Isolation and Characterization of Novel Plasmid-Encoded umuC Mutants

ROGER WOODGATE,1* MAHIPAL SINGH,1 OLGA I. KULAEVA,1 EKATERINA G. FRANK,1 ARTHUR S. LEVINE,1 AND WALTER H. KOCH2

Section on DNA Replication, Repair and Mutagenesis, National Institute of Child Health and Human Development, Bethesda, Maryland 20892, and Molecular Biology Branch, Food and Drug Administration, Washington, D.C. 20204

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Most inducible mutagenesis in Escherichia coli is dependent upon the activity of the UmuDC proteins. The role of UmuC in this process is poorly understood, possibly because of the limited number of genetically characterized umuC mutants. To better understand the function of the UmuC protein in mutagenic DNA repair, we have isolated several novel plasmid-encoded umuC mutants. A multicopy plasmid that expressed UmuC at physiological levels was constructed and randomly mutagenized in vitro by exposure to hydroxylamine. Mutated plasmids were introduced into the umu tester strain RW126, and 16 plasmids that were unable to promote umuC-dependent spontaneous mutator activity were identified by a colorimetric papillation assay. Interestingly, these plasmid mutants fell into two classes: (i) 5 were expression mutants that produced either too little or too much wild-type UmuC protein, and (ii) 11 were plasmids with structural changes in the UmuC protein. Although hydroxylamine mutagenesis was random, most of the structural mutants identified in the screen were localized to two regions of the UmuC protein; four mutations were found in a stretch of 30 amino acids (residues 133 to 162) in the middle of the protein, while four other mutations (three of which resulted in a truncated UmuC protein) were localized in the last 50 carboxyl-terminal amino acid residues. These new plasmid umuC mutants, together with the previously identified chromosomal umuC25, umuC36, and umuC104 mutations that we have also cloned, should prove extremely useful in dissecting the genetic and biochemical activities of UmuC in mutagenic DNA repair.

The ability of Escherichia coli DNA polymerase III holoenzyme to replicate across bulky misinstructional lesions in DNA, such as cyclobutane pyrimidine dimers or 6-4 pyrimidine-pyrimidone photoproducts, is dependent upon the functional activity of the RecA and UmuD'C proteins (4, 14, 43, 50, 58). In addition, studies with a defined lesion in phage M13 DNA have shown that the extent and mutagenic consequences of translesion DNA synthesis vary and depend upon the specific type of DNA lesion encountered (3, 4, 25, 32-34).

Both genetic and biochemical experiments have shown that the RecA and UmuD proteins are induced as part of the cellular SOS response to DNA damage (53, 58). Furthermore, RecA mediates the processing of UmuD to a shorter but mutagenically active form, UmuD' (8, 40, 46). Mutants of umuD or recA that are unable to promote cleavage are rendered phenotypically nonmutable (2, 18, 31, 46). RecA also participates directly in the mutagenic process by targeting the Umu-like mutagenesis proteins to DNA (13, 20). Although recent studies have shown that DNA polymerase III holoenzyme can bypass a synthetic abasic lesion in vitro only in the presence of the highly purified UmuD'C and RecA proteins (43), the biochemical action of these proteins remains to be resolved. In particular, very little is known about UmuC's role in this process. To some extent this is due to the limited number of umuC mutants that have been characterized genetically. The E. coli umuC gene consists of 1,269 base pairs, and to date, only eight umuC mutants have been reported: four nonsense mutants (three chromosomal and one plasmid) (28, 31, 36, 48), two frameshift mutants that result in nonsense mutations (both plasmid encoded) (41), and two insertional mutants (both chromosomal) (1, 16).

In an attempt to better characterize UmuC function both genetically and biochemically, we attempted to isolate novel umuC mutants. We have recently developed an Escherichia coli strain that is particularly useful in identifying UmuC activity by way of a colorimetric papillation assay (24). We have used this assay to screen for randomly mutated umuC plasmids that were unable to promote normal SOS mutagenesis functions. In this report, we present a molecular and genetic characterization of some of these mutants. Our findings suggest that the activity of certain regions of the UmuC protein may be critical for activity and that changes in the levels of wild-type UmuC can also affect the efficiency at which mutagenesis functions are promoted.

MATERIALS AND METHODS

Bacterial strains. The E. coli strains used in this study are listed in Table 1. All have been reported elsewhere (1) except RW222, which was constructed by a series of P1 transductions as follows. The suAL211 mutation was introduced into TK603 (28) by initially transducing in the suAL100::Tn5 and closely linked pyrD mutations from DE124 (24) and then replacing them with suAL211 pyrD" from DE1776 (18). The Δ(umuDC) 595::cat, recA718 srlC300::Tn10, and lea71::Tn5 mutations were subsequently transduced from RW82 (55), DE1918 (24), and JL1047 (35) into this strain by selecting for chloramphenicol resistance, tetracycline resistance and UV sensitivity, and kanamycin resistance, respectively.

Plasmids. The E. coli umuD and umuC genes overlap by 1 base pair (30, 42), and mutations in either umuD or umuC render bacteria phenotypically nonmutable. To facilitate the specific identification of umuC mutants, we have manipulated
the umuD and umuC genes to physically separate them and place them on different but compatible plasmids.

