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Mismatch Repair in *Escherichia coli* Cells Lacking Single-Strand Exonucleases ExoI, ExoVII, and RecJ

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In vitro, the methyl-directed mismatch repair system of *Escherichia coli* requires the single-strand exonuclease activity of either ExoI, ExoVII, or RecJ and possibly a fourth, unknown single-strand exonuclease. We have created the first precise null mutations in genes encoding ExoI and ExoVII and find that cells lacking these nucleases and RecJ perform mismatch repair in vivo normally such that triple-null mutants display normal mutation rates. ExoI, ExoVII, and RecJ are either redundant with another function(s) or are unnecessary for mismatch repair in vivo.

The methyl-directed mismatch repair (MMR) system of *Escherichia coli* is a key enforcer of genetic stability. The MMR system corrects DNA polymerase errors (reviewed in reference 22) and prevents the recombination of partially diverged DNA sequences (18, 19, 26, 39). *E. coli* strains lacking any essential component of this system display a mutator phenotype in which mutation rates are 100- to 1,000-fold above normal (22, 30) and are better able to recombine partially diverged DNA sequences (18, 26, 39). Both the elevated mutation rate and the relaxed sequence stringency of recombination of mutator strains may contribute to the pathogenicity of *E. coli* (14, 17). Homologs of the *E. coli* MutS and MutL MMR proteins have been identified in yeasts, mice, and humans and, as predicted from studies with *E. coli*, their absence results in increased mutation, genome instability, and, in mammals, cancer (reviewed in references 12, 23, and 24).

The molecular mechanism of methyl-directed MMR in *E. coli*, as defined biochemically, includes the following steps (reviewed in references 15, 22, 23, and 28). Repair is initiated by the binding of MutS to the mismatch, of MutL to MutS, and of MutH to a nearby d(GATC) sequence. An incision is made by MutH 5′ to the d(GATC) sequence on an unmethylated DNA strand. The nicked DNA strand is displaced by the coordinated activities of MutS, MutL, and MutU (helicase II) and is degraded by exonucleases specific for single-strand DNA. The exonuclease required depends on the position of the incision relative to the mismatch: if the incision is located 3′ of the mismatch, repair requires the 3′ to 5′ exonucleolytic activity of exonuclease I (ExoI) in a purified system and/or that of an unidentified component in crude extracts (7); if the incision is located 5′ of the mismatch, repair requires the 5′ to 3′ exonucleolytic activity of either RecJ or exonuclease VII (ExoVII). The final steps in MMR require the activities of a single-strand DNA-binding protein, DNA polymerase III, and DNA ligase for filling the single-strand gap left by excision.

Of the components required biochemically, it is clear that MutS, MutL, MutH, and MutU are also needed to perform their respective functions in MMR in vivo. Null mutations in the genes encoding any one of these disable MMR, resulting in cells displaying a mutator phenotype (30–32) and decreased recombination sequence stringency (18, 26, 39). For the single-strand exonucleases, their roles in vivo have been less obvious, in part because of the absence of precise null alleles of the genes encoding them. Razavy et al. (27) constructed the first known precise null allele of the gene encoding ExoI in *E. coli*, a deletion-insertion allele called Δ*xonA300*::*cat*. Previous *E. coli* alleles either are altered-function and dominant alleles (e.g., *sbcB15*) (27), have not been demonstrated to be null alleles (e.g., *xonA2* and *xonA6*) (25), or remove a large segment of the *E. coli* chromosome such that phenotypes cannot be attributed unambiguously to the lack of the ExoI-encoding gene [Δ(*sbcB-his*); cited in reference 7]. Similarly, for ExoVII, the only previously known null alleles remove not only the *xseA* gene encoding the enzyme's large subunit but also a neighboring guanosine biosynthesis gene causing a guanosine requirement (37). This could alter normal DNA metabolism, which could be relevant to DNA MMR. A useful *recJ*-null allele has been described (16). Here we report the construction of the first precise null allele of ExoVII, Δ*xseA18*::*amp* (Table 1), and the first strains carrying precise null mutations in genes encoding all three known *E. coli* single-strand-dependent exonucleases. These strains are described and used to examine the role of the exonucleases in MMR in vivo.

