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Measuring chromosome dynamics on different time scales using resolvases with varying half-lives

Richard A. Stein, **Shuang Deng**, and **N. Patrick Higgins***
Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294, USA.

Abstract

The bacterial chromosome is organized into multiple independent domains, each capable of constraining the plectonemic negative supercoil energy introduced by DNA gyrase. Different experimental approaches have estimated the number of domains to be between 40 and 150. The site-specific resolution systems of closely related transposons Tn*3* and γδ are valuable tools for measuring supercoil diffusion and analysing bacterial chromosome dynamics *in vivo*. Once made, the wild-type resolvase persists in cells for time periods greater than the cell doubling time. To examine chromosome dynamics over shorter time frames that are more closely tuned to processes like inducible transcription, we constructed a set of resolvases with cellular half-lives ranging from less than 5 min to 30 min. Analysing chromosomes on different time scales shows domain structure to be dynamic. Rather than the 150 domains detected with the Tn*3* resolvase, wild-type cells measured over a 10 min time span have more than 400 domains per genome equivalent, and some gyrase mutants exceed 1000.

Introduction

Defining the compact structure of the bacterial chromosomes inside a living cell has been a long-standing goal of molecular genetics. A variety of assays have been developed to probe this structure including visualization by electron microscopy (Delius and Worcel, 1973; Griffith, 1976; Kavenoff and Bowen, 1976; Kavenoff and Ryder, 1976), UV-induced psoralen cross-linking (Pettijohn and Pfenninger, 1980; Sinden *et al*., 1980; Sinden and Pettijohn, 1981), measuring output from supercoil-sensitive promoters (Miller and Simons, 1993; Pavitt and Higgins, 1993; Spirito *et al*., 1994), site-specific recombination between distant chromosomal sites (Higgins *et al*., 1996; Staczek and Higgins, 1998; Higgins, 1999) and sequence-specific DNA localization (Glaser *et al*., 1997; Webb *et al*., 1997; Teleman *et al*., 1998; Niki *et al*., 2000; Gordon *et al*., 2004; Viollier *et al*., 2004). These assays illuminate different aspects of chromosomal behaviour, but understanding the nature of domain structural changes over time has been an especially daunting challenge (Deng *et al*., 2004).

One method to analyse time-dependent supercoil dynamics involves recombination between *res* sites spaced at different intervals (Staczek and Higgins, 1998). After the induction of the Tn*3* resolvase, cellular DNA can be isolated at different times and the recombinant products can be detected by Southern blot and polymerase chain reaction (PCR) analyses. In stationary-phase cells, a major fraction of the recombination (60%) occurred within 15 min but a significant segment of recombination (40%) happened slowly, i.e. much later than 4 h after induction of the recombination protein and then only after cells were diluted in fresh medium (Staczek and Higgins, 1998). Following recombination by isolating cellular DNA and carrying out either Southern blot or quantitative PCR analyses is tedious and requires a significant

*For correspondence. E-mail nphiggin@uab.edu; Tel. (+1) 205 934 3299; Fax (+1) 205 975 5955..

investment in both time and reagents, especially if one desires statistically significant data from many chromosomal sites and from bacteria carrying multiple combinations of genetic mutations.

To make site-specific resolution assays time sensitive, we devised a method to alter the life span of the recombinase. A 11-amino-acid sequence was added to the natural C-terminus of the γδRes protein. This sequence corresponds to a degradation 'tag' that the Tm or *ssrA* RNA system appends to proteins when a ribosome stalls at rare codons or when an mRNA lacks a termination codon (Keiler *et al*., 1996; Hayes *et al*., 2002a,b). The SsrA tag changed the half-life of resolvase in exponentially growing *Escherichia coli* or *Salmonella enterica* serovar typhimurium from well over 40 min to less than 5 min. By modifying amino acid residues in the SsrA tag sequence (Flynn *et al*., 2001), enzymes were also made with half-lives of 15 and 30 min. Recombination assays performed with this family of time-sensitive enzymes demonstrate a dynamic aspect of chromosome structure. More than 400 domains per genome equivalent are predicted to exist in exponentially growing bacteria rather than the 40 or 150 domains estimated by time-insensitive methods. Moreover, in cells harbouring mutations in gyrase, the number of domains can rise to 1000.

Results

Modifying resolvase half-life

One assay to monitor supercoil diffusion in living bacterial chromosomes is based on site-specific recombination catalysed by the Tn*3* resolvase (Higgins *et al*., 1996; Staczek and Higgins, 1998; Deng *et al*., 2004). Plasmid pJBRes*cI* has the Tn*3* *tnpR* gene expressed from the bacteriophage λ P_L promoter under control of the temperature-sensitive *cI857* repressor. In this system, resolvase protein expression is induced by a 10-min temperature shift from 30° C to 42°C. *res* sites are 114 bp DNA sequences that recombine in the presence of either the Tn*3* or γδ Res proteins (Krasnow and Cozzarelli, 1983; Benjamin *et al*., 1996). A burst of resolvase expression will catalyse site-specific recombination between two directly repeated chromosomal *res* sites if they reside in the same supercoil domain. If resolvase persists in cells at recombination-active concentrations for long time spans, short-lived barriers could be missed. For example, Deng showed that induction of or repression of a strong promoter caused a domain barrier to appear or disappear over a 20 min period (Deng *et al*., 2004).

To measure the time span over which resolution activity persists after thermoinduction, we developed a plasmid-based assay. The 5.9 kb pRR51 plasmid carries Amp and Tet resistance genes. Two *res* sites flank the *tet* gene so that cells become Tet sensitive after the plasmid undergoes resolution. Cells were induced for Res expression by 10 min of growth at 42°C, then returned to 30°C and maintained in exponential phase. At intervals a 1 ml aliquot of the culture was quickly washed in 10% ice-cold glycerol and subjected to electroporation to introduce plasmid pRR51. Cells were spread onto Amp-containing plates to select for cells transformed with plasmid DNA, and then individual transformants were patched onto Tet-containing plates to measure resolution efficiency. Cells had a reproducible resolution pattern. For the first 60 min after the return to 30°C, Tn*3* resolution was close to 100% efficient. Then an exponential decay of resolution activity followed with a half-life of about 40 min (Fig. 1, solid triangles). The Tn*3* and γδRes proteins are closely related (81% identity and 93% similarity at the amino acid level) and functionally interchangeable (Benjamin *et al*., 1996). However, the γδ resolvase was consistently more potent, resolving more than 95% of pRR51 DNA for more than 4 h after induction (Fig. 1, closed circles; and data not shown). In previous work, due to an data entry error, we mistakenly thought that a plasmid we obtained encoded the γδ resolvase, but it was the Tn*3* resolvase instead (Higgins *et al*., 1996;Staczek and Higgins, 1998;Scheirer and Higgins, 2001). We discovered the error by sequencing the plasmid and

then tested the γδ resolvase, and based on its increased persistence *in vivo*, chose to modify the C-terminus of this enzyme for chromosome studies.

