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REPORT

Construction of consecutive deletions of the *Escherichia coli* chromosome

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The minimal set of genetic information necessary and sufficient to sustain a functioning cell contains not only *trans*-acting genes, but also *cis*-acting chromosomal regions that cannot be complemented by plasmids carrying these regions. In *Escherichia coli* (*E. coli*), only one chromosomal region, the origin of replication has been identified to be *cis*-acting. We constructed a series of mutants with long-range deletions, and the chromosomal regions containing *trans*-acting essential genes were deleted in the presence of plasmids complementing the deleted genes. The deleted regions cover all regions of the chromosome except for the origin and terminus of replication. The terminus affects cell growth, but is not essential. Our results indicate that the origin of DNA replication is the only vital, unique *cis*-acting DNA sequence in the *E. coli* chromosome necessary for survival.
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Introduction

The experimental identification of essential genes has been carried out in some bacteria and yeast (Akerley *et al*, 2002; Forsyth *et al*, 2002; Giaever *et al*, 2002; Gerdes *et al*, 2003; Kobayashi *et al*, 2003; Baba *et al*, 2006; Glass *et al*, 2006). The methods often used to disrupt genes to determine whether they are essential or not included transposon mutagenesis and targeted disruption by homologous recombination. Using transposon mutagenesis, whole regions of chromosomes can be examined; however, the results are inconclusive, because not all regions are inactivated by random insertion. Targeted disruption, which can identify essential genes expressed in the diploid stage or expressed conditionally, is a suitable method to show whether annotated genes are essential. But intergenic regions have not been investigated. Although most essential *trans*-acting genes have been identified through gene disruption studies, the necessity of the intergenic regions has not been sufficiently clarified. For example, some genetic information in the intergenic regions is transcribed, whereas other genetic information is not. The former sites are *trans*-acting, and the latter sites are *cis*-acting.

In bacteria, chromosomes are generally uni-replicons; therefore, the origin of replication (*oriC*) is *cis*-acting and essential. In *Escherichia coli*, other *cis*-acting sites have also been reported. The *dif* is a *cis*-acting site, which is important for cell proliferation (Sherratt, 2003). This site was identified because a mutant with a very large deletion around the replication terminus (*terC*) grew slowly, and the chromosomal region responsible for this growth defect was identified and termed deletion-induced filamentation or *dif* (Kuempel *et al*, 1991). This *dif* site was eventually shown to be a site for recombination catalyzed by the XerC-XerD recombinase, which resolves chromosome multimers resulting from homologous recombination between replicated sister chromosomes. Although this site affects cell growth, its deletion leads to a relatively minor growth defect (Cui *et al*, 2007). Another *cis*-acting site, *migS*, was identified as being responsible for the polar movement of *oriC,* but this site was not essential for cell growth (Yamaichi and Niki, 2004).

To understand the essential genetic information of prokaryotic chromosomes, a genomic survey of *cis*-acting essential regions is necessary. An efficient way to identify essential factors, particularly *cis*-acting chromosome regions, is thought

to be the systematic construction of large-scale chromosomal deletions. If unique and essential *cis*-acting regions are on a chromosome, the deletion mutants of these regions are no longer viable even in the presence of complementing plasmids. Previously, we constructed long-range deletions of the *E. coli* chromosome, which led to the reduction of the genome size (Hashimoto *et al*, 2005; Kato and Hashimoto (in press)). First, we constructed 75 deletions (medium-scale deletions (MD)) in regions lacking the essential genes, which were identified through a survey of the published literature, using the *E. coli* homologous recombination system. We then constructed a second series of deletions (large-scale deletions (LD)) and combined them to construct an engineered strain lacking 29.7% of the parental chromosome. In this study, we constructed deletion mutants for other chromosomal regions, particularly those containing essential genes, to identify additional essential *cis*-acting chromosome regions, while maintaining the viability of the mutants with complementing plasmids expressing the deleted genes.

