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Note

Chromosomal Fragmentation Is the Major Consequence of the *rdgB* Defect in *Escherichia coli*

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ABSTRACT

The *rdgB* mutants depend on recombinational repair of double-strand breaks. To assess other consequences of *rdgB* inactivation in *Escherichia coli*, we isolated RdgB-dependent mutants. All transposon inserts making cells dependent on RdgB inactivate genes of double-strand break repair, indicating that chromosomal fragmentation is the major consequence of RdgB inactivation.

VARIOUS modifications of the four canonical DNA bases are called base analogs (Negishi *et al.* 1994). The base analogs hypoxanthine and xanthine are not normally present in RNA or DNA, but can be found in significant amounts in nucleoside monophosphate pools, serving as intermediates in the synthesis of canonical RNA precursors (Neuhard and Nygaard 1987). These nucleoside monophosphates may be converted with a low frequency into deoxynucleoside triphosphates and used by DNA polymerases as noncanonical DNA precursors. Since the pools of DNA precursors are constantly contaminated by noncanonical deoxyribonucleotides, the cell employs specialized NTPases that hydrolyze specific noncanonical nucleoside triphosphates to monophosphates (Bessman *et al.* 1996). In the absence of such "cleansing" of dNTP pools, the incorporation of base analogs into DNA may cause DNA damage or mutations (Michaels *et al.* 1992; Bradshaw and Kuzminov 2003; Kouzminova and Kuzminov 2004).

We study how the cell copes with the base analog toxicity by employing the *rdgB* mutants, originally isolated in *Escherichia coli* as synthetic lethals in combination with *recA* inactivation (Clyman and Cunningham 1987; Kouzminova *et al.* 2004) and later shown to be also synthetically lethal with *recBC* and *ruvABC* mutations (Bradshaw and Kuzminov 2003). The *rdgB* mutants experience increased levels of double-strand breaks in their chromosome, implicating the RdgB protein in avoidance of chromosomal fragmentation (Bradshaw and Kuzminov 2003; Kouzminova *et al.* 2004). Both the chromosomal fragmentation and the synthetic lethality of *rdgB rec* mutants are suppressed by inactivation of Endo V (Bradshaw and Kuzminov 2003), which nicks DNA near hypoxanthine and xanthine residues (Yao *et al.* 1994; Yao and Kow 1995; He *et al.* 2000). Moreover, DNA in *rdgB* mutants accumulates EndoV-recognized modifications (Bradshaw and Kuzminov 2003). Biochemically, the RdgB protein is an NTPase with a 100-fold preference for the noncanonical nucleotides ITP, XTP, and dITP (Chung *et al.* 2001, 2002). It is likely that RdgB hydrolyzes noncanonical DNA precursors dITP and dXTP to monophosphates, preventing incorporation of the base analogs hypoxanthine and xanthine into the chromosomal DNA (Bradshaw and Kuzminov 2003). There is still a possibility that RdgB also works in excision of hypoxanthines or xanthines from DNA after the EndoV-catalyzed nicking.

To reveal all the consequences of *rdgB* inactivation, we identified mutants dependent on RdgB for survival (synthetically lethal with *rdgB* inactivation). To reveal synthetic-lethal interactions, we utilize a color screen for inability to lose a plasmid (Kranz and Holm 1990; Kouzminova *et al.* 2004). To build a genetic system for isolation of RdgB-dependent mutants, an *rdgB lacZ* double-mutant strain was first complemented with $rdgB^+$ and $lacZ^+$ genes from a plasmid that has a temperature-sensitive origin of replication (Figure 1). The resulting strain is $rdgB^+$ $lacZ^+$ when grown at 28° but becomes $rdgB^-$ $lacZ^-$ mutant when grown at temperatures 37° or higher (Figure 1A). When grown at 34° on MacConkey plates, the plasmid is lost from the cells at a rate of ~5% per generation. Since it takes ~25 generations for a cell to grow into a 3- to 4-mm colony, this seemingly low rate of plasmid loss nevertheless results in