A 36 bp sequence (described in *Experimental procedures*) was added to the C-terminus of the γδ resolvase in plasmid pγδRes-SsrA. This sequence encodes 11 amino acids that were discovered when the overexpression in *E. coli* of murine interleukin-6 (IL-6) was found to generate a population of proteins of different sizes all sharing the same C-terminal sequence (Ala–Ala–Asn–Asp–Glu–Asn–Tyr–Ala–Leu–Ala–Ala–COOH) (Tu *et al*., 1995). The 11 amino acid residues are encoded by a stable RNA (*tm*RNA) and tagged proteins are efficiently degraded by the ClpXP protease *in vivo* and *in vitro* (Keiler *et al*., 1996; Welty *et al*., 1997; Gottesman *et al*., 1998).

When pRR51 was introduced into cells that were harvested immediately after thermoinduction of the γδRes-SsrA protein, 32% of cells transformed with pRR51 plasmid DNA contained recombinant plasmid (Fig. 1, Table 1). Resolution efficiency fell rapidly and after 10 min of growth at 30°C, no cell transformed with pRR51 was recombinant. Thus, the *in vivo* time-course of resolution can be changed substantially by expressing an SsrA-tagged resolvase.

To confirm the short half-life of γδRes-SsrA, cellular proteins were extracted with SDS at different times after induction and subjected to SDS-polyacrylamide gel electrophoresis. SsrA-tagged proteins were visualized on Western blot transfers using rabbit antibodies to the SsrA tag, generously provided by Tania Baker's lab. A protein band corresponding to the predicted size of γδRes-SsrA appeared after thermoinduction, then disappeared from cell extracts in less than 5 min after cells were returned to 30°C growth conditions (Fig. 2A). Thus, protein half-life and recombination activity were closely correlated.

One potential problem with the SsrA tagging strategy is that catalytic properties of resolvase might be altered by a C-terminal modification. This could be caused by interference with DNA binding or by impeding conformational changes that are required for recombination. To determine whether the enzymatic properties of resolvase were altered by the SsrA tag, three experiments were carried out.

First, ClpP is the protease-active subunit of the ClpAP and the ClpXP proteases. ClpXP is the major cellular degradation pathway for proteins carrying the SsrA tag. Eliminating this protease should stabilize the γδRes-SsrA protein. The *clpP* gene was deleted from the *Salmonella* chromosome and replaced with a Kan-resistant marker. Strain NH3566 carries the *clpP* deletion in combination with plasmid pγδRes-SsrA and cells from this strain are viable with growth properties very similar to wild type (WT). Cultures were heat shocked at 42°C for 10 min and subsequently transformed at different intervals with pRR51 to monitor resolution activity. One problem with *Salmonella* strains lacking ClpXP protease is that they had a constitutive low level of Res protein expression even at 30°C. The presence of the γδRes-SsrA protein could be detected on a Western blot before heat shock (Fig. 2B), and 35% of the plasmid transformed cells were recombinant even in the absence of heat shock induction. Nonetheless, a temperature-associated increase in the γδRes-SsrAg band was seen and the elevated level of this band persisted for 1 h after thermoinduction. Moreover, the resolution efficiency stayed near 100% for 2 h when plasmid DNAs were introduced by electroporation into an induced strain (Table 1, Fig. 2B).

Second, the two terminal alanine residues of the SsrA tag are crucial for recognition by the Clp system (Keiler *et al*., 1996). Many proteins tagged with a 11-amino-acid sequence containing two terminal aspartic acid residues (DD) have half-lives comparable to untagged proteins. A variant Res protein was made with both alanines changed to aspartates by two point mutations (γδRes-SsrA-DD). The time-course of resolution for the γδRes-SsrA-DD protein was

examined in cells with WT levels of ClpXP and ClpAP. Resolution activity persisted in these cells for >4 h after induction (Fig. 1, open squares).

Third, amino acid residues within the SsrA tag affect ClpXP proteolytic activity (Flynn *et al*., 2001). Two positions known to reduce ClpXP-dependent degradation are positions at leucine 9 and alanine 8. In plasmid pγδRes-SsrA-L9D and in pγδRes-SsrA-A8D the leucine and alanine residues, respectively, were changed to aspartate. Strains harbouring both plasmids were thermoinduced and tested for resolution activity using the pRR51 electroporation procedure. The half-life of γδRes-SsrA-A8D was 15 min (Fig. 1, open triangles) and γδRes-SsrA-L9D had a 30 min half-life (Fig. 1, solid squares). Together, these experiments show that in cells with active ClpXP protease, a family of resolvase enzymes can be used to measure the chromosome dynamics for intervals varying from a few minutes to several hours using the same thermoinduction protocol.

A 400-domain chromosome

The number of supercoil domains represents a basic unit of chromosome organization inside living cells. Estimates based on WT resolvase assays would underestimate the domain number if they appear and disappear in less than 4 h. The greater than 4 h time span for which γδ resolvase remains active is longer than six cell lives. If *res* sites occupy the same domain even briefly during this period, a resolution product could be produced.

To compare the efficiency of resolvases that span the widest range in time, nine strains (Higgins *et al*., 1996) were used with *res* site distances varying from 14 to 90 kb. Each strain was tested with the γδRes and the γδRes-SsrA proteins. The slope of the first-order decay curve gives the half-distance value (1/2D), which is the interval of separation that results in a 50% drop in recombination efficiency. Data from at least three independent experiments for each point are plotted (Fig. 3, Table 2). The 1/2D of γδRes protein was 36 kb whereas the γδRes-SsrA protein value was 9 kb (Fig. 3A). The correlation coefficient was good for both curves (R^2 = 0.841 and 0.974 respectively). This change in slope for the WT and γδRes-SsrA resolvases shows that a stable resolvase assay would underestimate the domain number by more than twofold.

Once cells are shifted from 30°C to 42°C to induce resolvase expression, about 6 min are required to detect resolution products for all resolvases (data not shown). During this 6 min interval transcription and translation must generate sufficient enzyme to saturate the *res* sites and the four strand exchanges necessary for recombination must be completed. Cellular persistence of γδRes-SsrA protein lasted for 6–10 min after induction (Fig. 1). Therefore, the γδRes-SsrA enzyme detects chromosome dynamics over a 10 min time span.

How do the intermediate half-life resolvases behave? Strains carrying the plasmids pγδRes-SsrA-A8D, pγδRes-SsrA-L9D and pγδRes-SsrA-DD were also tested for the same nine intervals. The 1/2D values were 12 kb, 13 kb and 23 kb, respectively, for these enzymes (Fig. 3B). The number of barriers detected in WT cells decreased gradually over time periods from 10 to 40 min, extending up to 5 h. In our previous experiments, the Tn*3* resolvase would have catalysed resolution after cells begin to enter stationary phase. In stationary-phase domain barriers disappear (Staczek and Higgins, 1998). Assuming a Poisson distribution of domain barriers, our new data with resolvases having half-lives of 30 min or less converge on a median domain in a WT bacterial chromosome of 13 kb. One genome equivalent of *Salmonella* DNA would contain from 350 to 450 domains. This more than doubles the prior estimate of 150 supercoil domains (Higgins, 1999;Scheirer and Higgins, 2001).