Results and discussion

First, for the chromosomal regions that were not deleted during the construction of the first MD series, the identified essential genes were cloned and the chromosomal regions containing these genes were deleted. We cloned the essential genes into mini-F plasmid vectors (Supplementary Figure 1 and Supplementary Information) either *in vitro* using restriction digestion and ligation methods or *in vivo* using the *red* recombination system (Supplementary Figure 2 and Supplementary Table I). Seven and 34 MDs were obtained in the absence and presence of the complementing plasmids, respectively. We also constructed one new LD. However, we did not succeed in constructing MD and LD deletions for the entire chromosome. Second, we investigated whether or not there were any essential *cis*-acting chromosome regions in the regions not deleted in the MD and LD. Therefore, we developed a system of moving chromosome regions into mini-F plasmids *in vivo* using the yeast FLP-FRT site-specific recombination system 1 (FLP-FRT1) (see Kato and Hashimoto, in press, for details). Figure 1 shows the improved system 2 (FLP-FRT2), which is essentially the same as FLP-FRT1. Using this system, 30 additional chromosome deletions were constructed, indicating that these regions have no essential *cis*-acting chromosome regions. Third, for these regions and the other regions that were not deleted, small-scale deletions (SD) were constructed using lambda (λ)-phage and the *red* recombination system (see Kato and Hashimoto, in press, for details.). Two hundred thirty-eight and 116 small-scale deletions (SD) were obtained using these methods in the absence and presence of complementing plasmids, respectively. Fourth, we tried to construct the deletions again for the other undeleted regions using improved systems. We obtained 5 and 15 deletions with system 2 (FLP-FRT2) and system 3 (FLP-FRT3), respectively. In total, 551 chromosome deletions mutations covering all of the *E. coli* genome, except for *oriC* and *terC* were constructed (Figures 2 and 3 and Supplementary

