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Mutational analysis of the 3′→5′ proofreading exonuclease of *Escherichia coli* DNA polymerase III

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ABSTRACT

The ε subunit of *Escherichia coli* DNA polymerase III holoenzyme, the enzyme primarily responsible for the duplication of the bacterial chromosome, is a 3′→5′ exonuclease that functions as a proofreader for polymerase errors. In addition, it plays an important structural role within the pol III core. To gain further insight into how ε performs these joint structural and catalytic functions, we have investigated a set of 20 newly isolated *dnaQ* mutator mutants. The mutator effects ranged from strong (700–8000-fold enhancement) to moderate (6–20-fold enhancement), reflecting the range of proofreading deficiencies. Complementation assays revealed most mutators to be partially or fully dominant, suggesting that they carried an exonucleolytic defect but retained binding to the pol III core subunits. One allele, containing a stop codon 3 amino acids from the C-terminal end of the protein, was fully recessive. Sequence analysis of the mutants revealed mutations in the Exo I, Exo II and recently proposed Exo IIIε motifs, as well as in the intervening regions. Together, the data support the functional significance of the proposed motifs, presumably in catalysis, and suggest that the C-terminus of ε may be specifically involved in binding to the α (polymerase) subunit.

INTRODUCTION

The structural and functional organization of DNA polymerase III holoenzyme, the chromosomal replication enzyme in *Escherichia coli* (1,2), is of significant current interest. The holoenzyme is composed of a total of 18 subunits, of which 10 are distinct (for a review, see 3). The enzyme is dimeric, containing two pol III core subassemblies, each containing three tightly-bound subunits in a linear arrangement, α–ε–θ. The dimeric structure of the pol III holoenzyme allows simultaneous replication, by a single pol III holoenzyme molecule, of both the leading and the lagging strand at the replication fork. The α subunit, the product of the *dnaE* gene, contains the polymerase. The ε subunit, the product of the *dnaQ* (or *mutD*) gene, contains the 3′→5′ exonuclease that functions as a proofreader for replication errors. The role of θ, the product of the *holE* gene, is as yet unknown. Additional subunits within the pol III holoenzyme include the τ subunit, which functions in dimerizing the two cores; the β subunit, which forms sliding clamps that tether the core polymerases to the DNA, thereby conferring high processivity; and the γ complex (γ,δ,δ′,χ,ψ), which functions in loading the β-clamps onto the DNA. The precise functioning of the various subunits in the complex as well as their interactions are being actively studied (e.g. 4–8).

The present study focuses on the structural and functional properties of the ε subunit within the pol III core. The 3′→5′ exonuclease of the ε subunit serves as the proofreader for DNA replication errors and thereby contributes to the high fidelity of DNA replication. This fidelity is generally considered the product of three serial steps: base selection, proofreading and post-replicative mismatch repair, resulting in an observed error rate of ~10^{-10} per base pair replicated (9,10). To this, proofreading may contribute a factor of ~10^{-2}–10^{-3} (9,11). In addition to its proofreading activity, ε likely plays an important structural role within the pol III core or holoenzyme, as predicted from its tight interactions with the α and θ subunits. This prediction is supported by observations that the ε subunit stimulates both the polymerase activity and processivity of the α subunit (12,13) and that conversely, α greatly stimulates the 3′ exonuclease activity of ε (12). Recessive *dnaQ*(Ts) mutants exist that are inviable at high temperatures in salt-free media. This has been ascribed to the intrinsic instability of the holoenzyme or α subunit in the absence of ε (14). Finally, strains containing deletion of *dnaQ* are essentially inviable, requiring the presence of stabilizing suppressor mutations in the α subunit (15). These combined data suggest that ε plays an important structural role in pol III holoenzyme, likely in stabilizing α and, possibly, serving as a bridge to the θ subunit, although the function of the latter subunit is still unclear (16).

Both structurally and functionally, little is known about ε or its interactions with other subunits. In contrast to most DNA polymerase-associated 3′→5′ exonucleases, which are part of the same polypeptide as the polymerase, ε is a separate subunit. Amino acid alignments among polymerase-associated exonucleases have revealed several homologous regions containing conserved amino acid residues (Exo motifs). Blanco *et al*. (17) defined three such conserved Exo motifs: Exo I, Exo II and Exo III, each containing several highly conserved residues. The importance of these residues within the motifs has been supported by site-specific mutagenesis experiments on a variety of polymerases from different organisms (18–26). However, studies on the 3′-exonuclease of

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Bacillus subtilis DNA polymerase III suggested that the Exo III motif, as defined by Blanco *et al*. (17), is absent in this enzyme (27). Instead, the authors identified an alternative motif, which they termed Exo III ε. This name was chosen as alignments identified a similar motif in the *E.coli* pol III ε subunit (27). While the importance of the conserved residues in *B.subtilis* Exo IIIε was supported by site-specific mutagenesis experiments on this enzyme, no corresponding studies have yet been performed on the ε subunit. To date, only limited information exists with regard to the important residues in *E.coli* ε and their functions (28,29). More recent, extensive alignments (30) encompassing a total of 148 3′ exonucleases, including both DNases and RNases, have placed ε, along with the proofreading exonucleases of the gram-positive pol IIIs and, interestingly, a series of RNases T, in a distinct subgroup of exonucleases characterized by the Exo IIIε motif.