The gyrase impact on domain number

Gyrase and TopoIV mutations influence domain structure (Staczek and Higgins, 1998). Gyrase mutants vary from exerting a mild impact on resolution for the *gyrA205* allele to severe effects for the *gyrB652* and *gyrB1820* alleles. Using the Tn*3* resolvase to probe genome structure, some severe gyrase mutants had approximately twice as many domains as WT (Staczek and Higgins, 1998). To confirm the results obtained with Tn*3* resolvase using a tagged enzyme, experiments were carried out with the γδRes-SsrA-DD protein (Fig. 4). Intervals ranging from 14 to 90 kb were tested in strains with the WT gyrase, with the mild *gyrA209* gene, and with the severe *gyrB1820* allele (Fig. 4). Strains with WT gyrase had 1/2D of 23 kb ($R^2 = 0.91$), the *gyrA209* allele had a 1/2D of 18 ($R^2 = 0.91$), while the *gyrB1820* mutation gave a 1/2D of 12 ($R^2 = 0.89$). These results confirmed our previous conclusions.

If the median domain in a WT cell is 13 kb when assayed with a time-sensitive resolvase, how many domains exist in a *gyrB1820* mutant? Doubling the domain number should cause the fraction of cells capable of resolving a 14 kb interval to be small. The resolution of a 14 kb interval was tested with the γδRes-SsrA, γδRes-SsrA-L9D, γδRes-SsrA-A8D or γδRes-SsrA-DD proteins. Each enzyme was tested in combination with one of the five Ts gyrase alleles (Fig. 5). Gyrase had a dramatic impact on 14 kb resolution reactions. Even rather weak *gyrA* alleles showed a significant decline in resolution efficiency with enzymes having half-lives of 30 min or less. For example, resolution with the *gyrA213* allele decreased fivefold relative to a WT strain whereas the *gyrA209* allele depressed recombination by 10-fold. The most dramatic effect was obtained with the *gyrB1820* mutation where resolution efficiency fell to less than 1% of WT levels using all three time-restricted proteins.

The rank for each gyrase mutant from mild to severe for the effect on the change in the distance rule correlated well with its effect on the efficiency of producing a 14 kb deletion. We previously interpreted this correlation to indicate that supercoil movement is more restricted in the gyrase mutants. However, one class of promoters responds to altered gyrase activity with lower transcriptional output. If synthesis from the λ P_L promoter was strongly decreased by gyrase mutations, this could contribute to a decline in the resolution efficiency. To test how gyrase mutations affect the ability of Res protein to saturate *res* sites, a short interval containing only the *tet* gene in plasmid pRR51 was tested for resolution efficiency. Tet resistance could not be scored directly because all gyrase mutants are linked to a Tet marker in the chromosome. To circumvent this problem, cells harbouring pRR51 were induced for resolvase synthesis in gyrase mutants, and after 30 min plasmid was extracted from each strain and transformed into the LT2 strain. All enzymes resolved the plasmid interval at 65% efficiency or greater, except for the γδRes-SsrA protein (52%) (Table 3). Therefore, impact of gyrase mutants on resolution of the 14 kb chromosomal domain could have a small (50%) expression component, but most of the 10- to 100-fold effects on recombination efficiency resulted from inhibition of synaptic pairing of chromosomal sites. Another possibility is that the half-life of SsrA-tagged resolvases are changed in gyrase mutants. We tested resolvase activity in all four gyrase mutants used in this study, and resolvase half-life was unaltered (data not shown). Thus, in severe gyrase mutants, the chromosomes become balkanized into > 1000 domains per genome equivalent.

Discussion

Time-sensitive protein expression

To develop a new tool for measuring chromosome dynamics within specific windows of time, we designed proteins with cellular half-lives varying from 5 min to several hours by attaching different 11-amino-acid tags to the C-terminus of the γδ resolvase. Using a thermoinducible λ regulatory system cloned on a low-copy plasmid and various resolvase derivatives, this system allowed us to synchronously express resolvases in cell populations of *E. coli* and

Salmonella and to measure supercoil dynamics for intervals varying from 10 min to 6 h. Compared with other induction systems that we've tested, the λ control system is exceptional both for the level and uniformity of protein expressed in a short time and for the tightness of repression at 30°C. The *cI857* repressor dissociates from DNA at 42°C, but re-folds rapidly and binds DNA when cells are returned to 30°C (Gaitanaris *et al*., 1990). This performance is superior to systems we've developed using *lac* and *ara* regulatory elements. One difference may have to do with the simplicity and ease of temperature shift versus complications involved in inducer transport and added effects of c-AMP on arabinose-modulated expression. We suggest that this general strategy is ideal for analysing temporal effects of many cellular proteins that can be modified at the C-terminus without altering protein function.

Many experiments have been designed to study the structure of DNA inside living cells. Assays include gel analysis of plasmid linking number (Wang, 1986; Vologodskii, 1992), formation of left-handed Z-DNA conformation (Klysik *et al*., 1982; Peck *et al*., 1982; Mirkin *et al*., 1987), extrusion of cruciforms in special plasmids (Murchie and Lilley, 1987; McClellan *et al*., 1990; Dayn *et al*., 1991), quantitative measurements of trimethylpsoralen intercalation (Sinden *et al*., 1980; Sinden and Pettijohn, 1981; Mojica and Higgins, 1997) and measures of transcription from promoters that respond to superhelical tension (Miller and Simons, 1993; Pavitt and Higgins, 1993; Figueroa-Bossi *et al*., 1998). Although these experiments yield important information about DNA conformation, they are largely uninformative about DNA structure in many specific segments of the bacterial chromosome. The advantage of the Tn*3* and γδ resolution assay is that the technique is non-invasive, requires a minimum level of supercoiling and can be carried out for almost any segment of the chromosome (Deng *et al*., 2004).

Previous work from this lab showed that for about 5% of the *Salmonella* genome, the efficiency of recombination is a first-order function of physical distance (Higgins *et al*., 1996). The probability of two sites forming a plectonemic synapse behaves in a stochastic manner, i.e. barriers to supercoil diffusion vary in position from cell to cell and can change within a single cell over time. Like other first-order functions (radioactive decay) the slope of resolution efficiency curves gives a number that we call the 1/2D, which is the distance that causes resolution efficiency to fall by 50%. For the Tn*3* resolvase, which is mistakenly called γδ resolvase in previous works (Higgins *et al*., 1996; Staczek and Higgins, 1998; Scheirer and Higgins, 2001), this distance was about 15 kb.