The viabilities of strains of two different genetic backgrounds carrying the null alleles of *xonA*, *xseA*, and *recJ* (Table 1) are normal (Table 2), and their growth curves are not markedly different from those of their Exo^{+} parents (Fig. 1). It was shown previously that strains defective for all three exonucleases display decreased homologous recombination and Chi activity when Chi stimulates recombination opposite heterologous DNA (27). The latter phenotype was observed only in the triple mutant and not in any of the double-exonuclease mutants. As predicted on the basis of their recombination-depressed phenotypes, our strains carrying the null alleles of *xonA*, *xseA*, and *recJ* also display elevated UV light sensitivity. In both genetic backgrounds, their sensitivities are greater than that of any of the single mutants and less than those of *recA* recombination-deficient strains (data not shown).

Studies of MMR in vitro were inconclusive as to whether the absence of the three exonucleases should be sufficient to block MMR in cells. On the one hand, in a purified system, the

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TABLE 1. *E. coli* K-12 strains and plasmids

Strain or plasmid	Relevant genotype or characteristics	Reference, source, or construction
E. coli strains		
AB1157	F^- *thr-1 ara-14 leuB6* Δ(*gpt-proA*)*62 lacY1 tsx-33 supE44 galK2 hisG4 rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1*	1
JC11450	AB1157, spontaneous Su^-	A. J. Clark
FC40	*ara* Δ(*lac-proAB*)$_{XIII}$ *thi* Rif^r [F′ *proAB*$^+$ *lacI33*Ω*lacZ*]	3
CAG12176	*zef-3189*::Tn*10kan*	33
CAG18604	*zgf-3156*::Tn*10kan*	33
STL160	AB1157 Δ(*xseA-guaB*) *zfh-3139*::Tn*10kan*	S. T. Lovett
SMR91	*mutL211*::Tn*5*	Laboratory collection
SMR423	*recD1903*::Tn*10*	Laboratory collection
SMR690	FC40 *recJ284*::Tn*10*	10
SMR838	JC11450 Δ*xonA300*::*cat his*$^+$	Laboratory collection
SMR839	JC11450 Δ*xonA300*::*cat*	27
SMR1403	JC11450 Δ*xonA300*::*cat recJ284*::Tn*10*	27
SMR2597	FC40 Δ(*xseA-guaB*) *zfh-3139*::Tn*10kan*	Laboratory collection
SMR3070	FC40 Δ*xonA300*::*cat*	FC40 × P1(SMR839)
SMR3404	FC40 *mutL211*::Tn*5*	FC40 × P1(SMR91)
SMR3465	*recD1903*::Tn*10* Δ*xseA18*::*amp*	This study[a]
SMR3472	FC40 Δ*xseA18*::*amp*	SMR2597 × P1(SMR3465)[b]
SMR3481	FC40 Δ*xonA300*::*cat recJ284*::Tn*10* Δ*xseA18*::*amp*	FC40 × P1(SMR838), P1(SMR690), P1(SMR3472)[c]
SMR3488	JC11450 Δ*xonA300*::*cat recJ284*::Tn*10* Δ*xseA18*::*amp*	SMR1403 × P1(SMR3472)[c]
SMR3524	JC11450 *mutL211*::Tn*5*	JC11450 × P1 (SMR3404)
SMR4035	FC40 Δ*xonA300*::*cat zef-3189*::Tn*10kan*	SMR3070 × P1(CAG12176)
SMR4036	FC40 *recJ284*::Tn*10 zgf-3156*::Tn*10kan*	SMR690 × P1(CAG18604)
SMR4037	FC40 Δ*xseA18*::*amp zfh-3139*::Tn*10kan*	SMR3472 × P1(STL160)
Plasmids		
pMJ3	pACYC184 derivative containing *xseA* and *guaBA*	This study[d]
pMJ6	pMJ3 derivative containing Δ*xseA18*::*amp*	This study[e]

[a] Constructed by transforming SMR423 with the 5-kb *Ava*I-*Bam*HI fragment of pMJ6 which contains Δ*xseA18*::*amp* and selecting ampicillin-resistant transformants (29).