A low-copy-number spectinomycin-resistant plasmid, pRW66, that expresses UmuD'C, the mutagenically active form of UmuD, was constructed by ligating an appropriately modified 1-kb umuD' BagII fragment from pGW123 (40) and a SalI-digested, pSC101-derived vector, pGB2 (10).

The starting vector for the UmuC plasmid was pDS110 (11), a ~10 kb CoElI-based plasmid that expresses UmuDC and confers resistance to kanamycin and ampicillin. Donnelly and Walker (11) engineered this plasmid to create a novel EcoRI restriction site between the LexA binding site and the Shine-Dalgarno ribosome binding site of the umuDC operon (Fig. 1). Plasmid pRW116 was constructed by replacing a 4-kb EcoRI fragment from pDS110, encoding kanamycin resistance and the structural umuDC operon, with a 2-kb EcoRI fragment carrying the genetically engineered umuD'C operon from plasmid pEC42 (20) (Fig. 1). Although this plasmid does not contain a bona fide ribosome binding site, expression of UmuD'C from this plasmid was sufficient to fully restore mutagenesis functions to ΔumuDC strains (data not shown).

The UmuC plasmid pRW124 was constructed by removing the Neo-MluI umuD' interval from pRW116 and replacing it with two synthetic oligonucleotides which, when annealed together, reconstructed the start of the umuC gene (Fig. 1). Like pRW116, this construct does not contain any obvious Shine-Dalgarno binding site, yet expression of UmuC from pRW124 was sufficient to fully restore mutagenesis functions to umuC36 mutants (see below).

Both the medium-copy-number plasmid pRW30 (55) and the low-copy-number plasmid pRW154 (24) that express the UmuDC proteins from their natural promoter have been described previously.

In vitro mutagenesis of pRW124 and screening for umuC mutants. Randomly mutated pRW124 was obtained following the hydroxyamine mutagenesis procedure described by Isackson and Bertrand (26). Briefly, 2 μg of purified plasmid DNA was incubated in 100 μl of 800 mM hydroxylamine HCl (Sigma)-50 mM sodium phosphate, pH 6.0-1 mM EDTA for 48 h at 37°C. The mutated DNA was recovered in TE (10 mM Tris HCl, pH 8.0, 1 mM EDTA) by repeated dilution and concentration cycles in a Centricon 30 ultrafiltration cartridge (Amicon). Potential umuC mutants were identified by introducing the mutagenized plasmid DNA into the umu tester strain RW126 harboring the low-copy-number umuD' plasmid pRW66. Transformants were selected on Luria-Bertani (LB) plates supplemented with spectinomycin (50 μg/ml) and ampicillin (100 μg/ml) and then restreaked with a sterile toothpick onto MacConkey galactose (1%) indicator plates supplemented with spectinomycin and ampicillin. After 10 days of incubation at 37°C, colonies that produced less than half of the number of Gal⁺ papillae produced by the control strain were analyzed further by the qualitative His⁺ mutagenesis assay.

Qualitative mutagenesis assay. Bacterial cultures were grown overnight in LB medium containing the appropriate antibiotics. Aliquots (1 ml) were centrifuged and resuspended in an equal volume of SM buffer (38). The ability of particular plasmid-bearing strains to promote Umu-dependent SOS mutator activity in the absence of exogenous DNA damage was judged by plating 100-μl aliquots on Davis and Mingioli minimal agar plates supplemented with a trace amount of histidine (1 μg/ml) (7). Umu-dependent chemically induced mutagenesis was determined essentially as described above, except that 5 μl of a 1:5 dilution of dimethyl sulfoxonate (MMS) (Sigma) in dimethyl sulfoxide (Sigma) was applied to a small sterile disk in the center of the plate. Both spontaneously arising and MMS-induced His⁺ mutants were scored after 4 days of incubation at 37°C.

Quantitative mutagenesis assays. Plasmids that partially restored mutagenesis functions based upon the qualitative assay were further analyzed by quantitative UV- and MMS-induced mutagenesis assays. Bacterial cultures were grown to a cell density of ~1 × 10⁸/ml in LB broth with appropriate antibiotics, harvested, and resuspended in SM buffer. For UV mutagenesis assays, cells were irradiated and appropriate dilutions were plated on the low-histidine medium containing the indicated volume of MMS for 30 min at 37°C, after which cells were pelleted and washed in SM buffer; the appropriate dilutions were then plated onto His⁺ indicator plates and His⁺ mutants were scored after 4 days of incubation at 37°C, and induced mutation frequencies were calculated as described by Sedgwick and Bridges (45).

UV survival. Early-log-phase bacteria grown in LB medium plus appropriate antibiotics were harvested, resuspended in SM buffer, and irradiated with 254-nm UV light. Dilutions...
were plated in triplicate on LB agar plates, and surviving colonies were counted after overnight incubation at 37°C.

**Steady-state levels of wild-type and mutant UmuC proteins.** Polyclonal antibodies were raised in rabbits to affinity-purified UmuC protein (57) by Hazleton Laboratories (Washington, D.C.). UmuC protein was detected in whole-cell E. coli extracts by the Western-light chemiluminescent assay (Tropix) essentially as described previously (23, 56). Briefly, whole-cell extracts from ~10^10 cells were separated by electrophoresis in a 15% acrylamide–sodium dodecyl sulfate gel. Proteins were transferred to a nitrocellulose support membrane and incubated overnight with a 1:10,000 dilution of UmuC antisera. After appropriate washes, the membranes were incubated with a 1:3,000 dilution of a goat anti-rabbit antibody conjugated with alkaline phosphatase (Bio–Rad). UmuC was visualized after the membrane was incubated with chemiluminescent substrate and exposed to X-ray film for an appropriate period of time. The relative level of UmuC protein was obtained by densitometric analysis of films. In most cases, values were obtained from multiple exposures so that the desired bands were in the linear range of the film (56).