Domain numbers estimates

Two significant findings about chromosome structure are reported in this work. First, individual cells of WT strains of *Salmonella* have more than 400 domains that constrain negative supercoiling for each genome equivalent of DNA. This doubles the domain number we estimated from previous experiments employing the WT Tn*3* resolvase (Higgins *et al*., 1996; Scheirer and Higgins, 2001). Two factors contributed to this revision and to the need for time-sensitive enzymes in evaluating genome structure. (i) The WT resolvase from Tn*3* or γδ transposons remains in cells at recombination-active concentrations for up to 6 h after expression (Fig. 1 and R.A. Stein, unpublished data). In stationary cells, supercoil domains expand (Staczek and Higgins, 1998). Because cells enter stationary phase before the resolvase activity disappears, a significant amount of recombination can occur in stationary phase. The differences in 1/2D for the γδRes, γδRes-SsrA-DD and Tn*3* resolvases (36, 23 and 15 kb respectively) reflect how long these proteins persist as cells enter stationary phase (data not shown.) (ii) Recent work from this lab showed that the induction of transcription from a strong promoter causes a new domain to appear in the bacterial chromosomes (Deng *et al*., 2004). Transcription-induced domains can appear and disappear over a 20 min time span. The fact that resolvases with half-lives of 5–30 min all give similar 1/2D value (9–13 kb) indicates that

barriers are relatively stable over this reference frame, at least in the intervals that were studied in this work.

A 400-domain chromosome agrees with new measurements made by independent techniques in *E. coli* (Postow *et al*., 2004). Postow and co-workers introduced chromosome breaks using controlled expression of rare-cutting restriction enzymes. By measuring the distance from a break to a supercoil-responsive promoter, they used genome-wide microarray technology to measure supercoil diffusion. They also analysed the loop sizes of nucleoids spread on electron microscope grids. The model of chromosome structure that best fit their data and Monte Carlo computer simulations was a family of loops with a median size of 10 kb having variable length and sequence determinants. These measurements are in close agreement with the conclusions we reached with tagged resolvases.

Controlling domain number

Gyrase mutants cause the number of independent domains to increase, exceeding 1000 domains per genome equivalent in rare severe gyrase mutants (Fig. 5). This trend was seen previously in assays using Tn*3* resolvase (Staczek and Higgins, 1998), but time-sensitive enzymes made the effect more obvious. With time-shortened enzymes, nearly all gyrase mutants increased the number of domains. The impact of gyrase on domain size was more striking than the impact of these same mutations on superhelix density monitored by plasmid linking numbers (Staczek and Higgins, 1998) (Fig. 5). Most models of chromosome structure focus on torsional aspects of negative supercoiling. These models consider the impact of gyrase on the helical repeat, which can influence protein binding to complex sites, the influence on helix melting, which can alter initiation kinetics, and the waves of negative and positive supercoils that attend processes like replication and transcription (see Higgins and Vologodskii, 2004).

We propose that the domain number is a critical factor in prokaryotic genome structure. If domains are too large, DNA compaction and efficient segregation is at risk; if there are too many domains problems might arise during transcription and replication. Three observations are consistent with this view. First, genetic selections to find genes involved in chromosome condensation and partitioning uncovered proteins closely related to eukaryotic cohesins and condensins (Hiraga *et al*., 1989; Hiraga, 1992). In *E. coli* the gene is *mukB* and in *Bacillus subtilis* and *Caulobacter crescentus* the gene is called *smc*. Defects in these proteins can cause a diffuse appearance of cellular nucleoids (Hiraga *et al*., 1989; Hiraga, 1992), influence supercoiling (den Blaauwen *et al*., 2001; Lindow *et al*., 2002; Adachi and Hiraga, 2003; Case *et al*., 2004) and result in poor segregation of chromosomes between dividing sister cells (Weitao *et al*., 2000). Second, a new technique for gene localization is based on ‘tagging’ chromosomes with either *lac*-operator or *tet*-operator modules (Lau *et al*., 2003). When such green fluorescent protein (GFP) or CFP-fused repressors are expressed in cells with tagged chromosomes, a focus is observed that localizes one particular genetic region. Viollier *et al*. (2004) measured GFP or CFP-tagged repressor foci in 112 tagged strains of *C. crescentus*, scoring thousands of images for each strain. Each tagged gene moved to a post-replication position that reflected its genetic linkage to the origin and terminus of the chromosome. Such high fidelity positioning has been explained by underpinning structure with many small domains (Breier and Cozzarelli, 2004).

Third, the down side of too many domains becomes obvious in severe gyrase mutants. Strains with the *gyrB1820* allele have >1000 domains per genome equivalent. However, the phenotype is complex, including constitutive SOS induction, synthetic lethality with RecA and RecBC, and Recless DNA degradation of nascent DNA (Gari *et al*., 1996; 2001). These are all facets that suggest frequent replication fork collapse (Bidnenko *et al*., 2002; Grompone *et al*., 2003; Michel *et al*., 2004).

A complete description of chromosome structure remains elusive. A level of organization beyond a 10 kb domain pattern is suggested by several experiments. Using a system based on site-specific inversions catalysed by the phage λ Int and Xis proteins, Garcia-Russell showed that some segments of chromosome are unable to make intimate contact with each other (Garcia-Russell *et al*., 2004). In a similar study, Valens *et al*. (2004) found a distance rule for lambda excisive recombination indicating colocalization of many different regions. Bacteriophage Mu, which is 37 kb, contains a central strong gyrase site that functions in an early stage of transposition (Pato *et al*., 1990; Pato and Karlock, 1994). Evidence points to the critical step being formation of a functional synapse (Pato *et al*., 1995; Pato and Banerjee, 1996; 1999), but the detailed structure of a Mu synapse (a five-noded plectoneme; Pathania *et al*., 2002) suggests an end-pairing mechanism more complex than slithering.

Finally, the similarity in domain structure between *E. coli* and *Salmonella typhimurium* may indicate that a common set of proteins and mechanisms contribute to domain structure in two organisms separated from a common ancestor for 140 million years. However, significant differences are also apparent. The deletion of *mukB* leads to a much more severe phenotype in *Salmonella* than it does in *E. coli* (N.P. Higgins, unpublished). Deletions of *hupA* and *hupB* in *Salmonella* have different effects on phage Mu biology than in *E. coli* (Hillyard *et al*., 1990) and specific gyrase mutations that are viable in *Salmonella* are lethal in *E. coli* (K.M. Champion and N.P. Higgins, unpublished data) Developing a model of how the small DNA-binding proteins like HU, H-NS, IHF, STPA, FIS, the ATP-requiring type II topoisomerases, the type I topoisomerases and the cohensin-related proteins organize bacterial chromatin remains a tough challenge, but the comparative approach involving *E. coli* and *Salmonella* promises to be very useful.

Experimental procedures

Bacterial strains

Derivatives of *S. typhimurium* LT2 used in the study are described in Table 4. Gene transduction with bacteriophage P22 was performed as previously described (Higgins *et al*., 1996), and single colonies were re-streaked on green plates (Smith and Levine, 1967) to identify phage-free transductants.