To gain further insight into functionally and/or structurally important residues of the ε subunit, we have investigated a series of *E.coli dnaQ* mutants. These mutants were detected based on their high spontaneous mutability (mutator phenotype), presumably resulting from defective or impaired proofreading. In this study, we correlate the amino acid sequence changes in the mutants with the strength of the mutator phenotype and with the dominant or recessive nature of the mutations. Together, these data provide independent evidence for the biological significance of the conserved residues, including those in the Exo IIIε motif. In addition, the data allow us to discuss the possible catalytic or structural roles of the affected residues.

MATERIALS AND METHODS

Strains and media

The *dnaQ* mutator alleles were isolated in three different experiments, each based on localized mutagenesis of the *dnaE–dnaQ* region of the chromosome using hydroxylamine-treated P1 phage as described (31) and papillation assays to detect mutators. The papillation assays were based on reversion of the *galK2* allele (32), yielding *dnaQ918* through *dnaQ924*, reversion of the *lacZ75* frameshift allele (J.-Y.Mo and R.M.Schaaper, unpublished data) yielding *dnaQ927* through *dnaQ933*, or reversion of the *lacZ* allele from strain CC105 (33) yielding *dnaQ942* through *dnaQ950* (unpublished data). For the mutagenesis experiments of Figure 1, the *dnaQ* alleles were transferred into strain NR9601 (32) by P1 transduction using P1*virA*. Selection was for the tetracycline resistance conferred by transposon *zae-502*::Tn*10* (~40% linkage) followed by scoring for mutator phenotype. Complementation experiments (see below) were performed in the mismatch-repair-deficient *mutL* strains NR9501 and NR9606, into which the *dnaQ* alleles were likewise transduced. NR9606 has been described (32); NR9501 is identical to NR9606, except it carries a different *lacZ* allele (*lacZ75*). NR10187 (carrying the *dnaE915* and *dnaQ49* mutations), used for cloning some of the *dnaQ* alleles, was constructed from strain NR10180 (32) by transfer of *dnaE915* by P1 transduction from NR9905 (34) using the double chloramphenicol–tetracycline (*zae*::*Tn10d-Cam*, *zae-502*::Tn*10*) selection (34). pBluescript (KS+) was obtained from Stratagene. Mini-F plasmid pOF216 (35) was obtained from the American Type Culture Collection (Rockville, MD). Plasmid pSTB101 is identical to pOF216 but carrying in its *Eco*RI site a 1.6 kb *Eco*RI fragment containing the *dnaQ*$^+$ gene derived from pFF588 (16). LB broth was standard recipe. Antibiotics were added as follows: ampicillin (amp) 100 μg/ml, rifampicin (rif) 100 μg/ml and spectinomycin (spec) 100 μg/ml. Solid media contained 1.5% bacto agar (Difco).

Mutant frequency determinations

To determine the mutant frequencies of Figure 1, eight independent colonies from each strain were toothpicked into 1 ml of LB medium and grown overnight at 37°C with agitation. Aliquots of appropriate dilutions were plated on LB–Rif plates to determine the number of Rif^r colonies and on LB plates to determine the number of total cells. Mutant frequencies were calculated by dividing the median number of mutants by the average total number of cells.

Complementation assays

The *dnaQ* alleles were transduced into *mutL* strains NR9501 or NR9606 as described above. The *dnaQmutL* double mutators were electroporated with pSTB101 (*dnaQ*$^+$) and plated on LB containing spectinomycin. Mutant frequencies were determined by growing *dnaQmutL*, with or without pSTB101, in LB overnight at 37°C, and Rif^r mutant frequencies were determined as described above. Comparison of mutant frequencies in the presence or absence of the plasmid resulted in classification of the *dnaQ* alleles as dominant, partially dominant or recessive (Fig. 3).

Sequencing of *dnaQ* genes

For *dnaQ918* through *dnaQ933*, the mutant genes were first cloned into pBluescript (KS+) as a 1.6 kb *Eco*RI fragment. Chromosomal DNA from each of the *dnaQ* mutator strains was isolated using the Easy DNA Kit (InVitrogen), restricted with *Eco*RI, size-purified on a 1% agarose gel and ligated into *Eco*RI-linearized pBluescript (KS+). The ligated DNA was transformed into strain NR10187 followed by plating on LB–Amp plates at 37°C. NR10187 is temperature sensitive due to the simultaneous presence of the *dnaQ49* and *dnaE915* (31) alleles; however, growth at 37°C is restored by temperature-resistant *dnaQ* alleles. Single-stranded DNA for DNA sequencing was generated by infecting selected transformants with helper phage VCM13 (Stratagene) as per Stratagene single-stranded rescue protocol. DNA sequencing was by the single-stranded protocol provided with ^{35}Sequetide (Dupont NEN). Seven different primers covering the gene were used and each *dnaQ* allele was sequenced in its entirety. Alleles *dnaQ942* through *dnaQ950* proved difficult to clone by the above procedure, presumably due to the very high mutation rates associated with their proofreading deficiencies. For these alleles, two *dnaQ* specific primers, 5′-TTCTCGCGTCCGCGATAGCG-3′ (forward primer) located at positions 1173–1154 and 5′-TGCCTCGACCTCGTCAACGG-3′ (reverse primer) located at positions 189–208 (numbering system as in 14) were used to PCR amplify the chromosomal DNA containing the *dnaQ* gene. Single-stranded DNA for DNA sequencing was generated in an asymmetric PCR reaction using the double-stranded PCR product as template. DNA sequencing on the single-stranded DNA was performed as described above for *dnaQ918* through *dnaQ933*.