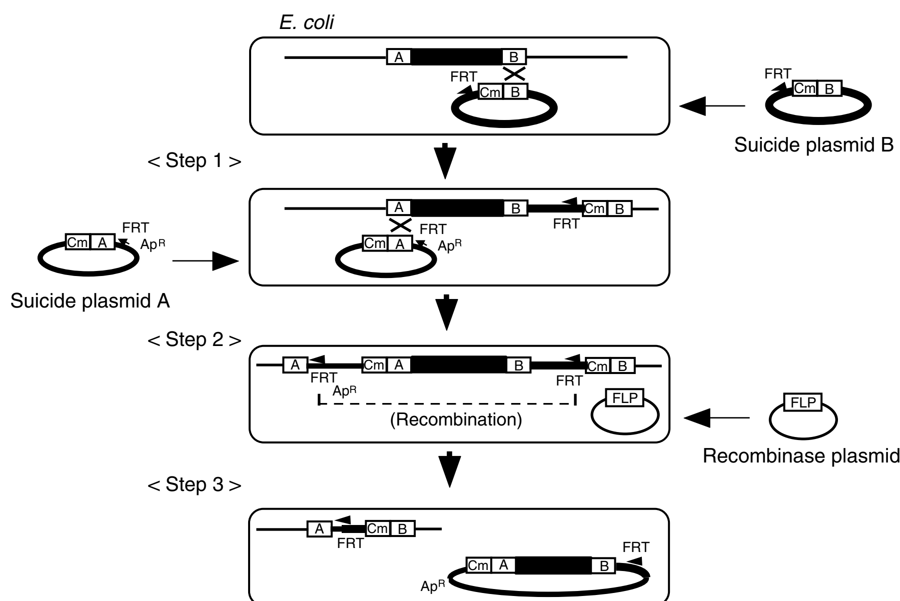

Figure 1 The FRT2 system. A schematic drawing of the transfer of a chromosomal region to a mini-F plasmid with the FRT2 system. The chromosomal region to be deleted is represented by the bold line. The two chromosomal regions (A and B) flanking the region to be deleted are cloned into two kinds of suicide vectors. One is a mini-F plasmid, which has an FRT site and is replication defective at 42°C. The other is a R6K derivative, which has an FRT site and is replication defective due to the absence the *pir* gene necessary for replication. In step 1, the suicide plasmid carrying B is introduced into an *E. coli* strain. The chloramphenicol-resistant (Cm^R) colonies are isolated, representing step 1 recombinants in which the plasmid is integrated into the chromosome by homologous recombination. Next, the other suicide plasmid carrying A is introduced into step 1 recombinants, and the ampicillin-resistant (Ap^R) transformants are obtained at 42°C, representing step 2 recombinants. To inhibit homologous recombination beyond this stage, *recA* is disrupted by P1 transduction. In step 3, the FLP-plasmid, which is replication defective at 35°C, is introduced into step 2 recombinants, and the expression of the FLP recombinase is induced, resulting in simultaneous plasmid excision and chromosome deletion. To obtain a strain that did not carry the FLP-plasmid, cells were incubated at 35°C, at which point the FLP-plasmid does not replicate, but the miniF ts replicon remains functional.

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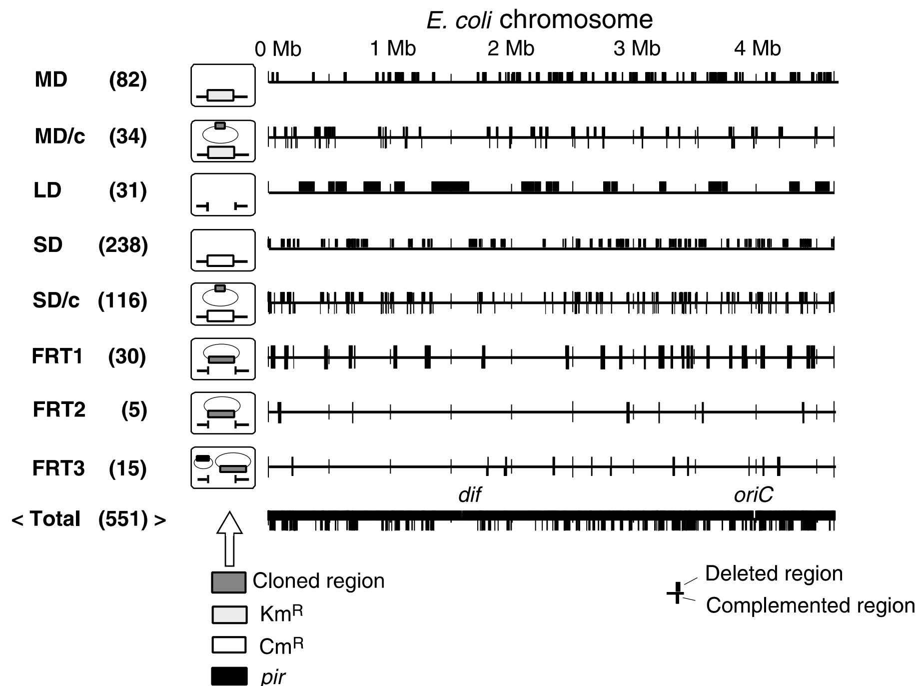

Figure 2 Summary of the *E. coli* chromosome deletions. Deleted and complemented regions are drawn using a linear chromosome map. Horizontal lines represent chromosomes, and upper boxes and lower boxes indicate deleted and complemented regions, respectively. MD, LD, SD, and FRT represent the systems used to construct the deletions and c indicates the presence of a complementing plasmid. Numbers in parentheses indicate the number of deletion mutations constructed using each system. Depictions of cells containing deletion mutations are provided to show markers inserted into the deleted chromosome regions and plasmids. For details and methods, see Supplementary information; Hashimoto *et al*, 2005, Kato and Hashimoto, in press.

Table I; see the profiling of *E. coli* chromosome (PEC) database (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp) for details).

