

Control of the endonuclease activity of type I restriction-modification systems is required to maintain chromosome integrity following homologous recombination

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Summary

A type I restriction-modification enzyme will bind to an unmethylated target sequence in DNA and, while still bound to the target, translocate DNA through the protein complex in both directions. DNA breakage occurs when two translocating complexes collide. However, if type I restriction-modification systems bind to unmodified target sequences within the resident bacterial chromosome, as opposed to incoming 'foreign' DNA, their activity is curtailed; a process known as restriction alleviation (RA). We have identified two genes in *Escherichia coli*, *rnhA* and *recG*, mutations in which lead to the alleviation of restriction. Induction of RA in response to these mutations is consistent with the production of unmodified target sequences following DNA synthesis associated with both homologous recombination and R-loop formation. This implies that a normal function of RA is to protect the bacterial chromosome when recombination generates unmodified products. For EcoKI, our experiments demonstrate the contribution of two pathways that serve to protect unmodified DNA in the bacterial chromosome: the primary pathway in which ClpXP degrades the restriction endonucleases and a mechanism dependent on the *lar* gene within Rac, a resident, defective prophage of *E. coli* K-12. Previously, the potential of the second pathway has only been demonstrated when expression of *lar* has been elevated. Our data identify the effect of *lar* from the repressed prophage.

Introduction

Restriction-modification (R-M) systems are widespread throughout the Eubacteria and Archaea, and of 339 sequenced genomes only 52 organisms have no detectable homologues (<http://rebase.neb.com>). R-M systems have been subdivided into different types (see Roberts *et al.*, 2003). Type I systems include the first, classical, R-M barrier described, namely EcoKI the system present in *Escherichia coli* K-12 (Bertani and Weigle, 1953). Type I R-M systems have been divided into families based historically on complementation tests and more recently on sequence similarity (see Murray, 2000). Currently, they are subdivided into four families, A, B, C and D (Titheradge *et al.*, 2001). EcoKI and its close relatives are referred to as members of the type IA family. Although type I systems were the first to be identified, type II systems may be more abundant. All classical R-M systems include two enzymatic functions active on specific nucleotide sequences: a methyltransferase that modifies the target sequences and an endonuclease that cuts DNA when a target sequence is unmodified. For type II systems, each activity is provided by a separate enzyme. In contrast, a type I R-M system is a single complex endowed with both activities. It is encoded by three genes (*hdsS*, *hdsM* and *hdsR*) and comprises five subunits (HsdS, HsdM₂ and HsdR₂). The endonuclease activity of the complex is achieved following an ATP-dependent pathway in which the R-M complex binds to an unmodified target and then 'pulls in' (translocates) the DNA from both sides simultaneously. Cleavage occurs when two translocating complexes collide (Studier and Bandyopadhyay, 1988), although alternative ways of impeding translocation can lead to cutting (Janscak *et al.*, 1999).

Restriction-modification systems may impose significant biological consequences on their hosts in terms of maintaining an intact genome. It has been shown that when *E. coli* loses a plasmid encoding the type II R-M system EcoRI, the cell dies (see Kobayashi, 2001). In this situation, as the cells grow and divide, the concentrations of restriction and modification enzymes will decrease and when unmodified targets appear in the presence of resid-

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ual endonuclease activity the bacterial chromosome becomes vulnerable. In contrast, loss of the genes that specify a type I R-M complex, such as EcoKI, is not associated with cell death (O'Neill *et al.*, 1997). Our current experiments concern the maintenance of chromosome integrity in a bacterial strain specifying a type I R-M system.

By 1984 it was well documented that DNA damage leads to the alleviation of restriction by EcoKI (Thoms and Wackernagel, 1984). This was originally detected (Bertani and Weigle, 1953), and is still quantified, by the decreased efficiency with which incoming unmodified phages are restricted. In 1999 it was shown that restriction alleviation (RA) in *E. coli* K-12 is the result of ClpXP-dependent degradation of one of the three types of subunits that make up the restriction and modification complex (Makovets *et al.*, 1999), namely, proteolysis of HsdR, the subunit that is essential for restriction but not modification. This leaves a complex of HsdS and HsdM that retains modification activity. The control of restriction activity at a post-translational level, rather than by gene expression, has the potential to provide a quick, protective response when an unmodified target sequence is generated within the bacterial chromosome (see Murray, 2000; 2002).

The effectiveness of ClpXP-mediated control over the potential self-destructive activity of EcoKI is illustrated by the demonstration that *E. coli* K-12 can survive when a mutation destroys only the modification activity of the complex. In this situation a restriction-deficient phenotype is generated following ClpXP degradation of the HsdR polypeptides. ClpXP permits survival of modification-deficient strains whether they are Rec⁺ or Rec⁻, but in its absence, RecA becomes essential (Makovets *et al.*, 1999). Experiments indicate that the HsdR subunits become susceptible to degradation when the EcoKI complex binds to an unmodified target sequence in the bacterial chromosome. Mutations in *hsdR* that block the early steps of the restriction process, i.e. the ATP-dependent binding of the complex to a target sequence and the consequent DNA translocation, remove not only the restriction activity of the complex but its vulnerability to ClpXP-dependent degradation (Doronina and Murray, 2001). These data imply that the EcoKI complex becomes vulnerable to proteolysis following the recognition of a target sequence and the initiation of translocation. Recent experiments have shown that RA for EcoR124I, a type IC R-M system, is independent of ClpXP and each of the proteases tested in earlier experiments (Makovets *et al.*, 2004).

2-Aminopurine (2-AP) has been used as a convenient means of inducing RA (Efimova *et al.*, 1988; Makovets *et al.*, 1999). Its role as a mutagen is predicted to generate new, and hence unmodified, target sequences. In the absence of ClpXP, failure to degrade the HsdR subunit of

EcoKI makes *E. coli* K-12 extremely sensitive to 2-AP (Makovets *et al.*, 1999). An *hsd^cclpX⁻* strain fails to form colonies on agar containing high (400 µg ml⁻¹) concentration of 2-AP.