[b] This transduction confirmed the chromosomal location of Δ*xseA18*::*amp*, as all Amp^r transductants were Gua^+ and the expected linkage to *zfh-3139*::Tn*10kan* (4) was observed.

[c] The presence of the null alleles in the triple mutants was confirmed by P1 transduction of each mutation into genetic backgrounds in which the following characteristic phenotypes were observed: *recJ284*::Tn*10* makes *recB21 recC22 sbcB15 sbcC201* strains extremely UV sensitive (16); *xonA*-null mutations decrease transductional recombination via the RecF pathway (2); and *xseA* mutations enhance sensitivity to low concentrations of nalidixic acid (4). The triple mutants also display UV light sensitivity (discussed in the text), as expected from their reduced-recombination phenotype (27).

[d] A 5-kb *Bgl*I-*Bam*HI fragment from Kohara phage λ427 (λ8E3) (11) containing *xseA* and *guaBA* was ligated into *Bgl*I-*Bam*HI-digested pACYC184 (see, e.g., reference 21). *Bgl*I 3′ overhangs were removed with T4 DNA polymerase (exonuclease activity) prior to ligation.

[e] The 690-bp *Eag*I-*Afl*II fragment of *xseA* (6) (GenBank accession no., J02599) was replaced with an *Eag*I-*Afl*II-digested 1,035-bp PCR fragment containing the *bla* gene of pBR322 (see, e.g., reference 21). *bla* was amplified with primers that create *Eag*I and *Afl*II sites (*Eag*I primer: 5′GTACGGCCGAGTAAACTTGGTCTGACA; *Afl*II primer: 5′ATGCTTAAGTAGACGTCAGGTGGCACT).

presence of ExoVII or RecJ was required for MMR with a d(GATC) sequence 5′ to the mismatch and ExoI was required for repair with the d(GATC) sequence 3′ to the mismatch (7). ExoVII could not substitute for ExoI on 3′ substrates in the purified system (7), despite the fact that ExoVII has been found to possess both 3′ and 5′ single-strand nuclease activity in other in vitro assays (5). On the other hand, in a crude system, MMR of 3′ substrates occurred in extracts of cells lacking ExoI, leading the authors to suggest the possible existence of a fourth single-strand exonuclease in *E. coli* which substitutes for ExoI in their crude system (7). However, ExoVII was present in the crude extract lacking ExoI. Because the ability of ExoVII to digest 3′ ends in the crude system is unknown, it remains possible that ExoVII was supporting the repair of 3′ substrates.

In vivo studies in which recombination was assayed suggested links between the single-strand exonucleases and MMR (8, 9). The authors described MMR protein-dependent recombination of UV-irradiated DNA. They found that single- and

TABLE 2. Viabilities of single-strand exonuclease-deficient strains

Strain	Relevant genotype	CFU/ml (10^9)[a]	No. of observed cells/ml (10^9)[b]	Viability (CFU/cell counted)
JC11450	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$ F^-	2.6 ± 0.13	2.9 ± 0.26	0.9
SMR3488	JC11450 Δ*xonA* Δ*xseA recJ*	2.7 ± 0.14	2.1 ± 0.28	1.3
SMR3524	JC11450 *mutL*	2.9 ± 0.11	2.4 ± 0.75	1.2
FC40	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$ F′	2.9 ± 0.09	2.8 ± 0.31	1.0
SMR3481	FC40 Δ*xonA* Δ*xseA recJ*	2.6 ± 0.18	2.3 ± 0.23	1.1
SMR3404	FC40 *mutL*	2.3 ± 0.07	1.2 ± 0.17	1.9

[a] Mean number of CFU per milliliter ± one standard error of the mean for five independent cultures grown to saturation in Luria-Bertani-Herskowitz (LBH) broth (see, e.g., reference 36).

[b] Mean number of cells per milliliter ± one standard error of the mean in the same five saturated cultures determined with a Petroff-Hausser counter.