The *terC* region does not contain a site that affects cell growth, other than *dif* (Kuempel *et al*, 1991); therefore, the results of this work indicate that there are no unique, *cis*-acting, and essential regions other than *oriC*. Eukaryotic chromosomes are multireplicons, and thus each origin of replication is not necessarily essential. Apart from the origin of replication, other *cis*-acting chromosome regions in eukaryotic cells include telomeric sequences, which are necessary for chromosome maintenance and centromeric regions, which are required for stable segregation of eukaryotic chromosomes. The centromere is a unique region in each chromosome: in theory, two centromeres on one chromosome can pull apart the chromosomal DNA between two daughter cells during mitosis (Mann and Davis, 1983). In prokaryotic cells, the mechanism underlying bacterial chromosome segregation is not understood. So far, a prokaryotic centromere has not been identified and it is not known if one exists. Low-copy number bacterial plasmids have their own partition systems, in which a *cis*-acting DNA region plays an essential role (Hayes and Barilla, 2006). But unlike eukaryotic centromeres, a plasmid carrying two copies of the *cis*-acting sequence is structurally stable. It is not known whether a eukaryote-like centromere functions in chromosome segregation in prokaryotes. Here, we did not identify any *cis*-acting and essential sites other than *oriC*. Because *oriC* and *dif* regions are not thought to contain a site for chromosome stability (Kogoma and Meyenburg, 1983; Ogura and Hiraga, 1983; Tecklenburg *et al*, 1995), our results suggest that a potential *cis*-acting site for chromosome segregation may be dispensable or redundant. Alternatively, prokaryotic cell sequences equivalent to the eukaryotic centromere may not exist. Global reorganization of chromosomes triggered by a loss of this cohesion resembles eukaryotic prometaphase (Sunako *et al*, 2001; Bates and Kleckner, 2005). It is suggested that the bacterial mechanism of chromosome segregation is a primordial one to which microtubule-based processes were added later.

Our results also show that all of the *trans*-acting essential genes were cloned on the complementing plasmids; however, the cloned genes (501 genes) are not necessarily essential. Baba *et al* (2006) reported 303 *trans*-acting essential genes by targeted disruption, but 35 of them were not cloned on our complementing plasmids, indicating that these genes are nonessential ('Class A' in the Supplementary Table II). The discrepancy between the two studies may be due to a difference in the strains and culture conditions used. For example, the culture media Antibiotic medium 3 (this work) contains a glucose, but LB (Baba *et al*, 2006) does not. Alternatively, it may be ascribed to the difference between a single gene knockout (Baba *et al*, 2006) and a large-scale deletion of genes (this work). For example, the anti-toxin genes *yefM* and *chpR*, which were identified as essential genes of the toxin-antitoxin system (Baba *et al*, 2006), were deleted with the toxin genes *yoeB* and *mazF*, respectively, in our study. In addition, the *dicA* gene encoding a repressor of a cell division inhibitor was deleted in our study with the *dicB*, the inhibitor gene, whereas the *dicA* gene disruptant was not obtained in a previous study (Baba *et al*, 2006). Furthermore, we identified 25 genes ('Class B') and 15 genes encoding small RNA