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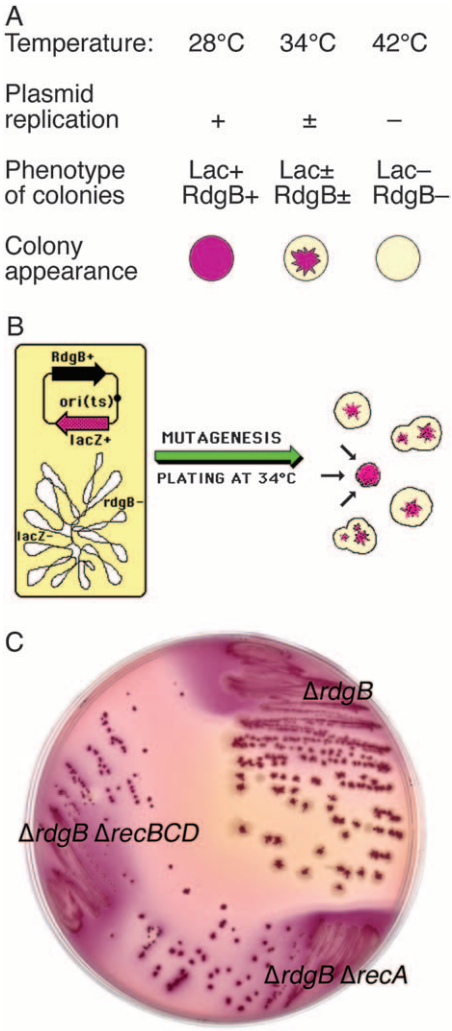

FIGURE 1.—The color screen for RdgB-dependent mutants. The screen looks for the inability to lose a temperature-sensitive plasmid carrying the *rdgB*$^{+}$ gene. (A) The properties of the strain for the screen [LL12 = Δ*lacZ* Δ*rdgB* p(*ori*-Ts)-*lacZ*$^{+}$-*rdgB*$^{+}$] at three temperatures, plated on MacConkey + lactose agar. (B) The scheme of the experimental strain (LL12) and the expected colony phenotype of RdgB-*in*dependent (sectoring) mutants, as well as a single RdgB-dependent mutant (converging arrows). (C) The colony phenotype of the parental color screen strain [LL12 Δ*rdgB* p*rdgB* + (Ts)] and its two derivatives, carrying either Δ*recA* (LL13) or Δ*recBCD* (LL14) mutations, on MacConkey–lactose at 34°. The parental strain forms sectoring colonies, whereas the double mutants form smaller nonsectoring colonies, serving as positive controls.

a colony in which only ~30% of the cells still keep the plasmid (0.95^{25} ~0.3). Therefore, when colonies of the strain are plated on MacConkey plates at 34°, they have dark-purple star-shaped centers surrounded by broad colorless borders (Figure 1A). This sectoring phenotype indicates loss of the plasmid in ~70% of the cells in the colony. However, if an RdgB-dependent mutant forms a colony, the cells become inhibited when they lose the plasmid (and the *rdgB*+ gene with it), so the colony is made up only of cells that still keep the plasmid. As a result, this colony is expected to be smaller and solidly colored without sectoring (Figure 1B). Plating of the original Δ*rdgB* strain and two positive controls (Δ*rdgB* Δ*recA* and Δ*rdgB* Δ*recBCD* double mutants) at 34° confirms the reasoning (Figure 1C).