**DNA sequence analysis.** The umuC gene in plasmid pRW124 (and mutant derivatives) was sequenced by Lark Sequencing Technologies Inc. (Houston, Tex.) by standard dideoxy sequencing protocols (44) and with seven unique oligonucleotide primers spaced about 200 bp apart.

**Subcloning multiple umuC plasmid mutations and cloning of** the previously identified chromosomal umuC25, umuC36, and umuC104 mutations. DNA sequence analysis revealed that plasmid pRW124-73 contained three mutations in umuC. A plasmid that contained only the most 5′ mutation (pRW124-73A; AT3) was constructed by replacing the MluI–PstI fragment of pRW124-73 with a similar fragment from an undamaged plasmid. The second mutation (pRW124-73B; GS24) was isolated as an MluI–HindIII fragment and was cloned into a similarly digested wild-type vector. The most 3′ mutation (pRW124-73C; QX399) was isolated as a BamHI–PstI fragment and was cloned into a similarly digested wild-type vector.

Plasmid pRW124-139 contained two mutations in umuC, one of which was silent. It also exhibited elevated levels of UmuC, suggesting that it may contain an additional mutation that affected plasmid copy number (data not shown). The mutation resulting in the structural change in umuC (pRW124-139B; QX372) was obtained as a HindIII–BamHI fragment and was cloned into the similarly digested wild-type vector.

The chromosomal umuC25 mutation was obtained from strain DE1878 (31) by PCR amplification of a 952-bp fragment with two oligonucleotide primers, 5′-GAGAAATTCGGCAACCGTGTTGTGA3′ (781s) and 5′-CTCTTGGCGCTTCTG3′ (1733a) (5′ nucleotides of the primers are shown in parentheses; s and a refer to sense and antisense strands, respectively [42]). Amplified DNA was digested with HindIII and BamHI, and cloned into HindIII–BamHI digested plasmid pRW124. The presence of the umuC25 allele was confirmed by allele-specific oligonucleotide colony hybridization analysis using a 22-mer end-labelled oligonucleotide probe (5′-GTATATCCGAGCTTTAAAG-3′) as previously described, omitting the psoralen cross-linking step (9).

**umuC104** was obtained from DE1882 (31) as a 685-bp PCR fragment by using primers 5′-GTGAAGATATCGGTATG-3′ (362s) and 5′-CTGTATCTGGACGGAC-3′ (1084a), and cloned into pRW124 after digestion with MluI and HindIII. The presence of the umuC104 mutation was confirmed by the presence of an extra AseI restriction site that results from the G→A transition found in umuC104 (31).

**umuC36** was obtained from TK610 (28, 31) as a 477-bp PCR fragment by using primers 5′-ATGGGGCGATCCCTGG-3′ (570s) and 5′-CTGTATCCGACGAGATG-3′ (1047a) and was cloned as a 78-bp BglII–SalI fragment into pRW134 (18). The umuC36 mutation was subsequently subcloned from pRW134 into pRW124 as an AseI–BamHI fragment. The presence of the umuC36 mutation was confirmed by the presence of an extra HindIII restriction site that results from the G→A transition found in umuC36 (31).

All PCR-amplified regions were subjected to DNA sequence analysis to confirm that no additional unwanted mutations were inadvertently cloned.

**RESULTS**

**Isolation of plasmid-encoded umuC mutants.** The aim of this study was to isolate new umuC mutants, characterization of which might provide some insight into the activities of UmuC. Since mutations in either umuD or umuC render E. coli nonmutable, our first step was to clone the umuC genes onto separate but compatible plasmids. The mutagenically active UmuD' protein was expressed from the low-copy-number plasmid, pRW66, whereas UmuC was expressed from the medium-copy-number plasmid pRW124 (Fig. 1). Both plasmids contain the same 5′ umu regulatory regions, and as a result, both the UmuD' and UmuC proteins are regulated by LexA protein and are expressed from the natural umuDC operon promoter. The exception is that pRW124 lacks an obvious ribosome binding site (Fig. 1). As a result, the amount of UmuC produced from the medium-copy-number plasmid, pRW124, is dramatically reduced compared with the amount of UmuC expressed from the umuDC operon cloned into a plasmid with a similar copy number (cf. pRW124 and pRW30; Fig. 2). In fact, the amount of UmuC produced from pRW124...
was comparable to that seen with the umuDC operon on the low-copy-number plasmid pRW154 and only 2.5-fold higher than that expressed chromosomally in a recA730 lexA(Def) strain (Fig. 2). On the basis of our previous calculations of 700 UmuC molecules per recA730 lexA(Def) cell (56), we estimate that the level of UmuC produced from pRW124 in strain RW126 corresponds to ~1,750 molecules per cell. By comparison, we have previously estimated that there are ~2,500 UmuD' molecules per recA730 lexA(Def) cell. Plasmid pRW66 produces roughly fourfold more UmuD' than that expressed from the chromosome (data not shown) and would therefore be expected to give rise to ~10,000 UmuD' molecules per cell. Given that UmuD(D') is a dimer under physiological conditions (6, 57), this equates to a ratio of approximately three UmuD' dimers to one UmuC molecule. Previously, we have noted that when expressed from the natural umuDC operon, UmuD(D') is produced in an approximately sixfold excess over UmuC (56). The two-plasmid system employed here, therefore, maintains close to physiological levels and ratios of the UmuD'C proteins and avoids most, if not all, of the problems that are commonly associated with overproducing the Umu proteins from multicopy plasmids (37, 52).