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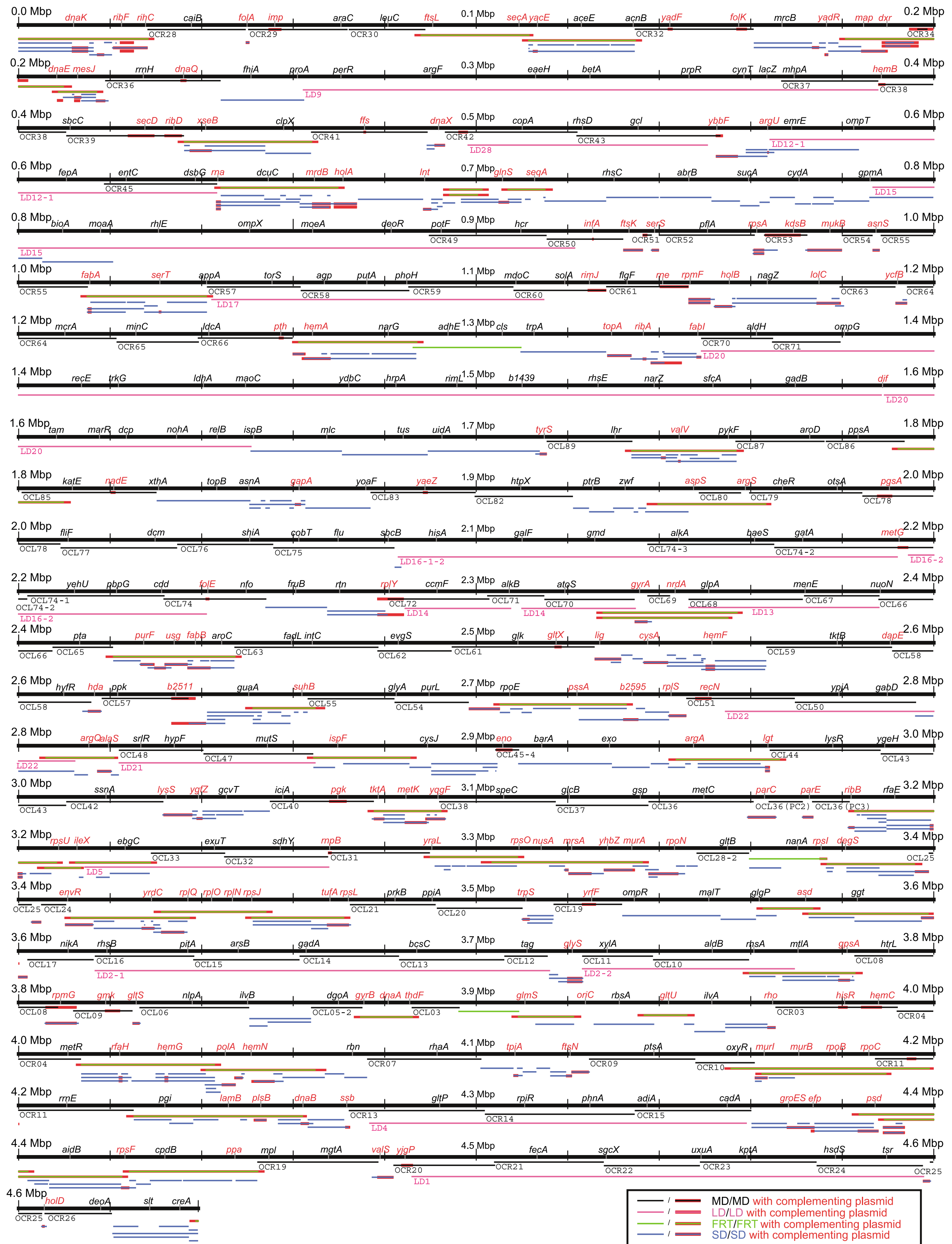

Figure 3 *E. coli* chromosome deletions. Deleted and complemented regions of each deletion mutant are shown using a linear chromosome map. Thick black horizontal lines indicate the chromosomes. Thin colored lines show the deleted and complemented regions. These colors are different due to the types of the deletion mutants and are indicated at the bottom of the Figure. The names of MD and LD deletions are shown below the thin lines. The gene names are shown above the line as a reference for the map locations. The black and red gene names indicate genes that are deleted and complemented, respectively. Details of deletions are shown in the profiling of *E. coli* chromosome (PEC) database (http://www.shigen.nig.ac.jp/ecoli/pec/index.jsp).

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('Class C') as essential genes and 2 genes as nonessential genes, which were determined from the results of our gene disruption experiments (J Kato, unpublished data, 2006) and other reports (We listed the relevant PMID number in the Supplementary Table II). In total, we identified 303 essential genes (Supplementary Table II). Sequence comparison of these essential genes with those of *Bacillus subtilis* (271 genes) revealed that 177 were conserved between these two genera (Supplementary Table II; Kobayashi *et al*, 2003). When functionally classified, genes involved in translation, protein translocation, and lipid synthesis were well conserved, whereas those involved in cell wall and membrane synthesis were not (Supplementary Table II), which may reflect structural differences in the cell wall and membrane.

