 1.13 | 1.86 | 1.13 | 1.13 | 1.04 | 1.44 | 2.36 | 1.64 | 1.09 |
| L64S | 1.16 | 1.21 | 1.21 | 1.12 | 1.04 | 1.26 | 1.21 | 1.08 | 1.17 |
| L64T | 1.55 | 1.35 | 1.72 | 1.48 | 1.29 | 1.94 | 2.82 | 0.91 | 1.33 |
| L64V | 1.26 | 1.55 | 1.55 | 1.06 | 1.13 | 1.42 | 1.42 | 1.45 | 1.36 |
| W70A | 1.94 | 1.24 | 1.82 | 0.97 | 1.07 | 1.29 | 1.48 | 1.28 | 1.71 |
| K88I | 1.30 | 2.26 | 2.87 | 1.13 | 1.05 | 1.54 | 1.48 | 2.00 | 2.73 |
| K88N | 1.10 | 1.42 | 1.42 | 1.03 | 0.97 | 1.31 | 1.36 | 1.38 | 1.46 |
| K88V | 1.21 | 1.40 | 1.25 | 1.25 | 1.09 | 1.35 | 1.40 | 1.12 | 1.14 |
| D136A | 1.21 | 0.92 | 1.35 | 1.35 | 0.92 | 1.92 | 1.35 | 0.68 | 1.47 |
| Q652E | 1.10 | 1.44 | 1.21 | 0.92 | 0.88 | 1.35 | 1.53 | 1.56 | 1.37 |
| Q652L | 1.54 | 1.95 | 1.61 | 1.19 | 1.09 | 2.18 | 1.76 | 1.63 | 1.48 |
| Q652N | 1.19 | 1.72 | 1.55 | 1.35 | 0.97 | 1.29 | 1.63 | 1.28 | 1.60 |
| R708D | 1.31 | 1.65 | 2.53 | 1.23 | 1.23 | 1.46 | 2.38 | 1.35 | 2.07 |
| R708V | 1.14 | 1.14 | 1.23 | 0.97 | 0.97 | 1.45 | 1.39 | 1.18 | 1.27 |

[a]Context-dependence is the ratio of clear/turbid plaques among $J^+R^+$ recombinants from crosses of type 2 (Fig. 3C) with the first indicated Chi allele ($\chi^+L1$) divided by the ratio of clear/turbid plaques among $J^+R^+$ recombinants from the crosses with the second indicated Chi allele ($\chi^+L2$). Filled cells (□) have values ≤ 1.2 indicating that the two Chi alleles have similar activity and therefore show little or no context-dependence in the indicated strain.

**Table S9. Chi activity of RecC tunnel mutants**

| $\chi^+$L allele[a] | Chi activity (c/t ratio)[b] in host strain *recBCD* allele[c] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *recBCD*$^+$ | *recB21* | *recD1013* | K88I | R708D | L64A | S39E | L64S |
| *$\chi^+$L259* | 13.1 ± 0.4 | 1.1 ± 0.049 | 0.95 ± 0.06 | 5.4 ± 0.62 | 4.8 ± 0.74 | 5.7 ± 0.26 | 1.67 ± 0.09 | 1.3 ± 0.13 |
| *$\chi^-$L259* | 2.1 ± 0.3 | 0.87 ± 0.09 | 1.0 ± 0.09 | 1.43 ± 0.09 | 1.19 ± 0.12 | 2.3 ± 0.48 | 1.27 ± 0.09 | 1.0 ± 0.04 |
| *$\chi^+$L262* | 8.2 ± 0.3 | 0.93 ± 0.09 | 0.85 ± 0.05 | 3.1 ± 0.6 | 2.6 ± 0.03 | 3.4 ± 0.37 | 1.4 ± 0.01 | 1.03 ± 0.13 |

[a] See Table 2 for sequences flanking the indicated *$\chi^+$L* allele.

[b] Chi activity is expressed as the ratio of clear (*cI857*) to turbid (*cI*$^+$) plaques among selected *J*$^+$ *R*$^+$ recombinants (Figure 3C). Data are the mean ± SEM from 3 or 4 experiments.

[c] Strains are transformants of strain V2831 (*ΔrecBCD2731*) with the indicated *recC* codon change on derivatives of plasmid pSA607 (*recBC*$^{2773}$*D*).

**File S1: Supplemental Materials and Methods**

***Preparation of a lambda library containing 25-bp inserts***

A library of phage containing inserts with 25 random bp flanked by *Xho*I and *Nhe*I cleavage sites was prepared in λ 1560 (*b1453 cI60*). Oligonucleotide 2932 was made double-stranded by primer extension in NEBuffer 2 using the Klenow fragment of DNA polymerase I (New England Biolabs) according to the manufacturer's protocol. The reaction contained 100 pmol of oligonucleotide 2931 and 100 pmol of the oligonucleotide template. The reaction was terminated by incubation at 75° for 20 min. The DNA was digested with *Xho*I and *Nhe*I and purified (QIAquick Nucleotide Removal Kit; Qiagen). The products of four separate reactions were pooled and used for ligation into λ DNA arms prepared as follows.