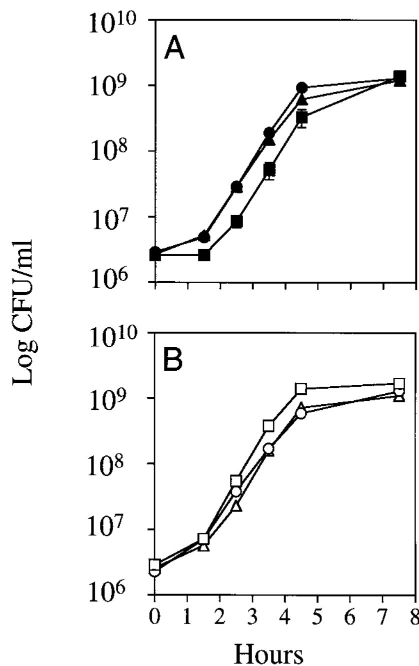

FIG. 1. Growth curves of strains carrying null mutations in *xonA*, *xseA*, and *recJ*. Each point represents the mean of the viable cell counts from three independent cultures. Error bars, one standard error of the mean. (A) JC11450 derivatives; (B) FC40 derivatives. Squares, Exo$^+$ strains; triangles, Δ*xonA300*::*cat* Δ*xseA18*::*amp recJ284*::Tn*10* strains; circles, *mutL* strains.

double-exonuclease mutants display small decreases in the frequency of such recombination, indicating roles for these nucleases in either MMR, recombination, or both (8). Because single- and double-exonuclease mutants have since been shown to have similarly small decreases in their frequencies of normal (MMR-independent) recombination (20, 27), it now seems likely that recombination rather than MMR was inhibited by exonuclease deficiency in the previous study (8). The observation of single-strand DNA formation in nuclease-proficient cells, but not in cells deficient for one of the single-strand exonucleases (8), similarly cannot distinguish whether such single-strand DNA was an intermediate in MMR or recombination or in both processes or neither process.

To test whether single-strand exonucleases are required for MMR in vivo, we asked whether cells lacking ExoI, ExoVII, and RecJ display the mutator phenotype characteristic of MMR-deficient cells. For two separate *E. coli* K-12 strain backgrounds, we found that cells lacking ExoI, ExoVII, and RecJ displayed mutation rates similar to those of their *xonA*$^+$ *xseA*$^+$ *recJ*$^+$ parents (Table 3). In contrast, isogenic strains lacking MutL, an essential component of MMR in vivo, showed greatly elevated mutation rates. Thus, the activities of ExoI, ExoVII, and RecJ appear not to be essential for MMR in vivo.

Could the triple-exonuclease mutants be MMR deficient but fail to display the mutator phenotype? For example, it might be that ExoI, ExoVII, and RecJ are necessary and sufficient exonuclease activities for MMR in vivo but that triple-mutant cells initiating MMR die from accumulation of the nicked but not displaced DNA intermediate. This would kill those cells that experienced polymerase errors and so prevent a mutator phe-

TABLE 3. Mutation rates[a]

Strain	Genetic background	Relevant genotype	Expt no.	Rate of mutation[b] (10^{-10}) phenotype indicated		
				Nal^r	Str^r	Arg$^+$
JC11450	JC11450	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$ *mutL*$^+$	1	1.5	NA[c]	12
			2	1.2	NA	11
			3	3.7	NA	45
SMR3488	JC11450	Δ*xonA300*::*cat recJ284*::Tn*10* Δ*xseA18*::*amp*	1	<1.6[d]	NA	44
			2	<1.2[d]	NA	36
			3	3.0	NA	59
SMR3524	JC11450	*mutL211*::Tn*5*	1	580	NA	740
			2	700	NA	430
			3	700	NA	220
FC40	FC40	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$ *mutL*$^+$	3	5.0	1.3	NA
			4	4.7	0.83	NA
			5	3.6	0.82	NA
SMR3481	FC40	Δ*xonA300*::*cat recJ284*::Tn*10* Δ*xseA18*::*amp*	3	2.4	0.74	NA
			4	<2.9[d]	<1.0[d]	NA
			5	<3.8[d]	<1.1[d]	NA
SMR3404	FC40	*mutL211*::Tn*5*	3	1,100	91	NA
			4	1,000	40	NA
			5	1,200	53	NA

[a] For each strain and experiment, the number of cultures was 25.