We previously used sensitivity to 2-AP to search for mutants deficient in RA for strains specifying the type IC system EcoR124I. A strain was isolated and shown to have impaired RA as a consequence of a mutation in *hsdR* (Makovets *et al.*, 2004). Our present analyses now show that a second mutation in this strain contributes to 2-AP sensitivity. This second mutation is within *rnhA*, the gene specifying RNase HI, an enzyme that contributes to removal of RNA primers from Okazaki fragments (Ogawa and Okazaki, 1984). Our experiments demonstrate that mutations in *rnhA* lead to RA of wild-type EcoR124I, but when combined with the mutation in *hsdR*, which reduces RA, the strain becomes hypersensitive to 2-AP.

We find that a mutation in *rnhA* also activates RA for EcoKI, but this effect is hard to detect in the presence of the Rac prophage. This defective prophage is known to include a gene (*lar*) that can alleviate restriction when expression is enhanced (Simmon and Lederberg, 1972). Our data suggest that constitutive expression of *lar*, within the context of Rac, contributes to the protection of the bacterial chromosome.

Results

Identification of a mutation in *rnhA*

The first report of a mutation that caused a deficiency in RA for EcoR124I identified an amino acid change (A957V) in the C-terminus of the HsdR subunit. The RA-deficient strain that contained this mutation (NK445) showed severe sensitivity to 2-AP, indicative of cell death following cleavage of the chromosome by the resident R-M system (Makovets *et al.*, 2004; see Fig. 1A). These published experiments used a strain derived from C600 that contained a large deletion that removed the *hsd* genes specifying EcoKI; the *hsd* genes specifying EcoR124I had been introduced within a cassette tagged with an antibiotic-resistance marker (Makovets *et al.*, 2004). To extend our analyses of RA for the EcoR124I system we have chosen to use the sequenced *E. coli* K-12 strain MG1655 (Blattner *et al.*, 1997). Inactivation of the resident R-M system was achieved by the introduction of either a deletion within *hsdR*, to generate an $r_k^-m_k^+$ strain, or a deletion removing *hsdS* to generate an $r_k^-m_k^-$ strain. The genes specifying wild-type, or mutated, derivatives of EcoR124I were then transferred by P1 transduction from our earlier strains to the restriction-deficient strains of MG1655.

A $r_k^-m_k^+$ derivative of MG1655 specifying wild-type EcoR124I generated the control strain and this was resistant to 2-AP, while introduction of the mutant *hsdR*

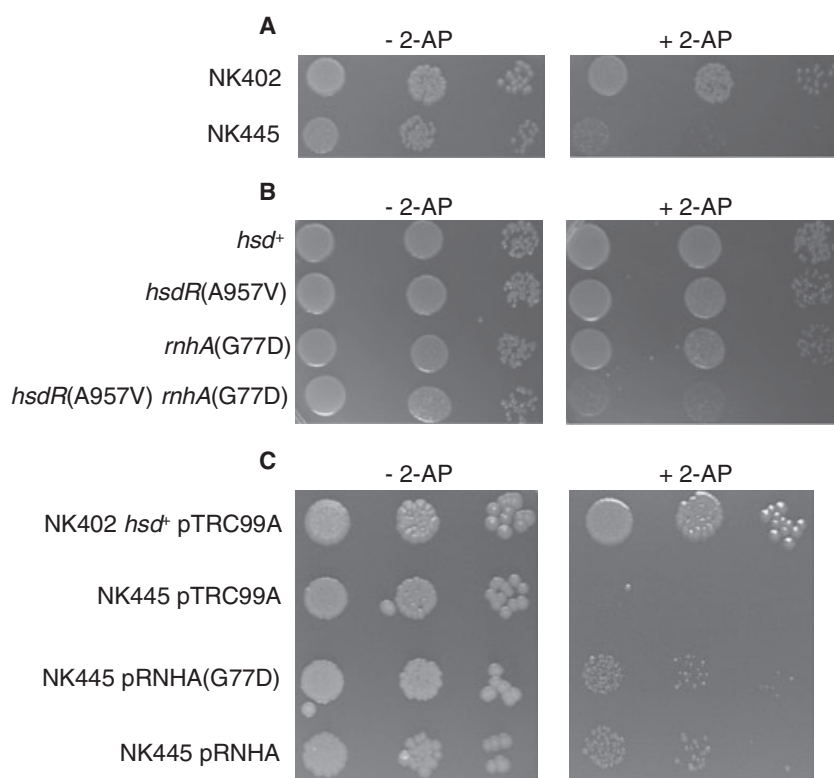


Fig. 1. Identification of two mutations that contribute to 2-AP sensitivity. Serial dilutions of bacterial cultures were spotted on L-agar and L-agar containing 2-AP (400 $\mu\text{g ml}^{-1}$) and incubated overnight.

A. NK402 is the parental strain from which NK445 was derived. Two mutations, *hsdR*(A957V) and *rnhA*(G77D), have now been identified in NK445.

B. Transfer of either of these mutations to the $\Delta\text{hsdR}_{\text{EcoK1}} \text{hsd}^{\text{EcoR1241}}$ derivative of MG1655 conferred some sensitivity to 2-AP. In contrast, the acquisition of both mutations generates a strain that, like the donor NK445, is extremely sensitive to 2-AP. The strains used, in descending order are: NM1104; NM1105; NM1075 and NM1110 (see Table 5).

C. The presence of either wild-type *rnhA* or *rnhA*(G77D) on multicopy plasmids confers partial resistance to 2-AP in NK445. Note that resistance is partial because NK445 retains the *hsdR*(A957V) mutation that impairs RA.

(A957V) allele resulted in a strain that was less sensitive to 2-AP (Fig. 1B) than the donor strain NK445 (Fig. 1A); the colonies were smaller than the control strain, while those of NK445 were barely visible. This observation indicated that our original 2-AP strain, NK445, might include an additional, relevant mutation. We searched for the putative second mutation by screening for its loss following transduction of NK445 using a P1 lysate generated from a library of strains carrying Tn10mini-*kan* insertions throughout the chromosome (Way *et al.*, 1984). Transductants were selected by their ability to grow on plates supplemented with kanamycin and 2-AP. Among the transductants was a strain in which a gene responsible for reduced sensitivity to 2-AP was linked to a Tn10mini-*kan* insertion. The location of this transposon was determined by sequence analysis. Using inverse polymerase chain reaction (PCR), the relevant DNA sequence adjacent to the transposon was amplified and determined. The site of insertion of the transposon was within the *yafU* open reading frame (bp238880 of the *E. coli* chromosome).