In our study, 50 chromosome regions were moved to a mini-F plasmid using the FLP-FRT systems. Forty-six of them were found to contain essential gene(s), whereas 2 regions had no essential genes and were deleted without complementing plasmids. The other two regions (OCL30 and OCL34) did not contain any essential genes, but these regions were essential and therefore were not deleted without the complementing plasmids. In these regions, there may be the functionally redundant genes; one of which may be at least essential. Thirty chromosome regions were first moved to a mini-F plasmid using system 1 (FLP-FRT1). When we tried to move the other 20 regions to the plasmids using the improved system 2, (FLP-FRT2), 5 regions were moved, but the other 15 regions were not. For the regions affected by these deletions, the chromosomes and plasmids of the strains obtained at each step of system 2 (FLP-FRT2) were analyzed. The results indicated that the chromosomal regions flanked by two FRT sites had been excised after induction of the FLP recombinase, but the resultant plasmids were not stably maintained even in the wild-type strain using the mini-F temperature-sensitive replicon. Analyses of some of the excised plasmids suggested a rearrangement of the plasmid structure (data not shown). Unexpected recombination between the cloned chromosomal regions and a mini-F plasmid may cause instability of the excised plasmids. Therefore, we developed an improved system, termed system 3 (FLP-FRT3), in which the excised chromosome region was maintained by an R6K replicon. This finally allowed us to construct the remaining 15 deletion mutations.

Developments in synthetic biology have made it possible to reduce the size of the genome of *E. coli* K-12 (Kolisnychenko *et al*, 2002; Yu *et al*, 2002; Hashimoto *et al*, 2005; Posfai *et al*, 2006), and recent work indicates that genome reduction can have unanticipated benefits (Posfai *et al*, 2006). To further engineer *E. coli* and to make useful improvements for industry and therapeutics, such as facilitating the production of metabolites and proteins, it is important to understand both the *cis*- and *trans*-acting essential genetic information. Further analyses are necessary to experimentally clarify the minimal set of genetic information necessary and sufficient to sustain a functioning cell.

Materials and methods

Strains and media

All *E. coli* strains used are derivatives of MG1655. The MD series was constructed in MG1655 *rpsL polA12*. The LD and SD deletion series were built using MG1655 rsh3 (*red:tet* (Δ(*recC ptr recB recD*)::*Plac-red*) *rpsL hsdR*:Ap), which was constructed using KM22 (Murphy, 1998). MG1655 *rpsL* was used to combine LD deletion units. Antibiotic Medium 3 (Becton Dickinson, USA) was used for all experiments except for those involving *sacB* selection, for which LB (-N) Suc was used (LB broth with 10% sucrose and lacking NaCl). The approximate formula per liter of the Antibiotic Medium 3 is beef extract 1.5 g, yeast extract 1.5 g, peptone 5.0 g, dextrose 1.0 g, sodium chloride 3.5 g, dipotassium phosphate 3.68 g, and monopotassium phosphate 1.32 g.

Construction of the complementing plasmids

Complementing plasmids were constructed *in vitro* or *in vivo*. *In vitro,* chromosome regions were amplified by PCR using primers flanked by restriction sites, digested with restriction enzymes, and ligated into mini-F vectors (Supplementary Figure 1 and Supplementary Information) with T4 DNA ligase. *In vivo* (Supplementary Figure 2), DNA fragments to be cloned were prepared and flanked by two DNA fragments, 'KmN' and 'mF', by two successive PCR reactions and introduced into the *E. coli* strain with the *red* gene of λ-phage and a mini-F vector, mFCm4-2. Introduced fragments were cloned into the mini-F vector by *red* recombination, resulting in kanamycin-resistant (Km^R) and chloramphenicol-sensitive (Cm^S) complementing plasmids.

MD mutant construction

The MD series was constructed with the *E. coli* homologous recombination system using ColE1-related plasmids and the *polA* mutant (Hashimoto *et al*, 2005; Kato and Hashimoto, in press). The vector 664BSCK2-4 has two positive selection markers (Cm^R and Km^R), two negative selection markers (*rpsL*$^+$ streptomycin-sensitive (Sm^S) and *sacB*$^+$), and multicloning sites flanking the Km^R marker. Both chromosomal regions flanking the targeted region were cloned into 664BSCK2-4, and the resulting plasmid introduced into MG1655 *rpsL polA12*. A Cm^R transformant, in which the plasmid was integrated by homologous recombination between the cloned region and the same region on the chromosome, was selected at 42°C. After incubation at 30°C, a Sm^R Km^R Cm^S colony, in which the plasmid was excised by another homologous recombination between the other cloned chromosomal region and the same region on the chromosome, was isolated and deletions were confirmed by PCR.