Plasmid construction

Plasmid pJBRes*cI* was originally derived from pACYC184 (Bliska and Cozzarelli, 1987). In this plasmid, the *tnpR* gene encoding Tn*3* resolvase is regulated by a temperature-sensitive (Ts) λ repressor, *cI857*. Due to a strain transfer recording error, we believed that we were using a plasmid with a copy of γδ resolvase (see Fig. 1). At permissive temperature (30°C) resolvase transcription is tightly repressed. Incubation at 42°C for 10 min results in reversible denaturation of the repressor (Gaitanaris *et al*., 1990) and a burst of the resolvase protein expression. Plasmid pJBRes*cI* was digested with *Bam*HI and *Bsa*HI to remove a 600 bp fragment containing the Tn*3 tnpR* gene. Digested plasmid was subjected to electrophoresis on a 1% agarose gel, and after extraction followed by nucleotide end-filling with T7 DNA polymerase (Promega), the product was blunt-end ligated to create the empty vector pJB.

The γδ *tnpR* gene was amplified from the F factor by carrying out PCRs using oligonucleotides RS01 (5′-GCTAGCCTTGGATCCGATAATAAAGAAGGAGATATCATAT GCGACTTTTTGGTTACGC-3′) and RS02 (5′-GTCCGCATCGGATCCGCTAACGTAATTAGTTGCTTTCATT-3′). PCR products were precipitated with ethanol, resuspended in enzyme buffer, digested with *Bam*HI (which cuts in the underlined sequence shown above), ligated to *Bam*HI-linearized pJB and transformed into chemically competent Top 10 *E. coli* cells (Invitrogen). Individual clones were sequenced to

confirm proper orientation and sequence and then introduced into the restriction-defective *S. typhimurium* strain NH0185 by electroporation. Plasmids isolated from NH0185 can be efficiently moved into any *S. typhimurium* strain derived from LT2.

To create pγδRes-SsrA, the γδ *tnpR* gene was first amplified with oligonucleotides RS03 (ATGCGACTTTTTGGTTA CGC) and RS04 (TTAAGCTGCTAAAGCGTAGTTTT*CGTC GTTTGCTGCGTTGCTTTCATTTATTAC*) where the segment that encodes the *ssrA* tag is italicized. The product was cloned into the TopoTA vector, yielding TopoTA-*tnpR-ssrA* plasmid encoding a resolvase tagged at its C-terminus with the signal for rapid degradation by the ClpXP proteosome (Levchenko *et al*., 2000; Joshi *et al*., 2004). Oligonucleotides RS01 and RS05 (5′-GTCCGCATCGGATCCGCTAACGTAAT TAAGCTGCTAAAGCGTAGTT-3′) were then used to PCR amplify a 580 bp *tnpR-ssrA* DNA fragment with *Bam*HI cleavage sites (underlined) at both ends. After *Bam*HI digestion and gel purification (Qiagen), the PCR product was ligated into pJB vector linearized with *Bam*HI. Clones were screened for inserts in the correct orientation and then subjected to sequence analysis to confirm the expected sequence.

To make pγδRes-SsrA-L9D, the *tnpR-ssrA* fragment was amplified from the Topo-tnpR-ssrA template by using oligonucleotides RS01 and RS06 (5′-GTCCGCATCGGAT CCGCTAACGTAATTAAGCTGC**GTC**AGCGTAGTT-3′) (L9D replacement indicated with bold characters). pγδRes-SsrA-A8D was generated with oligonucleotides RS01 and RS07 (5′-GTCCGCATCGGATCCGCTAACGTAATTAAGCTGCTAA**GTC**GTAGTTTTC-3′) using the strategy described above. To make a plasmid that is immune to ClpXP degradation, the same procedure was carried out with RS08 (5′-GTCCG CATCGGATCCGCTAACG TAATTA**GTCGTC**TTAAAGCGTA GTTTTC-3′) where the A10A11 and D10D11 mutations are indicated by bold type. DNA sequence analysis confirmed that each new encoded proper C-terminal tags: γδ-*ssrA* gene encoded AANDENYALAA, γδ-*ssrA-L9D* encoded AANDEN YA**D**AA, γδ-*ssrA-A8D* encoded AANDENY**D**LAA and γδ-*ssrA-DD* encoded AANDENYAL**DD.**

Media

Bacteria were grown in complex LB medium (5 g of NaCl, 10 g of bactotryptone, 5 g of yeast extract per litre). Plates used for recombination assays were prepared by using NCE salts (Davis *et al*., 1980) and casamino acids at 0.2% with X-Gal at a final concentration of 40 μg ml^{-1}. Antibiotic concentrations were 50 μg ml^{-1} for kanamycin, 20 μg ml^{-1} for chloramphenicol, 15 μg ml^{-1} for gentamicin and 10 μg ml^{-1} for tetracycline.

Polymerase chain reaction (PCR)

Recombinant Taq DNA polymerase and DNA extender (Fisher) were used in an equimolar ratio in thermocycling reactions. Reactions were performed in an air thermocycler (Idaho Technologies) (15 s denaturation at 94°C, 15 s annealing at 55°C, 45 s synthesis at 72°C, 30 cycles, S = 6).

Resolution assays

Recombination assays were performed as described previously (Higgins *et al*., 1996) with one modification to decrease the number of sectored colonies. Fresh colonies from an LB plate containing chloramphenicol, kanamycin and gentamicin were inoculated in 2 ml of LB medium containing chloram-phenicol and kanamycin and incubated with shaking at 30°C overnight. The next morning, 100 μl of aliquots were inoculated into 10 ml of LB with chloramphenicol and kanamycin in 125 ml Nephlo culture flasks (Bellco Biotechnology) and grown with shaking at 30°C. When cultures reached Klett 50, 500 μl were placed into a sterile glass tube and incubated for 10 min in a 42°C shaking water bath. Twenty microlitres of each induced

and uninduced culture were added to 180 μl of LB and incubated at 30°C overnight in 96-well microtitre plates (Greiner BioOne). The next day, serial 10-fold dilutions were made and cells from the 10^{-4} and 10^{-5} dilution were spread on plates containing chloramphenicol and X-Gal. To score resolution frequencies, the recombinant (white) and non-recombinant (blue) colonies were counted after plates had been incubated for several days at 30°C.

Measuring resolvase half-life

The half-life of different resolvase derivatives was measured using pRR51, a 5.9 kb plasmid with two directly repeated *res* sites flanking a gene encoding Tet resistance plus an Amp resistance gene linked to the replication origin (Reed, 1981). Strain NH3568 contains the compatible plasmid pJBRes*cI* encoding Tn*3* resolvase. NH3568 was grown in LB with chloramphenicol at 30°C and at a density of Klett 50 (mid-log phase), the culture was shifted to 42°C for 10 min and then returned to 30°C. Aliquots of thermoinduced bacterial cultures were quickly (within 2 min) pelleted three times, resuspended in 10% ice-cold glycerol, and then plasmid pRR51 was introduced by electroporation. Culture was spread on LB plates with chloramphenicol and ampicillin and incubated overnight (without shaking) at 30°C. The next day, 500 single colonies for each time point were picked and patched on LB Tet-plates to measure the fraction of recombinant (Tet-sensitive) colonies.