Two genes involved in DNA replication, *dnaQ* and *rnhA*, are adjacent to *yafU*, and were considered as possible candidates for the location of the mutation. Our tests indicated that NK445 did not have the mutator phenotype characteristic of *dnaQ* mutants (Echols *et al.*, 1983), but it appeared to behave like an *rnhA* mutant in that *recG* derivatives could not be made (Hong *et al.*, 1995). The

nucleotide sequence of the *rnhA* gene of NK445 revealed a transition that changed the specificity of codon 77 from glycine to aspartic acid. This substitution altered the sequence of RNase HI within helix 2, a structural feature previously implicated in binding the RNA/DNA hybrid substrate (Tsunaka *et al.*, 2001).

Individually the *rnhA*(G77D) and *hsdR*(A957V) alleles confer sensitivity to 2-AP, i.e. strains containing either of these mutations give smaller colonies than the control strain on agar containing 2-AP (Fig. 1B), while the double mutant resembles NK445 and is extremely sensitive to 2-AP (Fig. 1A and B). This demonstrates that one mutation exacerbates the effect of the other, i.e. the impairment of RA as the result of *hsdR*(A957V) enhances the effect of the *rnhA* mutation. We are unaware of any previous evidence that *rnhA* mutants are sensitive to 2-AP, but found that other *rnhA* alleles (*rnhA*339 and *rnhA*224) have the same phenotype (data not shown). The molecular basis of this observation remains to be determined (see *Discussion*).

Additional evidence that the chromosomal mutation in *rnhA* contributes to 2-AP sensitivity, was obtained using plasmids expressing either the wild-type or mutant alleles of *rnhA*. Expression of the plasmid-borne, wild-type *rnhA* gene in NK445 conferred partial resistance to 2-AP (Fig. 1C). Note that complete resistance is not possible because the strain retains the *hsdR*(A957V) mutation. Unexpectedly, overexpression of the mutant *rnhA* allele

Table 1. Restriction alleviation in response to 2-AP.

No.	Strain		Time (min)	Restriction ^a	RA
	EcoR124I genes				
NM1104	Wild-type		0	$(3 \pm 1.2) \times 10^4$	468 ± 145
			100	$(6.5 \pm 3.1) \times 10^1$	
NM1105	<i>hsdR(A957V)</i> M ⁺ S ⁺		0	$(2.3 \pm 0.2) \times 10^5$	18 ± 6.4
			100	$(1.4 \pm 0.4) \times 10^4$	
NM1133	<i>hsdR(A957K)</i> M ⁺ S ⁺		0	$(1.4 \pm 0.4) \times 10^5$	5 ± 2.5
			100	$(3.6 \pm 1.6) \times 10^4$	

a. Tests using modified λ vir indicated no significant differences in efficiency of plating, therefore the calculations are based on the titres of λ vir.0 on the test bacteria relative to the titre on a restriction deficient strain (NM1057).

also conferred some resistance to 2-AP. This suggested that the *rnhA(G77D)* mutation does not completely inactivate RNase HI, a finding substantiated by subsequent phenotypic comparisons with a null mutant.

Mutations *hsdR(A957V)* and *(A957K)* enhance restriction and impair RA

In tests using the mutagen 2-AP as an inducing agent, strain NK445 was shown to be impaired in RA (Makovets *et al.*, 2004). We have quantified RA in response to 2-AP for each of two mutations in *hsdR* following their transfer to the $r_{\kappa^-}m_{\kappa^+}$ derivative of MG1655 (Table 1). Restriction was assessed by the efficiency of restriction of unmodified λ vir. The tests show that the changes in HsdR enhance restriction ~10-fold, and they impair RA 25- to 100-fold after treatment with 2-AP for 100 min. These data confirm that each of the two *hsdR* mutations in codon 957 reduces RA.

The mutation in *rnhA* stimulates RA of *EcoR124I*

The effect of *rnhA* on restriction by *EcoR124I* was determined using unmodified phage (Table 2A). The mutation in *rnhA* stimulates RA for the *EcoR124I* system ~70-fold, but its effect is reduced to ~17-fold in the presence of the *hsdR(A957V)* mutation that impairs RA. RA in response to the mutation in *rnhA* occurred in a *lexA3* strain, indicating that RA in this strain was independent of the SOS pathway (data not shown).

Restriction alleviation and chromosome integrity

Loss of RNase HI function requires homologous recombination to maintain the integrity of the chromosome (Kogoma *et al.*, 1993). DNA synthesis associated with recombination intermediates in an *rnhA* strain may generate unmodified chromosomal DNA that will be a substrate for a resident type I R-M system. One of the reported phenotypes for cells containing mutations in

rnhA is inhibition of division in a subpopulation of cells following induction of the SOS response (Kogoma *et al.*, 1993). To further investigate the role of RA in maintaining chromosome integrity, we used cell filamentation as an index for SOS induction arising from DNA damage in an *rnhA(G77D)* background (Fig. 2A). In the absence of a functional type I R-M system (Δ *hsdS_κ*) the average cell length of a strain containing the *rnhA(G77D)* mutation was 6.1 μm. Introduction of the *EcoR124I* R-M system deficient in RA [*hsdR(A957V)*] into the *rnhA(G77D)* strain led to an increase in average cell length (8.7 μm), consistent with chromosome breakage and SOS induction. The percentage of cells greater than 8 μm in length (indicative of cells that had failed to undergo normal division) increased from 13% in the absence of an R-M system to 33% in the presence of *EcoR124I hsdR(A957V)* (Fig. 2A). Note that mass doubling times for both these strains were approximately 27 min. The experiments were repeated in the alternative background of a Δ *hsdR_κ* strain and demonstrated an equivalent increase in average cell length caused by the presence of *EcoR124I hsdR(A957V)* in combination with the *rnhA(G77D)* mutation (data not shown).

Introduction of the wild-type *EcoR124I* system into the strain containing *rnhA(G77D)* caused a minor increase in average cell length (6.9 μm), indicating that RA is generally effective in the presence of unmodified chromosomal DNA.

These data are consistent with an RA-deficient *EcoR124I* complex cleaving unmodified chromosomal DNA generated as a consequence of the *rnhA(G77D)* mutation.