By physically separating the umu genes on different plasmids, we were able to randomly mutate the umuC plasmid in the absence of umuD'. Potential mutants were easily identified by introducing the mutagenized plasmid into RW126/pRW66 and screening for SOS-dependent mutator activity with a colorimetric galactose papillation assay (24). A total of 1,500 separate colonies were assayed, and those that produced less than half of the normal number of Gal' papillae were chosen for further study. Of 140 potential mutants analyzed, 16 appeared to be either partially or completely deficient in SOS mutator activity as judged by their ability to revert the hisG4(Oc) mutation (Table 2). Generally, most plasmids were unable to promote both spontaneous and MMS-induced mutagenesis. The exceptions were pRW124-132, which appeared proficient at spontaneous mutagenesis but had a reduced capacity for MMS mutagenesis; pRW124-126, which was par-

### Table 2. Plasmid-encoded umuC mutants

<table>
<thead>
<tr>
<th>umuC plasmid</th>
<th>Mutagenesis functions (His' mutants/plate)*</th>
<th>Mutation in umuC*</th>
<th>umuC allele</th>
<th>Relative amt of UmuC</th>
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</thead>
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<tr>
<td>None&lt;sup&gt;i&lt;/sup&gt;</td>
<td>3 7 14</td>
<td>NA' NA NA</td>
<td>NA</td>
<td>NA</td>
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<td>123 444 250</td>
<td>NA NA NA</td>
<td>NA</td>
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<td>pRW124-10</td>
<td>14 248 167</td>
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<td>umuC251</td>
<td>ND&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>3 5 16</td>
<td>872G→A A1512</td>
<td>umuC252&lt;sup&gt;i&lt;/sup&gt;</td>
<td>ND</td>
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<td>umuC253</td>
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<tr>
<td>pRW124-65</td>
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<td>1256C→T RC279</td>
<td>umuC262</td>
<td>1.2</td>
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<tr>
<td>pRW124-73</td>
<td>3 5 11</td>
<td>426G→A AT3</td>
<td>umuC254&lt;sup&gt;i&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pRW124-77</td>
<td>3 9 16</td>
<td>1284T→C SP289</td>
<td>umuC255</td>
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</tr>
<tr>
<td>pRW124-92</td>
<td>7 116 29</td>
<td>837G→A A1512</td>
<td>umuC256</td>
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<tr>
<td>pRW124-100</td>
<td>5 7 20</td>
<td>1642C→T NA</td>
<td>umuC257</td>
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<td>pRW124-120</td>
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<tr>
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<td>84 163 137</td>
<td>NA 903G→A</td>
<td>umuC260</td>
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<tr>
<td>pRW124-134</td>
<td>12 84 20</td>
<td>NA</td>
<td>umuC260</td>
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</tr>
<tr>
<td>pRW124-138</td>
<td>6 6 25</td>
<td>845C→T Silent</td>
<td>umuC261&lt;sup&gt;i&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pRW124-139</td>
<td>6 12 19</td>
<td>1533C→T Silent</td>
<td>umuC261&lt;sup&gt;i&lt;/sup&gt;</td>
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<td>2 5 18</td>
<td>591C→T QX(Oc)58</td>
<td>umuC262</td>
<td>ND</td>
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</tbody>
</table>

<sup>*</sup>The data presented are the mean values from at least three separate cultures, with three plates per culture. RW126 was used to determine the ability of plasmids to promote spontaneous and MMS mutagenesis, while TK610 was used to assess MMS-induced umuC36 complementation.

<sup>i</sup>The numbering system used is based upon that previously described by Perry et al. (42). Wild-type umuC consists of 1,269 bp or 422 amino acids. Amino acid changes are noted by the single-letter code for the original residue followed by the residue in the mutant protein. X denotes a termination codon. The specific termination codon is indicated in parentheses, Am, amber; Oc, ochre; Op, opal.

<sup>j</sup>The data presented are representative and are based upon the densitometric analysis of multiple exposures of Fig. 4.

<sup>k</sup>The data presented for spontaneous and MMS mutagenesis are in the presence of the low-copy-number umuD' plasmid pRW66.

<sup>l</sup>NA, not applicable.

<sup>m</sup>ND, not detectable.

<sup>n</sup>The allele number refers to the combination of mutations found in each particular plasmid. Where subcloned, the individual mutations have been given separate allele designations.
mutagenesis (Table 2).

Our interest was in determining if any of the plasmid mutants could complement a missense umuC mutation. Since the umuC36 allele is the best characterized of the three chromosomal umuC mutants, all 16 plasmids were isolated and introduced into TK610 (umuC36 uvrA6) and analyzed for their ability to promote MMS-induced mutagenesis. Most plasmids failed to complement umuC36, and only plasmids pRW124-10, pRW124-92, and pRW124-132 showed any appreciable increase in inducible mutagenesis (Table 2).