TABLE 1

The color screen statistics

	Statistics for the:		
	First run	Second run	Third run
Colonies screened	30,250	42,100	20,000
Restreaked at 34°	190	367	Skipped
Streaked at 42°	49	78	140
UV tested	16	12	21
Sequenced from DH5α	6	10	4
Transduced into AB1157	9	2	None
Sequenced from AB1157	None	None	Not applicable

Insertional mutagenesis with a Tn*5*-based element was carried out as before (KOUZMINOVA *et al.* 2004) with subsequent screening for the nonsectoring phenotype of colonies on MacConkey medium as an indicator of potential dependence on RdgB. The primary candidates were then confirmed: (1) for the inability to sector upon restreaking on MacConkey-lactose at 34°; (2) for growth inhibition after plasmid loss at 42°; and (3) for sensitivity to DNA damage (UV light). The UV-sensitive mutants were sequenced directly from a DH5α background. UV-resistant mutants were first transduced into an AB1157 background to confirm RdgB dependence; however, none of the UV-resistant mutants proved to be dependent on RdgB in an AB1157 background. Since the insertions are supposed to completely inactivate the affected genes, we did not expect isolating partial inactivation mutants in indispensable genes. The screen statistics are in Table 1.

We expected to isolate RdgB-dependent mutants in at least four major categories:

1. Mutants defective in excision repair of DNA modifications that cause repair intermediates to accumulate. The details of the excision repair pathway for DNA hypoxanthines downstream of the EndoV-catalyzed incision step are still unknown. It was anticipated that mutants affecting the post-incision stages of the repair mechanism would be lethal with the *rdgB* deletion, similar to the lethality of *dut* mutants in combination with inactivation of exonuclease III (TAYLOR and WEISS 1982).
2. Mutants defective in recombinational repair of double-strand breaks. *recA*, *recBC*, and *ruv* mutants are known synthetic lethals with *rdgB* inactivation (BRADSHAW and KUZMINOV 2003), so their finding would be "proof-of-principle" for our screen. It was also possible that new genes, previously unknown to be involved in recombinational repair, would be identified.

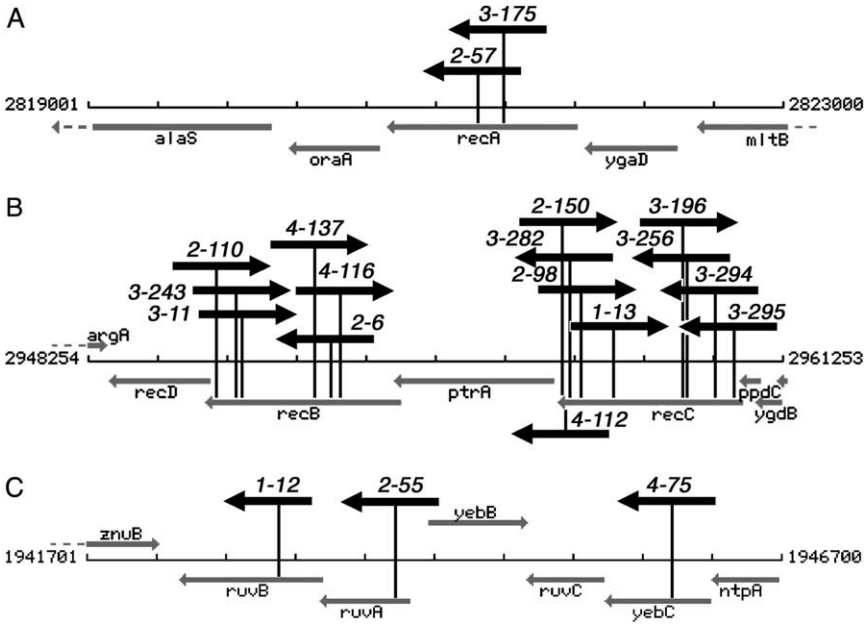

FIGURE 2.—The position and orientation of inserts, conferring RdgB dependence, in recombinational repair genes. The vicinity maps of the disrupted genes showing the chromosomal coordinates were generated with Colibri server (http://genolist.pasteur.fr/Colibri/genome.cgi). Open reading frames and their direction are shown by lightly shaded arrows, identified by gene names. The positions of pRL27 inserts are shown by darkly shaded arrows on sticks, identifying the orientation of the *kan* gene of the insert. The numbers near inserts identify individual mutants. Note that the three maps have different scales. (A) The 4-kbp *recA* region. (B) The 13-kbp *recBCD* region. (C) The 5-kbp *ruvABC* region.