Our earlier evidence for the partial activity of the *rnhA(G77D)* gene product (Fig. 1C) was further supported by phenotypic comparison with an *rnhA* null mutant (Fig. 2B). In the control strain MG1655, the average cell

Table 2. Restriction alleviation by *EcoR124I* in response to mutations in *rnhA* and *recG*.

Strain	Restriction ^a	RA
A. Effect of the <i>rnhA(G77D)</i> mutation		
NM1104 <i>hsdR⁺M⁺S⁺</i>	$(2 \pm 0.8) \times 10^4$	67 ± 23
NM1109 <i>hsdR⁺M⁺S⁺rnhA(G77D)</i>	$(3.2 \pm 1.5) \times 10^2$	
NM1105 <i>hsdR(A957V)M⁺S⁺</i>	$(1.7 \pm 0.8) \times 10^5$	
NM1110 <i>hsdR(A957V)M⁺S⁺rnhA(G77D)</i>	$(1 \pm 0.08) \times 10^4$	
B. Effect of a <i>recG</i> mutation		
NM1121 <i>hsdR⁺M⁺S⁺recG::kan</i>	$(2.1 \pm 1) \times 10^3$	9.8 ± 1.8
NM1122 <i>hsdR(A957V)M⁺S⁺recG::kan</i>	$(3.4 \pm 0.6) \times 10^4$	6 ± 3.2

a. Tests using modified λ vir indicated no significant difference in efficiency of plating, therefore the calculations are based on the titres of λ vir.0 relative to the titre on a restriction-deficient strain. RA in NM1121 is calculated relative to NM1104, while NM1122 is calculated relative to NM1105.

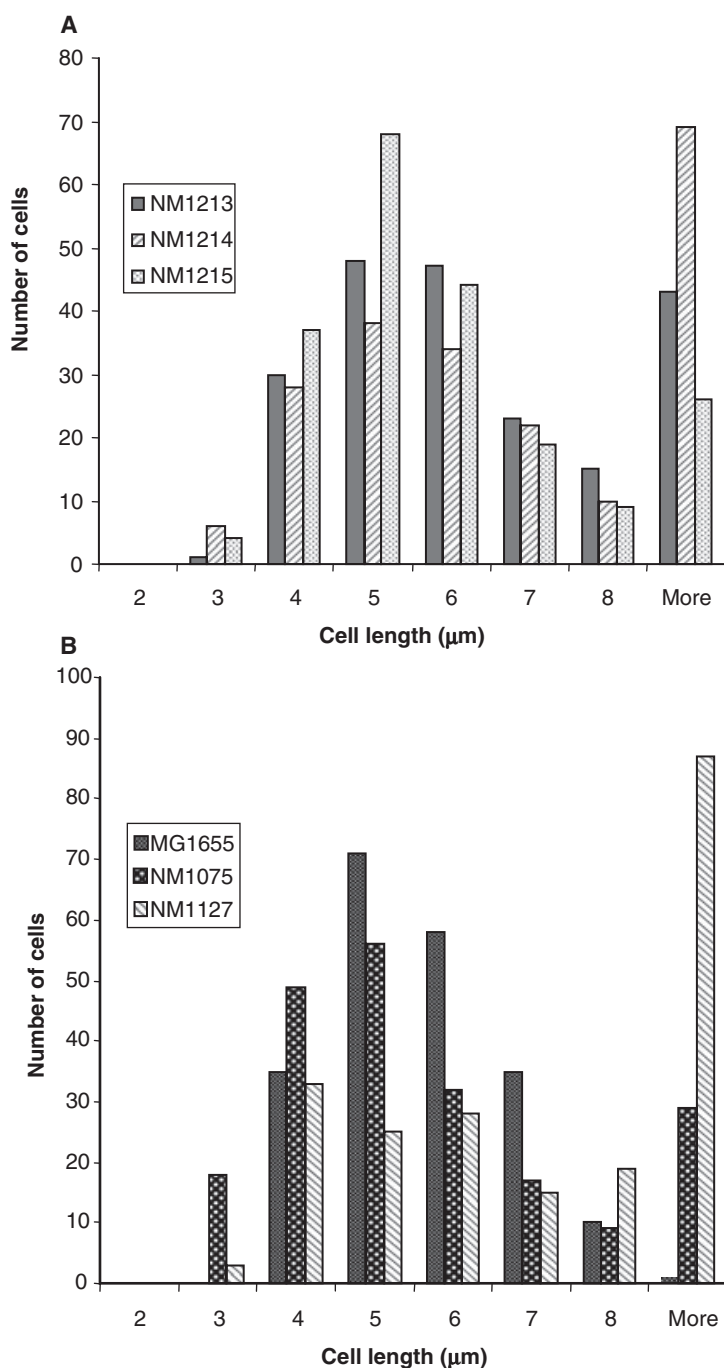


Fig. 2. Cell-length distributions for exponentially growing cultures in L-broth.

A. Induction of the SOS response, as an indicator of chromosome damage, in *rnhA*(G77D) strains was determined using cell-length measurements. The presence of EcoR124I with the mutation *hsdR*(A957V) (NM1214) increases the number of filamentous cells compared with strains specifying wild-type EcoR124I (NM1213) or restriction-deficient EcoR124I (NM1215).

B. The *rnhA*(G77D) mutation in the $r_{ik}^+m_{ik}^+$ strain (NM1075) causes cell filamentation due to SOS induction; however, the lower level of filamentation, in comparison with the *rnhA* null mutation (NM1127), indicates that the mutation does not destroy enzymatic activity.

length was 5.2 μm , with <1% of cells greater than 8 μm in length. The average cell length of an *rnhA::cat* strain was 12 μm , with 41% of cells greater than 8 μm in length, while the strain containing the *rnhA*(G77D) mutation had an average cell length of 5.9 μm , with 14% of cells greater than 8 μm in length. These observations suggest that a proportion of RNA/DNA hybrids present in the chromosome of the *rnhA*(G77D) strain are successfully removed and therefore do not lead to induction of the SOS response.