Phage λ DNA was prepared from pooled plate stocks ($2.5 \times 10^{10}$ to $4.4 \times 10^{10}$ pfu/ml) grown on strain JC8679 as described (ARBER *et al.* 1983). Phage were concentrated from approximately 300 ml of lysate by centrifugation at 12k x *g* for 2 hr, and suspended in TM buffer [10 mM Tris (pH 8.1), 10 mM $MgSO_4$]. Phage particles were layered on a CsCl step gradient prepared with 1 ml each of solutions in TM buffer with densities of 1.3, 1.5, and 1.7 g/cm$^3$ and purified by centrifugation at 48k x g for 2 hr. The band containing the phage was removed and further purified by centrifugation (125k x g for 18 hr) on a CsCl equilibrium gradient (1.5 g/cm$^3$) in TM buffer. The band containing the phage was dialyzed against 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and the $OD_{260}$ adjusted to 24 with the same buffer.

DNA was extracted from phage particles by the addition of 0.1 volume of 10% SDS and incubation at 65° for 15 min. Following incubation on ice, 0.11 volumes of 4.0 M KCl was added, and the suspension returned to ice. The material was centrifuged, and the DNA was recovered from the supernate by precipitation with ethanol. DNA (5 µg) was digested with *Xho*I and *Nhe*I in NEBuffer 4 and incubated at 80° for 20 min. Ligation of λ arms and inserts was as described (MANIATIS *et al.* 1982) using T4 DNA ligase (New England Biolabs). The products were packaged using Gigapack III Gold Packaging Extract (Agilent Technologies). The library contained $9.3 \times 10^4$ pfu as determined on strain JC8679. PCR amplification of 10 randomly selected plaques indicated that seven of ten had a single 25 bp insert; three had two 25 bp

inserts. The library was amplified on strain JC8679. Approximately 10,000 pfu (~750 per plate) were plated on lawns of *recBCD*$^+$, L64A, Q652L, R708D, or R708V cells on TB plates. Plaques noticeably larger than the majority were detected on all of these strains at a frequency of about 1 in 3000.

***Bacteriophage λ construction by recombineering***

We created various $\chi^+L$ alleles in *gam* using ss oligonucleotides bearing the desired mutation (Table S2) and λ 816 (*red-3*) by recombineering in *E. coli* strain DY378, which expresses λ Exo, Beta, and Gam after thermal induction (THOMASON *et al.* 2009). Recombination between the oligonucleotide and the infecting phage inactivated *gam* and allowed selection for the desired *red gam* mutants on a P2 lysogen of strain C600 (LINDAHL *et al.* 1970; ZISSLER *et al.* 1971). Briefly, *E. coli* strain DY378 was infected with λ 816 at a multiplicity of infection of 3. The infected cells were incubated at 42° to induce prophage Exo, Beta, and Gam functions and then prepared for electroporation. Mutagenic oligonucleotides targeting *gam* (Table S2; Integrated DNA Technologies, Inc.) were 70 – 90 nucleotides long with the desired changes approximately in the center. Oligonucleotides (100 ng) were introduced into infected cells by electroporation in 0.1 cm cuvettes at 1.8 kV (THOMASON *et al.* 2009). *red gam* mutant recombinants, selected on *E. coli* strain C600(P2), occurred at a frequency of approximately $10^{-3}$ to $10^{-6}$ depending on the oligonucleotide. Plaques were picked into SM, and streaked on strains V66 (*recBCD*$^+$) and V67 (*recB21*). Phage forming large plaques on V66, indicating the presence of Chi, were retested for growth on C600(P2); as expected, all formed large plaques on V67, which allows λ concatemer formation via rolling circle replication. After a second purification on V66, an isolated plaque was picked into SM for PCR amplification and sequence analysis and for the preparation of high-titer stocks on strain JC9387, JM1, or V66 (ARBER *et al.* 1983).

Lambda DNA was sequenced from small volume phage lysates prepared by spotting 50 µl of SM containing an isolated plaque on strain JM1 or JC9387 in TB top agar. The spots were harvested after overnight incubation at 37$^o$ into 300 µl of SM with 5 µl of chloroform. DNA in the lysate was amplified for sequencing using primers OL2938 and OL3009 (Table S2) and the following thermocycler program: segment 1, 1 cycle of 98$^o$ for 5 min; segment 2, 30 cycles of [94$^o$ for 30 sec, 55$^o$ for 30 sec, 72$^o$ for 1 min]; segment 3, 1 cycle of 72$^o$ for 3 min. Briefly, 5 µl of the phage lysate was added to a PCR tube containing 10 µl of $H_2O$; after segment 1, 15 µl of 2x PCR reaction mix (as described for Platinum Taq, Invitrogen) was added and the cycling continued. Analysis of the DNA sequence from approximately 500 bp centered on $\chi^+L$ confirmed the presence of Chi and the absence of any other nucleotide changes nearby.

We constructed *susJ6 cI857* or *cI*$^{+}$ *susR5* derivatives of each *red-3* $\chi^{+}$*L cI857* phage by lytic crosses between phage 2016 (*susJ6*) or phage 2017 (*cI*$^{+}$ *susR5*) as described (SCHULTZ *et al.* 1981). Details are available upon request.

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