Nucleotide changes in pRW124 derivatives unable to promote mutagenesis functions. Our strategy to isolate umuC mutants was based upon the assumption that any plasmid that failed to promote mutagenesis functions would probably contain a mutation in umuC. Although DNA sequence analysis proved this to be the case for 12 of the 16 plasmids (Table 2),

![Consensus alignment of five UmuC-like proteins and position of E. coli umuC mutants. The five previously characterized UmuC-like proteins were aligned by using the program GeneWorks (IntelliGenetics, Inc.) Areas that are boxed indicate regions that are 100% conserved in all five of the previously described UmuC-like proteins. Residues that are found in the consensus protein are indicated with a period (.) Residues that are different from the consensus are indicated by the single-letter code for that residue. umuC mutations and the plasmid from which they were identified are indicated below the alignment. Although mutations AT3 and GS24 cause only a slight loss of function (Table 3), they are noted in addition to the position of the four previously identified chromosomal umuC mutants (umuC25, umuC36, umuC104, and umuC122) and one plasmid-encoded umuC mutant (umuC125) for the sake of completeness.](attachment:Consensus_alignment.png)

![Consensus alignment of five UmuC-like proteins and position of E. coli umuC mutants. The five previously characterized UmuC-like proteins were aligned by using the program GeneWorks (IntelliGenetics, Inc.) Areas that are boxed indicate regions that are 100% conserved in all five of the previously described UmuC-like proteins. Residues that are found in the consensus protein are indicated with a period (.) Residues that are different from the consensus are indicated by the single-letter code for that residue. umuC mutations and the plasmid from which they were identified are indicated below the alignment. Although mutations AT3 and GS24 cause only a slight loss of function (Table 3), they are noted in addition to the position of the four previously identified chromosomal umuC mutants (umuC25, umuC36, umuC104, and umuC122) and one plasmid-encoded umuC mutant (umuC125) for the sake of completeness.](attachment:Consensus_alignment.png)
to our surprise, four plasmids that resulted in poorly mutable or nonmutable phenotypes (pRW124-40, pRW124-100, pRW124-132, and pRW124-138) did not contain any changes in the structural umuC gene. We have classified these plasmids as expression mutants.

In vitro, hydroxylamine almost exclusively produces C→T transitions. With the exception of pRW124-77, which contained a C→T transition, all of the mutations identified in the 12 plasmids with structural mutations in umuC could be attributed to a C→T transition in either the coding or noncoding strand of umuC (Table 2). (It is possible that the mutation identified in pRW124-77 occurred in vivo as a consequence of the normal spontaneous mutator activity exhibited by RW126/pRW66/pRW124 [Table 2].)

Although many of the 12 plasmids with a structural umuC mutation contained only a single-base-pair substitution, five plasmids, pRW124-34, pRW124-65, pRW124-73, pRW124-139, and pRW124-140, contained multiple changes. However, the two changes in pRW124-65 were in the same codon, and one of the two mutations in pRW124-139 and pRW124-140, respectively, was silent. Although hydroxylamine mutagenesis is generally thought to be random, most of the structural mutants identified in our screen for nonmutable umuC plasmids were localized to two regions of the UmuC protein; four missense mutations identified in plasmids pRW124-126, pRW124-92, pRW124-34, and pRW124-134 were found in a stretch of 30 amino acids (residues 133 to 162) in the middle of the protein (Table 2; Fig. 3), whereas four other mutations encoded by plasmids pRW124-139, pRW124-73C, pRW124-120, and pRW124-111 (the first three of which resulted in a truncated UmuC protein) were localized in the last 50 carboxyl-terminal amino acid residues (Table 2; Fig. 3).

Of particular interest was the C→T transition mutation found in pRW124-10 that changed the glutamine residue at position 172 to a TAG amber stop codon. Strain RW126 carries the amber suppressor mutation supE44 that encodes for a glutamine-inserting tRNA rather than a termination signal (22). The efficiency of extragenic suppression varies and, in the case of supE44, is estimated to occur somewhere between 0.8 and 20% of the time (15, 22, 39). Thus, since the wild-type residue in pRW124 already encodes for glutamine, if suppressed by SupE44, pRW124-10 would produce wild-type UmuC. Although this plasmid does contain a bona fide mutation in the structural umuC gene, it was considered a conditional expression mutant since it produces wild-type UmuC in an amber-suppressing background, albeit at somewhat lower levels. Interestingly, as noted above, the plasmid was unable to promote efficient spontaneous mutagenesis but was partially proficient for MMS-induced mutagenesis (Table 2; and below).

Steady-state levels of plasmid-encoded UmuC protein. Upon analysis of the UmuC protein expressed from all 16 plasmids, all four that lacked structural mutations exhibited elevated steady-state levels of UmuC compared to the wild type (Table 2; Fig. 4). The increase varied between twofold for plasmid pRW124-132 and eightfold for plasmid pRW124-40. We believe that the increased expression occurs through an increase in the copy number of the plasmid since the amount of plasmid DNA isolated from these derivatives was much higher than normal and was comparable to that of high-copy-number plasmids (data not shown). In contrast, plasmids with structural changes in umuC exhibited somewhat lower steady-state levels than wild type (Tables 2 and 3; Fig. 4 and 5). The exception was pRW124-139, which gave approximately 2.3-fold higher UmuC. However, like the other plasmids with increased UmuC levels, this probably occurs through an additional plasmid copy number mutation (see below). As might be expected from the nucleotide changes, plasmids pRW124-139, pRW124-73, and pRW124-120 produced truncated UmuC proteins. With the exception of the UmuCs encoded by pRW124-10, pRW124-34, pRW124-73, and pRW124-140, steady-state levels of all the plasmid encoded mutant UmuC proteins were comparable to, or greater than, the chromosomally expressed UmuC in a recA+ lexA(Def) strain (cf. Fig. 2 and 4). Since this amount is clearly sufficient to support cellular mutagenesis, these plasmid mutants are presumably phenotypically Umu- because of a loss of function rather than a simple reduction in UmuC protein (cf. pRW124-10).
TABLE 3. Separation of the multiple mutations identified in pRW124-73 and pRW124-139 and comparison with chromosomal umuC missense mutations cloned into pRW124