Increased crossing-over in RNase HI-deficient cells

In the absence of RNase HI, failure to process primers on Okazaki fragments could lead to production of double-strand breaks (DSBs) in subsequent rounds of replication. Some experiments have shown that DSB repair preferentially generates cross-over products from resolution of Holliday junctions (Cromie and Leach, 2000). To determine the extent of crossing-over in the absence of RNase HI, we used cell-length measurements to compare the

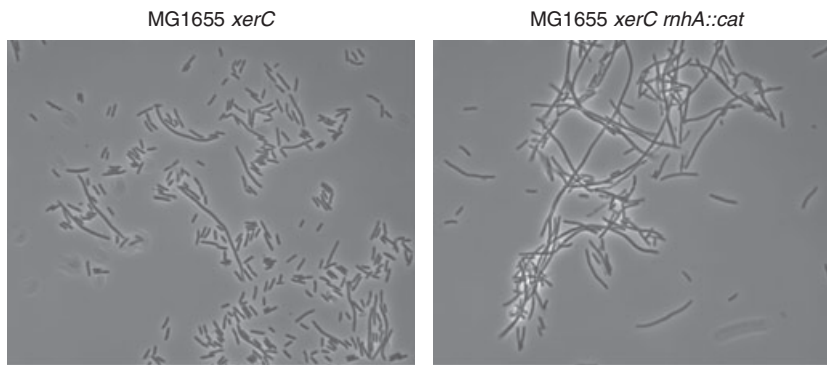


Fig. 3. Phase-contrast photomicrographs of *xerC* and *xerC rnhA* strains. The left panel shows the presence of a subpopulation of filamentous cells in an *xerC* strain due to the failure in chromosome dimer resolution. The right panel demonstrates extensive filamentation in the *xerC rnhA* strain, indicating that increased crossing-over has led to chromosome dimerization in many of the cells within the population.

extent of SOS induction in *xerC* and *rnhA xerC* strains. An odd number of cross-overs during homologous recombination will generate chromosome dimers that must be resolved by the Xer site-specific recombination system prior to segregation (Blakely *et al.*, 1991; 1993). A subpopulation of cells in the *xerC* strain were filamentous (average cell length of 6 μm with 10% of cells greater than 8 μm in length), consistent with induction of the SOS response following failure to resolve chromosome dimers (Fig. 3). Introduction of the *xerC* mutation into an *rnhA::cat* background produced a slower growing strain (doubling time of 36 min compared with 25 min for the *xerC* strain and 29 min for the *rnhA::cat* strain) that contained many filamentous cells (Fig. 3) (average cell length 15.4 μm with 60% of cells greater than 8 μm in length). These data are consistent with enhanced production of cross-overs in cells lacking RNase HI.

Inactivation of *RecG* also induces RA for *EcoR124I*

When deprived of RNaseHI, an RNase specific to RNA within RNA/DNA hybrids, cells constitutively produce R-loops from which origins of replication can be generated (see Kogoma, 1997). It has been shown that strains deficient in *RecG* behave similarly; and it was suggested that *RecG* normally acts as a junction-specific RNA/DNA helicase, thereby preventing the formation of R-loops (Hong *et al.*, 1995; Vincent *et al.*, 1996). With the knowledge that the *rnhA(G77D)* mutation leads to RA, we checked the effect of *recG::kan* and found that it, too, elicits RA for the *EcoR124I* system (Table 2B). In contrast, loss of *ruvA*, a

component of the Holliday junction resolvosome involved in homologous recombination, had no detectable effect on RA (data not shown). The RA response elicited by the *recG* mutation was smaller than that detected for *rnhA(G77D)*.

RA for *EcoKI* in response to *rnhA(G77D)* and *recG* is detected in the absence of *Lar*

Initially, we detected only a small effect of the *rnhA(G77D)* mutation on RA of *EcoKI* in MG1655 (approximately two-fold), but a greater effect was detected in a strain lacking the *Rac* prophage. We therefore made a derivative of MG1655 in which the *lar* gene of the *Rac* prophage was inactivated by a transposon. The product of *lar* is known to enhance modification and reduce restriction (Simmon and Lederberg, 1972; Toothman, 1981; King and Murray, 1995).

In *lar⁻* derivatives of MG1655, restriction of unmodified phage was enhanced by 15-fold (Table 3). The presence of *Lar* apparently reduces the need for RA of *EcoKI* by *ClpXP*. A mutation in *clpX*, thereby blocking *ClpXP*-dependent RA, enhanced restriction by more than 100-fold, a figure significantly greater than that previously reported by Makovets *et al.* (1999). We conclude that, in the earlier experiments using a *rac⁻* strain, the absence of *Lar* masks the full contribution of *ClpXP*.

In the *lar⁻* strains, RA was readily detected in the presence of either the *rnhA(G77D)* mutation or *recG::kan* (Table 4A). That RA, of approximately 20-fold, was due to the mutation in *rnhA* was confirmed by the demonstration that RA was lost when the *lar rnhA* strain was converted to *rnhA⁺* by P1 transduction, and that the *rnhA224* mutation also alleviated restriction in a *lar⁻* strain (data not shown). We note that the amplification of λ in *rnhA* strains is impaired; plaque sizes are generally smaller and the efficiency of plating slightly reduced. RA in the presence of *rnhA(G77D)* was shown to be dependent on *clpX* (Table 4B).

Our data extend to *EcoKI* the consequences of a deficiency in *rnhA*, i.e. RA in response to unmodified target

Table 3. Mutations in *lar* and *clpX* enhance restriction by *EcoKI*.

Strain	Restriction	Restriction enhancement
MG1655	$(5.4 \pm 2.3) \times 10^3$	
NM1049 MG1655 <i>lar::cat</i>	$(1.1 \pm 0.4) \times 10^5$	15 \pm 4.4
NM1041 MG1655 <i>clpX::kan</i>	$(7.7 \pm 0.3) \times 10^5$	146 \pm 26
NM1255 MG1655 <i>lar::cat clpX::kan</i>	$(1.4 \pm 0.08) \times 10^6$	350 \pm 20

Table 4. Mutations in *rnhA* and *recG* lead to alleviation of restriction by EcoKI.