3. Mutants defective in avoidance of chromosomal fragmentation. Usually, inactivating one system that avoids chromosomal lesions does not kill the cell (KOUZMINOVA *et al.* 2004); however, inactivation of two such systems simultaneously may result in an overwhelming amount of chromosomal damage (REID *et al.* 1999). Such damage would prevent the cell from replicating its chromosome and would make the double mutant a synthetic lethal.
4. Mutants with deregulated nucleotide metabolism that accumulate noncanonical DNA precursors (like dITP), normally detoxified by RdgB. For example, overproduction of *purA* suppresses *rdgB rec* synthetic lethality (CLYMAN and CUNNINGHAM 1991); therefore, inactivation of *purA* may cause synthetic lethality in combination with *rdgB* inactivation. In addition to these categories, there could be mutants inhibited by high levels of the ribonucleotides ITP and XTP.

After three runs of the screen, we isolated 20 RdgB-dependent mutants (Table 1). Unexpectedly, all of them turned out to be defective in recombinational repair of double-strand breaks. We obtained nine independent inserts in *recC*, six independent inserts in *recB*, two independent inserts in *recA*, and single inserts in *ruvA*, *ruvB*, and *yebC* genes just upstream of *ruvC* (Figure 2). Isolation of *recA*, *recB*, *recC*, *ruvA*, and *ruvB* mutants, all known synthetic lethals with *rdgB* (BRADSHAW and KUZMINOV 2003), proves that the screen works as designed. Isolation of these mutants, all deficient in recombinational repair of double-strand breaks, also provides a strong evidence for double-strand DNA breakage in *rdgB* mutants. If the combined length of *recA*, *recB*, *recC*, and *ruvABC* genes (10,119 bp) is divided by the total number of inserts in these genes (20), the density of the inserts in recombinational repair genes comes close to 1/500 bp. The high density of inserts in *recB* and *recC* genes and their complete absence in the neighboring *recD* and *ptr* genes suggests that (1) the latter two genes have no role in the double-strand break repair; (2) our insertion cassette does not have polar or antisense effects on neighboring genes. Our failure to isolate other known recombinational repair mutants as dependent on RdgB makes it unlikely that there are yet-to-be-identified nonessential genes 2 kbp or longer involved in the double-strand break repair in *E. coli*. Since double-strand break repair mutants compose the only class of RdgB-dependent mutants, double-strand DNA breaks must be the major consequence of *rdgB* inactivation.

Our inability to isolate other classes of RdgB-dependent mutants, if not a limitation of our specific protocol (see below), is an interesting result in itself. The absence of mutants inactivating steps in the excision of DNA hypoxanthines downstream from the initial EndoV-incision step suggests that either (i) no nonessential enzymes are involved in the subsequent steps (both DNA pol I and DNA ligase are essential under our screen conditions) or (ii) RdgB itself catalyzes this later step before DNA pol I and DNA ligase finish the repair. On the other hand, the absence of inactivational mutants in the nucleotide metabolism that would overproduce dITP suggests that there are no such mutants (at least in dispensable genes) and that there are no activities other than RdgB that detoxify dITP and dXTP. Indeed, our genetic findings from a different approach indicate that dITP is produced in the cell via multiple spurious and therefore difficult-to-control reactions, rather than via one major, tightly controlled reaction (B. BUDKE,

J. Bradshaw, S. Horrell and A. Kuzminov, unpublished results).

There is a possibility that our failure to isolate other classes of RdgB-dependent mutants is due to certain restrictions of our screen, for example, the confirmation of UV-resistant mutants in a different background in which double mutants happen to be viable. Also, our screen, by employing a P1 transduction step, is biased against slow-growing (low-viability) mutants. This may be the reason why we did not isolate low-viability recombinational repair-deficient mutants like *priA*, a likely candidate for another RdgB-dependent mutant. Employing less restrictive confirmation protocols in the future may broaden the scope of RdgB-dependent mutants.

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