SD mutant construction

The SD system has been described previously (Kato and Hashimoto, in press). Briefly, a linear DNA fragment encoding the Cm^R gene was generated by PCR using oligonucleotide primers with a 40-base-pair region of homology to regions flanking the targeted deletion. The frequency of recombination was low using primers containing a 40-base-pair region of homology, but improved upon attachment of an approximately 1-kb region of homology to either end of the Cm^R gene. Fragments were introduced into the *E. coli* strain MG1655 *red* by electroporation and Cm^R recombinants were isolated. Deletions were confirmed by PCR analysis.

LD mutant construction

The LD series was constructed by the 'CRS cassette method' using the *red* gene-mediated λ-phage homologous recombination system and linear DNA fragments (Hashimoto *et al*, 2005; Kato and Hashimoto, in press). The CRS cassette is approximately 5 kb and bears one positive selection marker, Cm^R, and two negative selection markers, *rpsL*$^+$ (Sm^S) and *sacB*$^+$. A DNA fragment in which chromosomal regions flanking the region to be deleted were joined to the ends of the CRS cassette was introduced into MG1655 rsh3. Cm^R colonies were selected and deletions confirmed by PCR. To remove the CRS cassette, a DNA fragment in which the same flanking chromosomal regions were directly joined to each other was introduced into Cm^R colonies. Sm^R and sucrose-resistant colonies were selected and deletions confirmed by PCR.

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FLP-FRT mutant construction

The FLP-FRT2 system is shown in Figure 1 (for details, see Kato and Hashimoto, in press). In our FLP-FRT 1 prototype system, A-Km and B-Km DNA fragments, which contain the Km^R gene joined to two (A and B) chromosomal regions flanking the region to be deleted, were prepared by PCR using 664BSCK2-4 derivative plasmids used to construct MD deletions described above. The A-Km DNA fragment was inserted into a mini-F plasmid, mini-FtsFA (suicide plasmid A with a Cm^R marker), which is replication-defective at 42°C, and the B-Km DNA fragment was inserted into a R6K-related plasmid, pSG76SA (suicide plasmid B with an Ap^R marker), which lacks *pir* necessary for replication (Posfai *et al*, 1994; Kato and Hashimoto, in press). First, pSG76SA carrying B-Km was introduced into the wild-type strain MG1655 and the Km^R recombinants in which the plasmid was integrated by homologous recombination were isolated. Second, the plasmid mini-FtsFA carrying A-Km was introduced into Km^R colonies obtained as described, and Cm^R colonies were obtained. Third, *recA* was disrupted by P1 transduction to inhibit homologous recombination beyond this stage. The FLP-containing plasmid (recombinase plasmid) was introduced and recombinase expression was induced, resulting in plasmid excision and chromosome deletion. To obtain a strain lacking the FLP-plasmid, cells were incubated at 35°C, at which the FLP-plasmid does not replicate but the miniF ts replicon remains functional.

The FRT2 system introduces improvements to the FRT1 system (Kato and Hashimoto, in press). Briefly, the two chromosomal regions (A and B) flanking the targeted region were joined to the Cm^R gene to create A-Cm and B-Cm by PCR. A-Cm was inserted into mini-FtsFAK, and B-Cm into pSG76SA. pSG76SA carrying B-Cm was introduced into the wild-type strain MG1655. Cm^R colonies in which the plasmid was integrated were isolated. Next, mini-FtsFAK carrying A-Cm was introduced into the Cm^R recombinants, and the ampicillin-resistant (Ap^R) recombinants were isolated at 42°C. To inhibit homologous recombination beyond this stage, *recA* was disrupted by P1 transduction. The FLP-plasmid was introduced into Cm^R Ap^R recombinants and plasmid excision and chromosome deletion was induced. In the FRT3 system, the plasmid 184 Km *pir*, encoding a functional copy of *pir*, was co-introduced with the FLP-plasmid, and the excised plasmid was maintained with the R6K replicon in addition to the miniF ts replicon (Kato and Hashimoto, in press). In all other aspects, the FRT3 system was the same as the FRT2 system.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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