Strain	Restriction	RA
A. Mutations in <i>rnhA</i> and <i>recG</i> lead to RA		
NM1049 MG1655 <i>lar::cat</i>	$(1.5 \pm 0.5) \times 10^5$	23 ± 9.8
NM1089 MG1655 <i>lar::cat</i> <i>rnhA(G77D)</i>	$(6.2 \pm 2) \times 10^3$	
NM1130 MG1655 <i>lar::cat recG::kan</i>	$(2.4 \pm 0.5) \times 10^4$	
B. RA by <i>rnhA</i> is dependent on ClpX		
NM1255 MG1655 <i>lar::cat clpX::kan</i>	$(1.5 \pm 0.2) \times 10^6$	1.2 ± 0.1
NM1256 MG1655 <i>lar::cat</i> <i>rnhA(G77D) clpX::kan</i>	$(1.3 \pm 0.2) \times 10^6$	

sequences created following homologous recombination or the formation of new origins of replication. Of additional, general interest is the implication that the Lar product of the resident Rac prophage serves to protect the bacterial chromosome; in a *lar^r* strain the minor effect of the *rnhA(G77D)* mutation indicates that Lar relieves the need for RA by the ClpXP-dependent pathway.

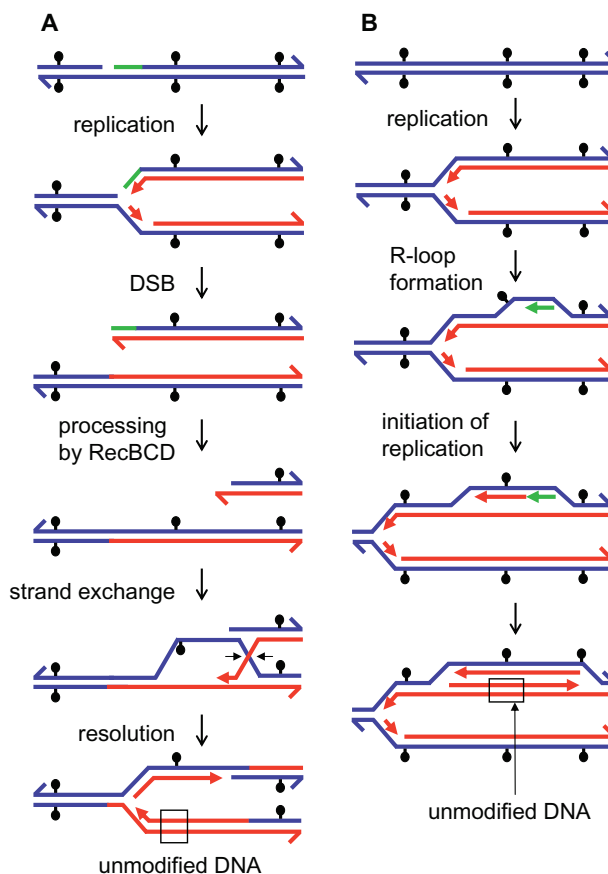
Discussion

Our current experiments emphasize the importance of controlling restriction activity of type I systems during replication of the bacterial chromosome. In *E. coli*, the primers for lagging strand DNA synthesis are primarily removed by the 5' exonuclease activity of DNA polymerase I. An additional catalytic function, provided by RNase HI, is also required for correct processing of 5' ends from longer RNA primers (Ogawa and Okazaki, 1984). While *polA* is an essential gene when *E. coli* is grown in a rich medium (Joyce and Grindley, 1984), mutations in *rnhA* only lead to a reduction in growth rate and constitutive SOS induction (Kogoma *et al.*, 1993). Survival of *rnhA* mutants is dependent on the recombination functions of both the RecBCD and RecFOR pathways (Kogoma *et al.*, 1993). This suggests that failure to process a subset of primers from Okazaki fragments leads to double- and single-strand breaks, which is consistent with the observed constitutive SOS induction.

A further consequence of mutations in *rnhA* is the initiation of DNA replication from DnaA-independent origins, *oriK1-5* (Ogawa *et al.*, 1984). This constitutive stable DNA replication mechanism is initiated following formation of an R-loop generated by a RecA-mediated invasion of a nascent transcript into a DNA duplex (Kogoma *et al.*, 1985; Zaitsev and Kowalczykowski, 2000). A second role for RNase HI is therefore the prevention of aberrant initiation of replication at ectopic origins by destruction of RNA/DNA hybrids.

Why does a mutation in *rnhA* lead to RA in the presence of either EcoKI or EcoR124I? It has been known for many years that DNA damage leads to RA (Thoms

and Wackernagel, 1984) and more recently it was suggested that DNA repair could lead to unmodified target sequences and the induction of RA (Makovets *et al.*, 1999; see Murray, 2000). The absence or reduction of RNase HI activity will lead to persistence of a subset of RNA primers from lagging strand DNA synthesis. Failure to remove primers may generate single-strand nicks or gaps that will produce DSBs and cause fork arrest in a subsequent round of DNA replication (Fig. 4). Restarting

**Fig. 4.** Two pathways that can lead to unmodified DNA in the absence of RNase HI.

A. Failure to remove an RNA primer (green) will lead to a nick or gap on the template for leading strand DNA synthesis in the subsequent round of replication. Newly synthesized DNA (red lines) will be unmodified. A replication fork encountering the nick will produce a DSB that is then processed by RecBCD, leaving a 3' overhang that allows RecA-mediated strand invasion of the sister duplex. Resolution of the Holliday junction by RuvABC will regenerate the replication fork. The cross-over product that would generate a chromosome dimer is shown. If an R-M target sequence is located near the DSB, degradation of a modified site (black circles) by RecBCD will lead to production of unmodified DNA (boxed) when the fork is re-established. B. Transcription at *oriK* sites on newly replicated DNA, containing hemi-methylated R-M target sites, may allow formation of an R-loop. The annealed RNA (green arrow) can act as a primer for DNA synthesis and allow initiation of a further round of replication. Replication using an unmodified strand as a template will generate unmodified duplex target sites (boxed) for the R-M system. Modified sites are indicated by black circles and newly replicated DNA strands are shown as red lines. (Adapted from the study by Kogoma, 1997).

the replication fork requires homologous recombination between the replicating sister chromosomes. DNA replication following strand exchange between duplexes that contain hemi-methylated target sequences for a type I restriction enzyme, will generate stretches of unmodified DNA that will be recognized as potential substrates for the endonuclease complex. Generation of unmodified target sequences by recombination does not only happen in *rnhA* mutants but also can occur when a replication fork encounters a single-strand nick or gap or during repair of any DSB. This implies that a normal function of RA is to protect unmodified products resulting from replication associated with recombination (Fig. 4). Binding and translocation of a type IA or IB R-M complex on unmodified chromosomal DNA will subsequently lead to loss of restriction activity as a result of ClpXP-mediated proteolysis of the HsdR subunit (Makovets *et al.*, 1999). For EcoR124I the mechanism of RA remains to be determined (Makovets *et al.*, 2004; Seidel *et al.*, 2005).