<table>
<thead>
<tr>
<th>umuC plasmid</th>
<th>Mutagenesis functions (His+ mutants/plate)*</th>
<th>Mutation in umuC*</th>
<th>umuC allele</th>
<th>Relative amt of UmuC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Spontaneous 3</td>
<td>MMS induced 5</td>
<td>umuC36 complementation 14</td>
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<td>pRW124(wt)</td>
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<td>389</td>
<td>223</td>
<td>426&lt;sup&gt;c&lt;/sup&gt;G→A</td>
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<sup>a</sup>The data presented are the mean values from at least three separate cultures, with three plates per culture. RW126 was used to determine the ability of plasmids to promote spontaneous and MMS mutagenesis, while TK610 was used to assess MMS-induced umuC36 complementation.

<sup>b</sup>Amino acid changes are noted by the single-letter code for the original residue followed by the residue in the mutant protein. X denotes a termination codon. The nucleotide and amino acid changes in the chromosomal umuC25, umuC36, and umuC104 mutants were obtained from the results of Koch et al. (31).

<sup>c</sup>The data presented are representative and are based on the densitometric analysis of multiple exposures of Fig. 6.

<sup>d</sup>The data presented for spontaneous and MMS mutagenesis are in the presence of the low-copy-number umuD' plasmid pRW66.

<sup>e</sup>NA, not applicable.

Ability of plasmid umuC mutants to promote IRR. In addition to their role in inducible mutagenesis, the Umu proteins (or functional homologs) are required for induced replisome reactivation (IRR) in recA718 and recA727 strains (24, 29, 49, 54). The ability to promote IRR can easily be monitored in vivo by the dramatic increase in UV resistance conferred by umu-complementing plasmids to a recA718 ΔumuDC strain (24, 54). Although the recA718 ΔumuDC lexA71::Tn5 strain RW126 was not as sensitive as its lexA<sup>+</sup> counterpart (24), there was a significant increase in UV resistance in the presence of pRW66 and pRW124 (Fig. 5). Two of the expression mutants, pRW124-132 and pRW124-138, that exhibited 2- and 2.4-fold increases in UmuC protein, respectively, yielded intermediate survival (Fig. 5, left panel), whereas plasmids that produced much higher (pRW124-40 and pRW124-100) or lower (pRW124-10) levels of UmuC had no effect on UV survival (Fig. 5, left panel). Of the structural umuC mutants, only pRW124-126 was partially proficient at restoring UV resistance (Fig. 5, right panel).

Separation of complex umuC mutations and comparison of chromosomal umuC mutants cloned into pRW124. As noted above and in Table 2, some of the plasmid mutants that we identified contained multiple mutations. The most notable was pRW124-73, which had three separate mutations. We have subcloned these mutations onto separate plasmids and compared their phenotypes. Both of the 5′-most mutations (pRW124-73A [AT3] and pRW124-73B [GS24]) resulted in only a slight loss of mutagenesis functions (Table 3). The AT3 change is in a block of residues that are 100% conserved in UmuC homologs, while the GS24 residue is more variable (Fig. 3). We conclude that either the conservative amino acid changes are readily accommodated at these positions or that at least the first 24 amino acids of UmuC are not critical for UmuC's mutagenesis functions. In contrast, the most 3′ mutation (pRW124-73C; QX399) was completely defective for UmuC functions (Table 3). The original parental plasmid

FIG. 5. UV survival curves of RW126/pRW66 and the same strain harboring pRW124 (wild-type) and mutant derivatives. Left panel, expression mutants; right panel, structural mutants; all strains carry the low-copy-number UmuD' plasmid pRW66. ○, pRW66 alone; ●, plus pRW124 (wild type); △, plus pRW124-132; ■, plus pRW124-138; ◆, plus pRW124-100; ▽, plus pRW124-40; □, plus pRW124-10; ▼, plus pRW124-126; ◆, plus pRW124-92. All of the other structural plasmid mutants failed to restore significant UV resistance and gave results similar to those with pRW124-92 (data not shown).

FIG. 6. Steady-state levels of plasmid and chromosomal umuC mutations cloned into pRW124. The level of UmuC protein expressed from pRW124 and its mutant derivatives was analyzed by the chemiluminescent immunoassay. The numbers above the individual lanes refer to the derivative of plasmid pRW124 from which the whole-cell extract was obtained. The position of wild-type UmuC is indicated on the left.
pRW124-73 yielded a low steady-state level of UmuC protein (Table 2; Fig. 4); however, this seems to have arisen as a combination of the three different mutations since the individual mutants showed a somewhat increased level of the mutant UmuC protein (Fig. 6; Table 3).