[b] Number of mutations per cell per generation. Nalidixic acid-resistant (Nal^r) and streptomycin-resistant (Str^r) colonies were selected on Luria-Bertani-Herskowitz (LBH) plates (see, e.g., reference 36) supplemented with 40 μg of nalidixic acid or 100 μg of streptomycin per ml. Arginine prototrophs (Arg$^+$) were selected on minimal M9 plates (21) with the addition of 0.1% glycerol and 5 μg of the appropriate amino acids per ml. Mutants were scored after ca. 24 (LBH) or 72 h (M9) of incubation at 37°C. Mutation rates were calculated by the method of the median as modified by von Borstel (38).

[c] Not applicable (JC11450 is Str^r and FC40 is Arg$^+$).

[d] In these cases more than half of the cultures produced no mutant colonies and the mutation rate was calculated using a median of <1; the rates are thus overestimates and are preceded by "<."

TABLE 4. Ability to construct triple-exonuclease mutants without direct selection[a]

Genetic background	Relevant genotype of: Donor	Recipient	No. of col.[c]	Frequency of cotransducing *exo* mutation with linked Tn*10kan* (mean ± SD)[b]
JC11450	Δ*xseA18*::*amp zfh-3139*::Tn*10kan*	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$	148	0.19 ± 0.03
		Δ*xonA300*::*cat recJ284*::Tn*10*	573	0.19 ± 0.03
	Δ*xonA300*::*cat zef-3189*::Tn*10kan*	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$	161	0.24 ± 0.05
		recJ284::Tn*10* Δ*xseA18*::*amp*	170	0.27 ± 0.05
	recJ284::Tn*10 zgf-3156*::Tn*10kan*	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$	123	0.13 ± 0.04
		Δ*xonA300*::*cat* Δ*xseA18*::*amp*	244	0.20 ± 0.05
FC40	Δ*xseA18*::*amp zfh-3139*::Tn*10kan*	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$	886	0.19 ± 0.01
		Δ*xonA300*::*cat recJ284*::Tn*10*	1,420	0.18 ± 0.01
	Δ*xonA300*::*cat zef-3189*::Tn*10kan*	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$	1,010	0.27 ± 0.01
		recJ284::Tn*10* Δ*xseA18*::*amp*	902	0.21 ± 0.01
	recJ284::Tn*10 zgf-3156*::Tn*10kan*	*xonA*$^+$ *xseA*$^+$ *recJ*$^+$	540	0.17 ± 0.01
		Δ*xonA300*::*cat* Δ*xseA18*::*amp*	576	0.15 ± 0.02

[a] If secondary suppressor mutations are necessarily selected when constructing triple-exonuclease null mutants, then most transductants of a double-exonuclease mutant with the final nuclease allele would be absent from the transduction progeny—only the rare double-nuclease mutant that already carries a secondary suppressor mutation would form a detectable transductant colony. The frequency of cotransduction of the third nuclease allele with a nearby selectable marker would then appear to be much lower when transducing into double-nuclease mutant recipient strains than into exonuclease-proficient recipients. These frequencies were compared for all of the possible orders of construction of triple-nuclease mutants in both strain backgrounds used in this study. In each case, a nearby selectable kanamycin resistance-encoding transposon, Tn*10kan*, was selected, and the number of colonies also carrying the linked nuclease allele was determined by assaying the (other) drug resistance encoded by the cassette disrupting each nuclease gene.

[b] Three separate P1 transductional crosses were performed. Standard transduction methods were used (21).

[c] The total number of transductant colonies screened.

notype. This possibility seems unlikely for two reasons. First, the viabilities of strains lacking ExoI, ExoVII, and RecJ are normal (Table 2), providing no evidence that those attempting MMR die. Second, *mutU* helicase mutants would be expected to accumulate a similar DNA intermediate (a nicked but not displaced strand) but these are viable and display a mutator phenotype (31, 32). This argues that failure to complete MMR after nicking is not a lethal event.