The second consequence of mutations in *rnhA*, R-loop formation, could also lead to RA. Transcription occurring at an *oriK* site following the passage of a replication fork originating from *oriC*, could form an R-loop that would subsequently produce a new replication fork and generate unmodified DNA (Fig. 4). A role for R-loop formation in activating RA is supported by the observed reduction in restriction activity of a *recG* mutant. The branch migration activity of RecG has been postulated to destabilize R-loops by removing RNA from the RNA/DNA hybrid (Kogoma, 1997). The induction of RA in a *recG* strain is not simply a consequence of failed processing of Holliday junctions as RA is not observed in a *ruvA* mutant.

Mutations in both *rnhA* and *recG* produce R-loops, yet RA is more apparent in *rnhA* strains. Recombination functions, such as RecBCD, are required for the viability of *rnhA* strains (Kogoma *et al.*, 1993), and we have demonstrated that crossing-over is increased in the absence of RNase HI. The higher levels of RA in the *rnhA* strains are potentially a consequence of DNA synthesis associated with both repair and restart of replication forks and the formation of R-loops, while the *recG* strains may predominantly display RA as a result of R-loop formation. The reported lethality of an *rnhA recG* double mutant may not simply be caused by excessive R-loop formation interfering with normal replication, but may be a combination of R-loops plus the inability to correctly process large numbers of DSBs generated by the loss of RNase HI. In support of this notion it has been reported that *polA recG* double mutants are not viable (Hong *et al.*, 1995). Similarly, RA following UV irradiation and in the presence of mutations, such as *dam*, *dnaQ* and *topA* (Thoms and Wackernagel, 1984; Makovets *et al.*, 1999),

is also likely to arise as a consequence of DNA synthesis following recombination between hemi-methylated templates.

Despite the fact that our original screen used 2-AP sensitivity to identify RA-deficient mutations, it also identified an *rnhA* mutation that stimulates RA. Together the two mutations generated a strain that was extremely sensitive to 2-AP. Nevertheless, all of the *rnhA* mutations that we have tested, both in the presence and absence of an R-M system, confer some sensitivity to 2-AP. While the molecular mechanism of this sensitivity is unknown, there are a number of possible explanations. If 2-AP causes DNA breaks in addition to mismatches, the elevated levels of homologous recombination in cells depleted of RNase HI may lead to insufficient enzymatic capacity to repair additional damage. We note that a *recA* mutant shows some sensitivity to 2-AP (our unpublished observations). Alternatively, the presence of 2-AP may induce ectopic replication of the chromosome, which generates more RNA primers for lagging strand synthesis that require RNase HI for correct processing. The sensitivity of *rnhA* mutants to 2-AP does not appear to be associated with the mismatch repair system of *E. coli* because an *rnhA mutS* double mutant is still sensitive to the mutagen (our unpublished observations).

Restriction alleviation for type II systems has not been reported. Tests with a strain specifying EcoRI demonstrate high sensitivity to the presence of 2-AP, consistent with cutting of the bacterial chromosome by the restriction enzyme (Makovets *et al.*, 2004). The loss of genes specifying the EcoRI R and M enzymes also leads to cell death (Kobayashi, 2001). The evidence indicates that as the concentrations of the two enzymes decreases during cell growth, there will be insufficient methyltransferase to modify all targets on the chromosome, and unmodified targets will become substrates for the residual endonuclease. For a type II system, transcriptional control of the gene specifying the endonuclease has been demonstrated, and this permits the transfer of R-M genes to a new strain (Ives *et al.*, 1992; Tao and Blumenthal, 1992).

For many years it has been known that Lar alleviates restriction. Its effect was detected in *sbca* strains (Simon and Lederberg, 1972), where expression of *lar*, *recE* and *recT* is elevated by fusion to a different promoter, similarly in λ rev phages in which *lar*, *recE* and *recT* replace the recombination genes of λ (Toothman, 1981). More recently, following the cloning of *lar* in a plasmid, Lar was shown to alleviate restriction and enhance modification (King and Murray, 1995).

Genome sequences of two *E. coli* 0157 strains identify Rac-like defective prophages that retain genes homologous to *lar*, *recE* and *recT* (Casjens, 2003). It has been suggested that defective prophages could provide a res-

ervoir of potentially useful genes (Botstein and Campbell, 1983). The role of *sbca* mutations in providing expression of the *recE* and *recT* genes to provide an alternative recombination pathway in *recBC* strains has been cited to support this concept. RecE and RecT can reduce the restriction of unmodified λ by the type III system EcoPI, when expression of *recE* and *recT* is enhanced in the absence of RecBCD nuclease (Handa and Kobayashi, 2005).

Our experiments indicate that the *lar* coding sequence, as part of the resident Rac prophage, has a detectable effect on the phenotype of the cell. It is well documented that EcoKI is a very inefficient methyltransferase if the substrate is unmodified (Suri and Bickle, 1985; Dryden *et al.*, 1993). It seems probable that the resident *lar* gene in MG1655 enhances the efficiency of methylation of unmodified target sequences in the bacterial chromosome.

Experimental procedures

Bacterial strains, phages, plasmids and general methods

Bacterial strains and phages are listed in Table 5. Media, general methods for cloning, handling of bacteria and their phages, were as described previously (Makovets *et al.*, 1999). Deletions Δ *hsdR*_{EcoKI} and Δ *hsdS*_{EcoKI}, described in the study by Sain and Murray (1980) were transferred to recipient strains from λ *hsd* phages (Gough and Murray, 1983). The *lar::cat* mutation was produced by ligation of the Agel *cat* fragment from pACYC184 to the Agel site of *lar* in pGK8 (King and Murray, 1995), followed by subcloning of the BamHI *lar::cat* fragment in λ for transfer to the bacterial chromosome. Transfer of *clpX::kan* into Kan^R strains was achieved by cotransduction with *tsx247::Tn10*. Plasmid-borne copies of *rnhA* and *rnhA*(G77D) under control of *P_{tac}* were generated by PCR amplification using Pfu polymerase (NEB) followed by ligation into the NcoI and HindIII sites of pTRC99a (Amersham Pharmacia Biotech).