Like pRW124-73C, the structural mutation in pRW124-139B (QX372) resulted in complete loss of mutagenic activity. This mutation results in a UmuC protein lacking the last 50 carboxyl amino acids, and although the level of the truncated UmuC was lower than that of the original parent plasmid, expression (and stability) of the truncated UmuC protein was similar to that of wild-type UmuC (Fig. 6; Table 3). These data, together with the nonmutable phenotypes of plasmids pRW124-120 and pRW124-111 (Table 2), suggest that the carboxyl terminus of UmuC is critical for mutagenic activity.

Three chromosomal missense umuC mutations have been reported (28, 31, 48), and we were interested in comparing the effects of the chromosomal mutations in our plasmid expression system. Neither umuC36 nor umuC104 promoted any significant spontaneous mutator activity; however, both exhibited a very limited ability to promote MMS-induced mutagenesis in the qualitative plate assay (Table 3). When these plasmids were assayed for their ability to complement the chromosomal umuC36 mutation in TK610, plasmid-encoded umuC104 failed to complement, but the plasmid-encoded umuC36 produced a modest but reproducible twofold increase in the number of His+ mutants (Table 3). This finding is consistent with a previous report suggesting that umuC36 may have a leaky phenotype and is able to promote mutagenic activity under certain conditions (5). By comparison, the umuC25 mutation appeared to be completely defective for all mutagenic activity (Table 3).

With respect to the steady-state levels of the mutant proteins, UmuC104 protein was similar to wild-type UmuC, UmuC36 was slightly reduced, and UmuC25 had the lowest steady-state level of the mutant UmuC proteins (Table 3; Fig. 6).

**Further characterization of plasmids pRW124-10, pRW124-92, and pRW124-126.** On the basis of the qualitative His+ reversion assay, the UmuC proteins encoded by plasmids pRW124-10, pRW124-92, and pRW124-126 were able to promote limited mutagenesis functions (Table 2). To determine the efficiency of mutagenic activity more accurately, we performed quantitative UV mutagenesis assays (Fig. 7). To our surprise, neither plasmids pRW124-10 nor pRW124-92 yielded any UV-induced mutants that were reproducibly above background (Fig. 7). In contrast, pRW124-126 yielded approximately one-fifth of the mutants seen with the wild-type plasmid. Since the qualitative mutagenesis experiment used the chemical mutagen MMS rather than UV light, we considered the possibility that the differences were due to the different types of lesion generated by each treatment. However, quantitative mutagenesis assays with MMS gave essentially similar results (Fig. 8).

The difference in the qualitative and quantitative assay may...
be explained by the fact that in the qualitative assay, growing cells are exposed to mutagen (from the disk in the center of the plate) for the duration of the experiment, whereas in the quantitative assay, cells are exposed to mutagen (UV light or MMS) for only a short period of time. Since RW126 is lexA(Def) uvr+ and therefore expresses nucleotide excision repair constitutively, we hypothesized that lesions might be rapidly removed via error-free excision repair before the Umu proteins could act to promote error-prone repair. To test this hypothesis, plasmids were introduced into RW222 (lexA71:: Tn5 recA718 ΔumuDC uvrA6). Indeed, in the excision-defective background, both pRW124-10 and pRW124-92 promoted much higher levels of mutagenesis than in the excision-proficient background, giving approximately one-third to one-quarter of the level of mutagenesis seen with the wild-type plasmid (Fig. 9). By comparison, plasmid pRW124-126 (which was partially proficient for mutagenesis functions in the excision-proficient strain) yielded only slightly more mutants in the excision-defective strain, with approximately one-quarter to one-third of the His+ mutants seen with the wild-type plasmid (cf. Fig. 7 and 9).

**DISCUSSION**

As part of our studies into the mechanisms of inducible mutagenesis in *E. coli*, we have isolated several *umuC* plasmids that have a reduced ability to promote UmuC's functions in SOS-dependent spontaneous and induced mutagenesis and also appear unable to promote IRR in a *recA718* mutant. Interestingly, these plasmids fell into two classes: those mutants that produced either too little or too much wild-type UmuC and those mutants that contained nucleotide changes in the structural *umuC* gene.

**Expression mutants.** We isolated five plasmids (pRW124-10, pRW124-40, pRW124-100, pRW124-132, and pRW124-138) that caused either a partial or complete loss of UmuC activity yet did not contain any structural change in the UmuC protein. Analysis of the UmuC protein expressed from these plasmids revealed that four produced elevated levels of UmuC, while the fifth (pRW124-10; pRW124-10; pRW124-11) produced significantly less and was not detectable.

The phenotype observed when a limited amount of UmuC is synthesized is relatively easy to rationalize. SOS mutagenesis is not a passive process but, rather, occurs after a series of inefficient reactions. For example, the *umuDC* operon is tightly regulated by LexA and would be expected to be one of the last operons induced by DNA damage (30). Likewise, posttranslational cleavage of UmuD to mutagenically active UmuD+ is also inefficient (8, 56). As a consequence of these inefficient reactions, the cell potentially has time to repair DNA damage via error-free pathways such as nucleotide excision repair or recombinational repair before being committed to error-prone translesion DNA synthesis (8, 56). Reducing the amount of available UmuC protein may simply further shift the balance towards error-free transcription-coupled nucleotide excision repair rather than error-prone repair (Fig. 7 and 8). When this pathway is genetically inactivated, the limited amount of UmuC can once more promote error-prone repair (Fig. 9).