We will discuss three possible explanations for the results presented. First, another as yet uncharacterized exonuclease(s) may be sufficient for MMR. Cooper et al. (7) postulated that another exonuclease must contribute after they found repair of 3′ but not 5′ substrates in cells lacking ExoI and RecJ. If such an activity catalyzes MMR in vivo in our assay, then it is interesting that apparently normal levels of MMR can be accomplished with only this 3′ nuclease.

Regardless of the polarity of a putative substituting nuclease(s), the existence of one or more is suggested by the discoveries of MMR-associated single-strand exonucleases in the yeasts *Schizosaccharomyces pombe* (34) and *Saccharomyces cerevisiae* (35). In these systems a single 5′ single-strand-dependent exonuclease associates with (35) and/or is required for proper function of (34) the MMR apparatus.

There are two uncharacterized open reading frames in the *E. coli* genome sequence that contain conserved exonuclease motifs, although neither gene's product has been tested yet for nuclease function (13). Either of these or an as yet unidentified gene might supply a function that substitutes for that of ExoI, ExoVII, and RecJ exonucleases in MMR in vivo.

Second, it is formally possible that the three single-strand exonucleases are normally required for MMR in vivo and that cells lacking them do not show an in vivo mutator phenotype because their absence induces the expression of a new substituting activity. This idea could be tested by in vitro analysis of MMR in crude extracts of our triple-exonuclease-defective strains, as both the crude and purified in vitro MMR assays require exonuclease, at least when 5′ substrates are used (7). If a new exonuclease-bypassing (or substituting) activity were expressed only in the triple mutant, one might expect 5′ substrates to be repaired in crude extracts of the triple-exonuclease mutants, but not in double-mutant extracts, as was reported previously for 5′ substrates (7). Also, any 3′ substrate repair detected would be unambiguously independent of ExoVII.

One specific version of this general idea is addressed by data shown in Table 4. If the triple-nuclease-defective strain grew slowly or were inviable, then cells already harboring a secondary (suppressor) mutation might usually be selected when constructing triple-exonuclease mutants. When this kind of problem occurs, most cells receiving the deleterious mutation during the strain construction (the third nuclease allele in our constructions) are lost, and the rare suppressor-carrying mutants predominate among progeny that carry the deleterious mutation. This possibility was tested by determining the efficiency of recovering the third nuclease mutation in P1 transduction experiments in which the third nuclease mutation is introduced by selection for a nearby, linked marker rather than by direct selection. We find that there is no bias against recovering the third exonuclease mutation in double-exonuclease mutants, as compared with exonuclease-proficient strains (Table 4). This argues strongly against the presence of secondary suppressor mutations in triple-exonuclease-deficient strains.

Finally, it could be that single-strand exonuclease activity is not required for MMR in vivo, even though it is required in vitro. Complete displacement of the unmethylated DNA strand by MutU helicase may be unfavorable in vitro, perhaps because the displaced single-strand DNA can reanneal. Exonuclease activity would then be required to degrade the displaced DNA strand. This requirement might be bypassed in vivo if MutU, MutS, and MutL could remove the unmethylated DNA strand completely. This might occur in vivo but not in vitro for any of several possible reasons including the following.

(i) In vivo, the displaced strand might not reanneal because it competes for reannealing with the (perfectly complementary) parental strand at the replication fork. A competitor strand is not provided in vitro.

(ii) A single nick was provided to direct MutU helicase in the experiments demonstrating nuclease requirements in vitro.

Perhaps the normal in vivo substrate is a mismatch flanked by two nicks, making complete removal of the displaced strand possible without exonucleolytic digestion.

(iii) The displaced strand might be stabilized and prevented from reannealing in vivo by single-strand binding proteins or other activities or conditions that might not have been optimized in the in vitro systems.

With any of these possibilities, the single-strand exonucleases might still degrade the displaced single strand, but this would not be an obligate step in MMR.

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