Table 5. Bacterial strains (derivatives of *E. coli* K-12).

Strain	Relevant genotype	Reference or origin
MG1655	F ⁻ λ - <i>ilvG rfb-50 rph1</i>	Blattner <i>et al.</i> (1997)
N3793	Δ <i>recG::kan</i>	Al-Deib <i>et al.</i> (1996)
N2057	<i>ruvA60::Tn10</i>	Shurvinton <i>et al.</i> (1984)
N1642	<i>lexA3 malE::Tn10</i>	Lloyd <i>et al.</i> (1987)
CAG18436	<i>zae-502::Tn10</i>	Nichols <i>et al.</i> (1998)
VC48	NK301 <i>clpX::kan tsx247::Tn10</i>	Makovets <i>et al.</i> (2004)
DS981	AB1157 <i>recF xerC::kan</i>	Blakely <i>et al.</i> (1993)
GJ3129	MC4100 <i>rnhA339::cat</i>	Kogoma <i>et al.</i> (1993)
NK402	Δ <i>hsd</i> _{EcoKI} <i>lac::(hsd</i> ⁺ _{EcoR1241} <i>cat)</i>	Makovets <i>et al.</i> (2004)
NK420	Δ <i>hsd</i> _{EcoKI} <i>lac::(hsdM</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat) proC zaj-3053::Tn10</i>	Makovets <i>et al.</i> (2004)
NK439	C600 <i>lar::cat</i>	see <i>Experimental Procedures</i>
NK445	NK402 <i>hsdR</i> (A957V) _{EcoR1241} [<i>rnhA</i> (G77D)] ^a	Makovets <i>et al.</i> (2004)
NM996	NK402 <i>hsdR</i> (A957K) _{EcoR1241}	Makovets <i>et al.</i> (2004)
NM1041	MG1655 <i>clpX::kan</i>	MG1655 + P1.VC48
NM1049	MG1655 <i>lar::cat</i>	MG1655 + P1.NK439
NM1056	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>)	MG1566 + λ Δ <i>hsdS</i>
NM1057	MG1655 Δ <i>hsdR</i> _{EcoKI}	MG1566 + λ <i>hsdR</i> Δ 4
NM1073	NK445 <i>yafU::Tn10</i> mini- <i>kan</i>	see <i>Experimental procedures</i>
NM1075	MG1655 <i>rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan</i>	MG1655 + P1.NM1073
NM1089	MG1655 <i>lar::cat rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan</i>	NM1049 + P1.NM1073
NM1104	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsd</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1057 + P1.NK402
NM1105	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsdR</i> (A957V) <i>M</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1057 + P1.NK445
NM1109	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsd</i> ⁺ _{EcoR1241} <i>cat) rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan</i>	NM1104 + P1.NM1075
NM1110	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsdR</i> (A957V) <i>M</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat) rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan</i>	NM1105 + P1.NM1075
NM1121	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsd</i> ⁺ _{EcoR1241} <i>cat) ΔrecG::kan</i>	NM1104 + P1.N3793
NM1122	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsdR</i> (A957V) <i>M</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat) ΔrecG::kan</i>	NM1105 + P1.N3793
NM1127	MG1655 <i>rnhA339::cat</i>	MG1655 + P1.GJ3129
NM1130	MG1655 <i>lar::cat recG::kan</i>	NM1049 + P1.N3793
NM1133	MG1655 Δ <i>hsdR</i> _{EcoKI} <i>lac::(hsdR</i> (A957K) <i>M</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1104 + λ <i>hsdR</i> (A957K)
NM1204	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>proC zaj-3053::Tn10 lac::(hsdM</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1056 + P1.NK420
NM1205	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>lac::(hsd</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1204 + P1.NK402
NM1207	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>lac::(hsdR</i> (A957V) <i>M</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1204 + P1.NK445
NM1208	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>lac::(hsdM</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1204 + P1.NK402
NM1213	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>lac::(hsd</i> ⁺ _{EcoR1241} <i>cat) rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan</i>	NM1205 + P1.NM1073
NM1214	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>lac::(hsdR</i> (A957V) <i>M</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat)</i>	NM1207 + P1.NM1073
NM1215	MG1655 Δ (<i>hsdS</i> _{EcoKI} - <i>mcr</i>) <i>lac::(hsdM</i> ⁺ <i>S</i> ⁺ _{EcoR1241} <i>cat) rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan</i>	NM1208 + P1.NM1073
NM1255	MG1655 <i>lar::cat clpX::kan tsx247::Tn10</i>	NM1049 + P1.VC48
NM1256	MG1655 <i>lar::cat rnhA</i> (G77D) <i>yafU::Tn10</i> mini- <i>kan clpX::kan tsx247::Tn10</i>	NM1089 + P1.VC48
GB2001	MG1655 <i>xerC::kan</i>	MG1655 + P1.DS981
GB2002	MG1655 <i>rnhA339::cat xerC::kan</i>	NM1127 + P1.DS981

a. The *rnhA*(G77D) mutation in NK445 was identified in the current work.

Identification of the rnhAG77D mutation

A P1^{vir} lysate was produced from a pooled library of kanamycin-resistant colonies that had been generated by infecting MG1655 with λ NK1316 Tn10mini-kan (Kleckner *et al.*, 1991). P1-mediated transductants of NK445 containing Tn10mini-kan were selected on kanamycin and 400 $\mu\text{g ml}^{-1}$ of 2-AP (NM1073). Colonies obtained in the presence of 2-AP were screened using P1 transduction into NK445 to determine linkage between 2-AP^R and Tn10mini-kan. Genomic DNA from 2-AP^R colonies was used as the template for inverse PCR using primers specific for Tn10mini-kan (Ochman *et al.*, 1988). Briefly, 0.5 μg of genomic DNA digested with PstI was ligated in a 500 μl reaction to favour intramolecular products. An inverse PCR product was generated using Pfu polymerase (NEB) that was sequenced using the BigDye Terminator 3.1 on an ABI Prism 3100 automated sequencer (Applied Biosystems).

Cell-length measurements

Mass doubling times were determined for at least six generations from triplicate exponential cultures kept below an OD₆₀₀ of 0.2. Phase-contrast images of cells were captured using a Zeiss Axioplan II microscope and a Hamamatsu Orca CCD camera, with cell-length measurements obtained using Improvise OpenLab software.

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