Western blots

To measure γδRes-SsrA protein stability, strain NH3546 was grown at 30°C to mid-log phase (Klett 50) then shifted to 42°C for 10 min. Immediately after induction, a 1.5 ml sample was removed, centrifuged for 20 s and the pellet was fast-frozen in a dry ice-ethanol bath. The induced culture was placed back to 30°C and samples were similarly removed after different incubation times, pelleted and snap frozen. Frozen pellets were resuspended in 50 μl of SDS sample buffer and boiled for 10 min in a water bath and centrifuged to remove insoluble cell debris. Independent samples (15 μl) were applied to a 12% SDS-PAGE gel and the protein bands were transferred to a PVDF membrane as previously described (Maniatis *et al*., 1982). PVDF membranes were processed by using the Western Breeze Chemoluminescence Detection Kit (Invitrogen) according to manufacturer's instructions. Polyclonal antibodies against the *ssrA* tag, a gift from Tania Baker, were diluted to 1:1000 before use.

Gyrase mutants

The pRR51 reporter plasmid was used to examine the persistence of the various resolvases in the presence of the various gyrase alleles used in this study. WT LT2 and strains harbouring the *gyrA 209*, *gyrA 213*, *gyrB 1820* or *gyrA 205* alleles were transformed with the pRR51 reporter plasmid as well as the plasmid encoding various half-life resolvases (Table 4). Single colonies were grown up to stationary phase at 30°C overnight and the next morning cultures were diluted 1:100 and grown to Klett 50. After heat induction at 42°C for 10 min, plasmids were isolated from 1 ml of cultures (Promega), electroporated into LT2 strains and the cultures were then grown for several hours at 30°C and plated on Amp plates. The next day, individual colonies were patched on Amp and Tet plates and the deletion efficiencies were calculated (Table 3). Three independent experiments were performed for each strain.

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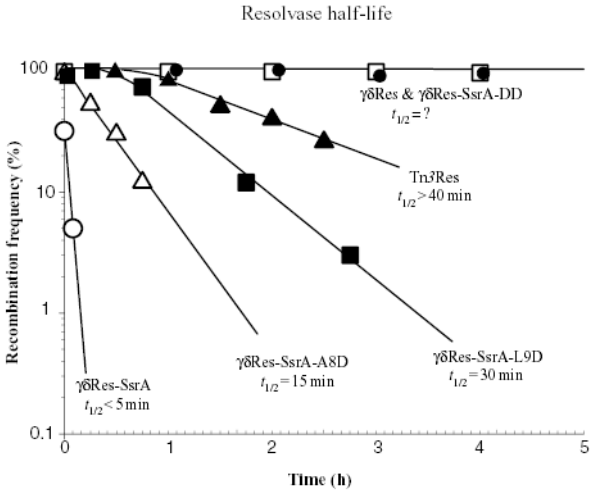

Fig. 1.

Half-life ($t_{1/2}$) measurement of modified resolvases. Strains with plasmids expressing different cloned resolvase proteins were grown to mid-log phase (Klett 50) at 30°C, induced at 42°C for 10 min, returned to 30°C and samples were subsequently periodically harvested by centrifugation. After cells were resuspended in 10% ice-cold glycerol, plasmid pRR51 was introduced by electroporation. All experiments were performed in triplicate with at least 300 bacterial colonies examined in each test to determine one standard deviation.

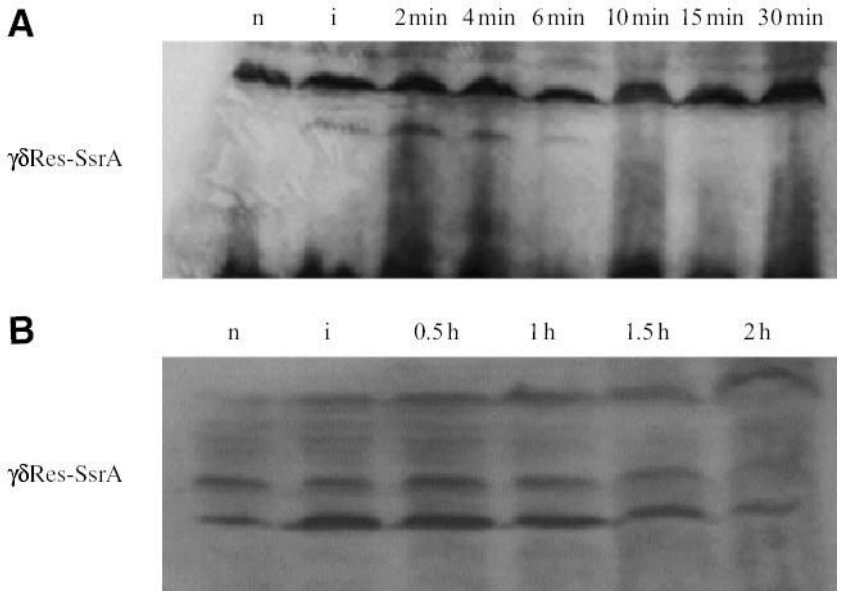

Fig. 2.

Western blot analysis of resolvase stability. Bacterial cultures (1.5 ml) in mid-log phase (Klett 50) were harvested by centrifugation and lysed in a final volume of 50 μl. Clarified lysate (15 μl) from each preparation was loaded on the gel. Anti-SsrA antibodies identify a band at the predicted size. A non-specific band of higher molecular weight served as the loading control.

A. γδRes-SsrA is a short-lived resolvase. The protein is detected at induction (lane 2) and 2 and 4 min after induction (lanes 3 and 4 respectively). After the 6 min post-induction point (lane 5) the protein is at or below detection limits (lanes 6, 7 and 8). The lanes are: n, non-induced; i, induction; 2 min, 4 min, 6 min, 10 min, 15 min and 30 min are post-induction time points. Sample preparation is described under *Experimental procedures*.

B. In a *clpP*$^{-}$ background (*clpP* replaced with a *kan* gene), γδRes-SsrA becomes a very stable protein, detectable for hours after heat induction. The lanes are: n, non-induced; i, induced; 0.5 h, 1 h, 1.5 h and 2 h are post-induction time points. Sample preparation is described under *Experimental procedures*.