The phenotypes produced by overexpression of UmuC are not as easy to explain. Plasmids pRW124-40 and pRW124-100, which produced 8-fold and 4.5-fold more UmuC than wild type, respectively, were phenotypically UmuC− in all of the assays tested. In contrast, pRW124-138, which produced ~2.4-fold more UmuC, was defective for mutagenesis functions but partially proficient at IRR. Plasmid pRW124-132, which yielded only about twofold more UmuC, was partially proficient at IRR and induced mutagenesis and almost fully proficient at spontaneous mutagenesis. If our earlier calculations are correct, RW126/pRW66/pRW124-132 will contain approximately 3,500 UmuD/C molecules per cell. This amount therefore appears to be close to the uppermost limit of UmuD/C that the cell can tolerate before expressing an aberrant phenotype. Overproduction of the UmuDC proteins from multi-copy plasmids has previously been shown to cause cold sensitivity in *lexA*(Def) cells (37). However, none of the four plasmids that expressed elevated UmuC exhibited a cold-sensitive phenotype (data not shown). Evidently, the cold-sensitive phenotype requires overproduction of both UmuD and UmuC proteins.

Marsh et al. (36) previously reported a plasmid *umuC* mutant (*umuCI25*) that resulted in increased sensitivity to UV light. At the present time, we have not directly determined if our overproducing plasmids cause UV sensitization. Since strain RW126 is already UV sensitive, it is difficult to determine if the UV survival of the strain is simply due to an inability of the *umuC* plasmid to restore UV resistance or is due to UV sensitization (Fig. 5A). It is possible, therefore, that the UmuC+ phenotype may occur as a result of increased cell lethality.

UmuC is thought to physically interact with RecA protein (21), and very recently Sommer et al. (47) have presented evidence that overproduction of the UmuD/C proteins inhibits the ability of RecA to promote homologous recombination. It is conceivable that the elevated level of UmuC produced from our mutant plasmids not only inhibits RecA recombinational activities but also compromises its roles in SOS mutagenesis.

It has been difficult to test many of the hypotheses presented above because of the nature of the overproduction. All of the UmuC overproducers appeared to be expressed from plasmids with an increased copy number. These plasmids were somewhat unstable, and when introduced into different genetic backgrounds, many of the survivors had reverted back to a lower copy number and a UmuC+ phenotype (data not shown). The simple conclusion that can be drawn from the studies with the expression mutants is that either too much or too little UmuC can result in the same loss of function phenotype.

**Structural *umuC* mutants.** Our initial screen for *umuC* plasmid mutants identified 11 plasmids with structural changes in the UmuC protein. One of these plasmids, pRW124-73, was complex and contained three separate changes. We subcloned these mutations onto individual plasmids and cloned the chromosomal *umuC*25, *umuC*36, and *umuC*104 mutations into pRW124, giving us a total of 17 plasmids expressing a mutant UmuC protein. While we have clearly not identified all of the possible changes that can lead to a loss of UmuC function, most of the mutations in our plasmid mutants were localized to particular regions of the protein. For example, four mutations were found in a stretch of 30 amino acids (residues 133 to 162). Three of these plasmids (pRW124-92, pRW124-126, and pRW124-134) exhibited somewhat similar phenotypes in that they were partially leaky for some, or all, of UmuC activities (Table 2). The fourth mutation (AT152/pRW124-34A) may also exhibit a similar phenotype but is masked by the second mutation in pRW124-34 (EK228).

Another region where mutations were localized was the carboxyl terminus of the protein. Plasmid pRW124-111, with a Ser→ Leu change just 15 residues from the end of UmuC, resulted in a complete loss of function, as did those that resulted in truncated UmuC, lacking 50, 24, and 18 residues (plasmids pRW124-139, pRW124-73C, and pRW124-120, re-
spectively). We conclude, therefore, that the carboxyl-terminal region is critical for function.

If we include the chromosomal umuC25 mutation together with those found in plasmids pRW124-65 and pRW124-77, then a third smaller region (residues 279 to 290) also appears important for activity.

In contrast, we found few mutations in the very amino terminus of UmuC. Mutations were recovered at residues 3 (AT3) and 24 (GS24) but were only identified because they occurred in a plasmid (pRW124-73) with a mutation in the carboxyl terminus (QX399). When these mutations were subcloned into undamaged vectors, both AT3 and GS24 caused only a slight loss of activity. Indeed, had these mutations occurred independently, their modest change in phenotype would not have been detected in the initial screen for umuC mutants. On the basis of these observations, it would appear that amino acid substitutions in the first few residues of the protein may not be as critical as those located in the carboxyl terminus.

Although we believe that we may have identified potential functional domains of UmuC, the biochemical defect of each of these putative domains remains to be resolved. Umu-dependent error-prone translesion DNA synthesis is believed to occur as part of a multicomponent complex that requires protein interactions between UmuC and UmuD' (57), RecA (2, 20, 21, 50), and probably subunits of DNA polymerase III holoenzyme (19, 27, 43, 51). It is conceivable that the umuC mutants isolated here are defective in some, if not all, of the aforementioned interactions. Further characterization of these mutants should therefore provide insights into the functional domains of UmuC as well as its biochemical activities.

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