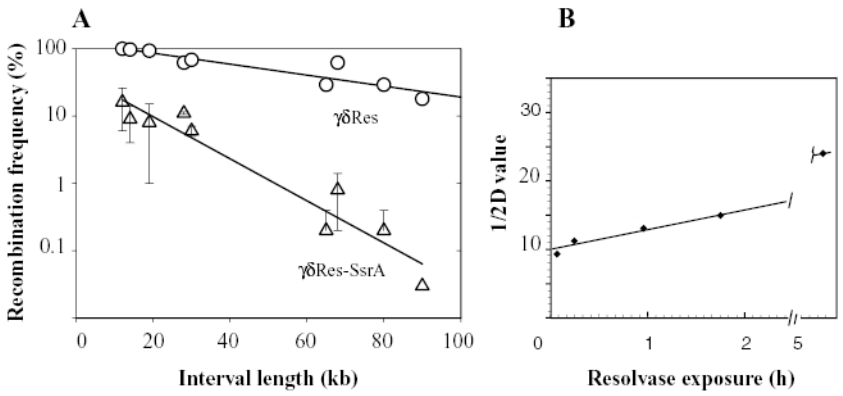

Fig. 3.

A. Resolution assays for nine intervals ranging in size from 12 to 90 kb in the *his-cob* region. Three independent experiments were averaged for each time point, and at least 300 colonies were counted for each time point. The distance penalty for recombination (the slope of the curve) is much lower for the WT γδ resolvase than for the short-lived, *ssrA*-tagged resolvase. The half-distance value calculated for the wild-type resolvase was 36 kb, and for the *ssrA*-tagged resolvase was 9 kb.

B. The bacterial chromosome changes over time. To illustrate the change, the first-order decay constants for different resolvases in Table 2 were plotted against the time that each enzyme persists in the cell. The resolvase exposure time is defined as the period it takes each type of resolvase to decay so that the resolution activity is half the maximum amount. Note that this value is not equivalent to the half-life for stable enzymes because saturating levels of resolvase persist for periods longer than the cell division time. The enzymes arranged in increasing exposure levels are γδRes-SsrA (1/2D = 9 kb), γδRes-SsrA-A8D (1/2D = 12 kb), γδRes-SsrA-L9D (1/2D = 13 kb), Tn*3* Res (1/2D = 15 kb) and γδRes-SsrA-DD (1/2D = 23 kb). Data for the γδRes protein (1/2D = 36 kb) is not included because it remains at saturating levels for more than 6 h after induction.

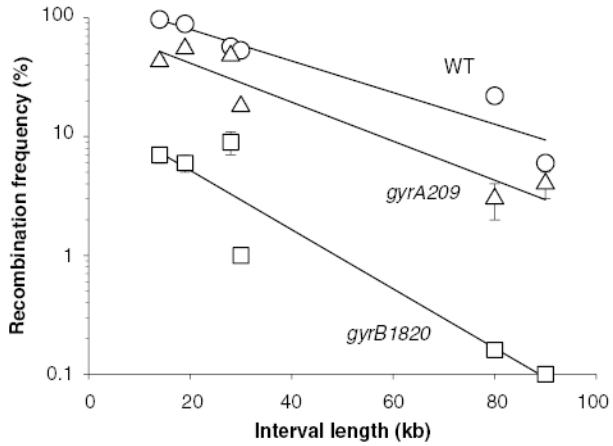

Fig. 4.

Resolution assays with the γδRes-SsrA-DD protein in the presence of wild-type (WT) gyrase (open circles) *gyrA209* (open triangles) and *gyrB1820* (open squares). The 1/2D values for recombination calculated for each curve were: 23 kb for the WT strain, 18 kb for a strain with *gyrA209* and 12 kb for a strain with the *gyrB1820* gene.

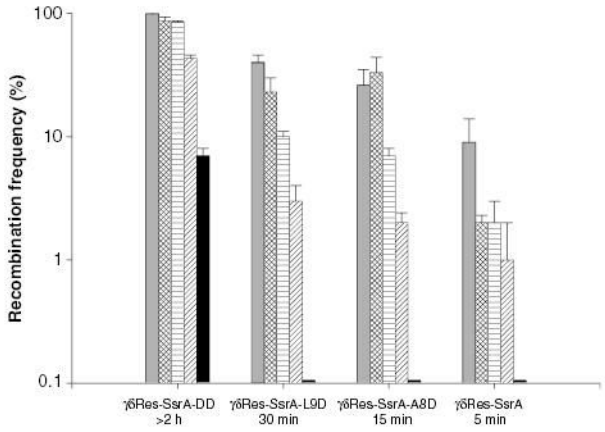

Fig. 5.

Resolution efficiency of a 14 kb domain in strains carrying different DNA gyrase mutations. A 14 kb interval was examined with resolvases having the indicated half-life. The recombination efficiency of each resolvase is reported for: wild-type gyrase, grey bars; *gyrA205*, hatched bars; *gyrA213*, horizontally striped bars; *gyrA209*, diagonally striped bars, and *gyrB1820*, black bars.

Table 1

Recombination activity of the γδRes-SsrA in a wild-type *Salmonella typhimurium* strain (column 2) and in a strain harbouring a *clpP::kan* mutation (column 3).

Time point	LT2/pγδRes-SsrA	LT2, *clpP::kan*/pγδRes-SsrA	LT2/pγδRes
TP_0 (induction)	32%	100%	100%
TP1 (1 h)	<1%	100%	99%
TP2 (2 h)	<1%	48 ± 6	99%
TP3 (3 h)	<1%	35 ± 8	98%
TP4 (4 h)	<1%	67 ± 20	98%

The *clpP::kan* mutation eliminates the ClpP subunit that is part of both the ClpAP and the ClpXP proteosome systems. As a control, recombination data with the wild-type resolvase are presented (column 4).

Table 2

Log-phase resolution data for nine intervals in the *his-cob* region (43–45 min) of the *Salmonella typhimurium* chromosome.

Interval size (kb)	γδRes A1/2 > 4 h	γδRes-SsrA-DD A1/2 > 4 h	Tn*3* Res A1/2 = 2 h	γδRes-SsrA-L9D A1/2 = 1 h	γδRes-SsrA-A8D A1/2 = 15 m	γδRes-SsrA A1/2 < 5 m
12	99 ± 1	97 ± 1	81 ± 7	35 ± 4	39 ± 10	16 ± 10
14	97 ± 1	99 ± 1	81 ± 10	40 ± 6	26 ± 9	9 ± 5
19	93 ± 1	89 ± 6	49 ± 17	19 ± 3	18 ± 10	7 ± 7
28	62 ± 2	57 ± 3	44 ± 11	17 ± 3	14 ± 15	10 ± 1
30	68 ± 6	53 ± 6	36 ± 14	24 ± 3	21 ± 4	6 ± 1
65	29 ± 5	16 ± 2	4 ± 3	3 ± 1	1 ± 1.4	0.2 ± 0.2
68	62 ± 7	30 ± 2	14 ± 3	3 ± 1	4 ± 2	0.8 ± 0.6
80	29 ± 5	22 ± 3	1 ± 1	1 ± 1	1 ± 1	<0.1
90	18 ± 1	6 ± 1	2 ± 2	1 ± 1	0.3	<0.1

Recombination assays from at least three independent experiments were used to calculate a mean with one standard deviation. The A1/2 represents the time required for cellular resolution activity to fall to half-maximum values. Note that this does not reflect a half-life for the long-lived enzymes because cells remain saturated for resolvase for different lengths of time before a first-order decay can be detected.

Table 3

Efficiency of time-sensitive resolvases in gyrase mutants.

	γδRes-SsrA-DD	γδRes-SsrA-L9D	γδRes-SsrA-A8D	γδRes-SsrA
Wild-type gyrase	99	99	99	52 ± 19
gyrA205	98	99	99	19 ± 10
gyrA213	99	96 ± 2	95 ± 2	6 ± 4
gyrA209	98	68 ± 8	76 ± 22	7 ± 1
gyrB1820	80 ± 10	64 ± 4	69 ± 26	7 ± 1

Table 4

Bacterial strains used in this study.

Strain	Interval size (kb)	Genotype	Plasmid
NH0185		*hsd*L6 *hsd*SA29(r_{LT}^- ml_T^+ r_s^- m_s^+) *metA22 metE551 ilv-452 trpB2 xyl404 rpsL*120(Str^r) *galE*496	
NH3568		LT2	pJBRes*cI* (Tn*3*)
NH3645		LT2	pγδRes
NH3546		LT2	pγδRes-SsrA
NH3578		LT2	pγδRes-SsrA-A8D
NH3643		LT2	pγδRes-SsrA-DD
NH3644		LT2	pγδRes-SsrA-L9D
NH3566		*clpP::kan*	pγδRes-SsrA
NH3569		*clpP::kan*	pJBRes*cI*
NH2124	12	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobU713*::Tn*10d*Gn	pJBRes*cI*
NH3618	12	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobU713*::Tn*10d*Gn	pγδRes
NH3561	12	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobU713*::Tn*10d*Gn	pγδRes-SsrA
NH3580	12	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobU713*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3573	12	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobU713*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3592	12	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobU713*::Tn*10d*Gn	pγδRes-SsrA-DD
NH2118	14	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobP712*::Tn*10d*Gn	pJBRes*cI*
NH3617	14	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobP712*::Tn*10d*Gn	pγδRes
NH3638	14	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobP712*::Tn*10d*Gn	pγδRes-SsrA
NH3552	14	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobP712*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3572	14	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobP712*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3594	14	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cobP712*::Tn*10d*Gn	pγδRes-SsrA-DD
NH2142	19	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *cobJ709*::Tn*10d*Gn	pJBRes*cI*
NH3616	19	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *cobJ709*::Tn*10d*Gn	pγδRes
NH3560	19	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *cobJ709*::Tn*10d*Gn	pγδRes-SsrA
NH3579	19	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *cobJ709*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3577	19	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *cobJ709*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3587	19	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *cobJ709*::Tn*10d*Gn	pγδRes-SsrA-DD
NH2119	28	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cob-708*::Tn*10d*Gn	pJBRes*cI*
NH3614	28	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cob-708*::Tn*10d*Gn	pγδRes
NH3556	28	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cob-708*::Tn*10d*Gn	pγδRes-SsrA
NH3551	28	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cob-708*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3575	28	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cob-708*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3588	28	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *cob-708*::Tn*10d*Gn	pγδRes-SsrA-DD
NH3590	30	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *pduF358*::Tn*10d*Gn	pγδRes-SsrA-DD
NH3642	30	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *pduF358*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3606	30	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *pduF358*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3621	30	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *pduF358*::Tn*10d*Gn	pγδRes
NH3639	30	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *pduF358*::Tn*10d*Gn	pγδRes-SsrA
NH2075	30	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *pduF358*::Tn*10d*Gn	pJBRes*cI*
NH2453	65	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *zec-8253*::Tn*10d*Gn	pJBRes*cI*
NH3613	65	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *zec-8253*::Tn*10d*Gn	pγδRes
NH3557	65	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *zec-8253*::Tn*10d*Gn	pγδRes-SsrA
NH3558	65	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *zec-8253*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3574	65	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *zec-8253*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3591	65	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *zec-8253*::Tn*10d*Gn	pγδRes-SsrA-DD
NH2223	68	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *phs-209*::Tn*10d*Gn	pJBRes *cI*
NH3619	68	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *phs-209*::Tn*10d*Gn	pγδRes
NH3640	68	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *phs-209*::Tn*10d*Gn	pγδRes-SsrA
NH3549	68	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *phs-209*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3571	68	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *phs-209*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3589	68	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *phs-209*::Tn*10d*Gn	pγδRes-SsrA-DD
NH2149	80	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *hisD10201*::Tn*10d*Gn	pJBRes*cI*

Strain	Interval size (kb)	Genotype	Plasmid
NH3620	80	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *hisD10201*::Tn*10d*Gn	pγδRes
NH3641	80	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *hisD10201*::Tn*10d*Gn	pγδRes-SsrA
NH3555	80	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *hisD10201*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3570	80	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *hisD10201*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3593	80	*cobT714*::MudJr2 *zea-3777*::Tn*10d*Tc *hisD10201*::Tn*10d*Gn	pγδRes-SsrA-DD
NH2226	90	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *zee-8251*::Tn*10d*Gn	pJBRes*cI*
NH3615	90	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *zee-8251*::Tn*10d*Gn	pγδRes
NH3559	90	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *zee-8251*::Tn*10d*Gn	pγδRes-SsrA
NH3554	90	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *zee-8251*::Tn*10d*Gn	pγδRes-SsrA-A8D
NH3576	90	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *zee-8251*::Tn*10d*Gn	pγδRes-SsrA-L9D
NH3586	90	*cobT714*::MudJr1 *zea-3777*::Tn*10d*Tc *zee-8251*::Tn*10d*Gn	pγδRes-SsrA-DD
NH3622	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA209*^{ts}	pγδRes-SsrA
NH3623	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA209*^{ts}	pγδRes-SsrA-A8D
NH3624	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA209*^{ts}	pγδRes-SsrA-L9D
NH3625	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA209*^{ts}	pγδRes-SsrA-DD
NH3626	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zib-748*::Tn*10 gyrB1820*^{ts}	pγδRes-SsrA
NH3627	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zib-748*::Tn*10 gyrB1820*^{ts}	pγδRes-SsrA-A8D
NH3628	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zib-748*::Tn*10 gyrB1820*^{ts}	pγδRes-SsrA-L9D
NH3629	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zib-748*::Tn*10 gyrB1820*^{ts}	pγδRes-SsrA-DD
NH3630	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA205*^{ts}	pγδRes-SsrA
NH3631	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA205*^{ts}	pγδRes-SsrA-A8D
NH3632	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA205*^{ts}	pγδRes-SsrA-L9D
NH3633	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA205*^{ts}	pγδRes-SsrA-DD
NH3634	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA213*^{ts}	pγδRes-SsrA
NH3635	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA213*^{ts}	pγδRes-SsrA-A8D
NH3636	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA213*^{ts}	pγδRes-SsrA-L9D
NH3637	14	*cobT714*::MudJr2, *cobP712*::Tn*10d*Gn *zeh-754*::Tn*10 gyrA213*^{ts}	pγδRes-SsrA-DD