Table 1. Mutations in mutant *dnaQ* alleles: DNA sequence changes and complementation phenotype

Allele	Base change[a]	Amino acid change	Complementation phenotype[b]	
mutD5	C967→T[c]	Thr 15 Ile	D	(4, 150)
dnaQ49	A725→C[d]	Val 96 Gly	R	(55, 1)
dnaQ918	G965→A	Thr 16 Ile	PD	(19, 5)
dnaQ920	G846→A	Arg 56 Trp	D	(5, 7)
dnaQ921	C473→T	Gly 180 Asp	PD	(25, 2)
dnaQ922	C473→T	Gly 180 Asp	PD	(15, 2)
dnaQ923	G816→A	His 66 Tyr	PD	(7, 4)
dnaQ924	G501→A	Leu 171 Phe	PD	(5, 3)
dnaQ927	C473→T	Gly 180 Asp	PD	(19, 2)
dnaQ928	C963→T	Gly 17 Ser	D	(5, 9)
dnaQ930	GG720,721→AA	His 98 Tyr	D	(6, 6)
dnaQ932	CC290,291→TT	Trp 241 STOP	R	(31, 1)
dnaQ933	CC473,474→TT	Gly 180 Asn	D	(5, 5)
dnaQ942	C513→T	Asp 167 Asn	PD[e]	
dnaQ943	C523→T	Ala 164 Thr	PD	(15, 22)
dnaQ944	C518→T	Asp 167 Asn	PD	(10, 88)
dnaQ945	G522→A	Ala 164 Val	PD	(17, 18)
dnaQ946	G522→A[f]	Ala 164 Val	PD	(8, 30)
dnaQ947	C972→T	Glu 14 Lys	PD	(23, 31)
dnaQ948	C978→T	Asp 12 Asn	D	(4, 53)
dnaQ949	C705→T	Asp 103 Asn	PD	(52, 4)
dnaQ950	G522→A	Ala 164 Val	D	(2, 55)

Each *dnaQ* allele was sequenced in its entirety to determine the mutation in the *dnaQ* gene.

[a]Base number 1 is the first base in the 3′ *Eco*RI cut site (3′ CTTAA) as in Maki *et al*. (14).

[b]D, dominant; PD, partially dominant; R, recessive. An example of the complementation data are shown in Figure 3. The criteria for classification of alleles as D, PD or R are given in the Figure 3 legend. The first number in parentheses represents the fold reduction in mutant frequency by pSTB101 (*dnaQ*$^+$) while the second number indicates the fold above the background mutant frequency in presence of pSTB101.

[c]As determined by Fijalkowska and Schaaper (28).

[d]As determined by Takano *et al*. (47).

[e]We were unable to construct the necessary *dnaQ942 mutL* double mutator strain; therefore, the PD character is inferred from *dnaQ944* which has the identical mutation.

[f]This allele also contained a G1087→A base change in the *dnaQ* promoter region.

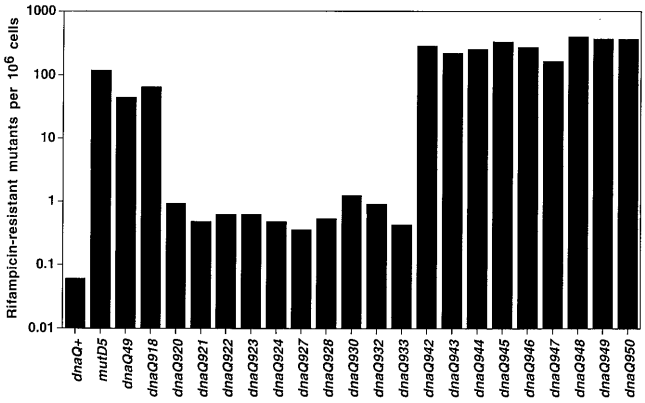

Figure 1. Mutant frequencies for *dnaQ* mutators. All *dnaQ* alleles were present in strain NR9601 (Materials and Methods), which also served as *dnaQ*$^+$ control. Frequencies for Rif^r mutations were determined as described in Materials and Methods.

RESULTS

To gain insights into the functional and structural properties of the pol III ε subunit, we have conducted an analysis of a total of 20 *dnaQ* mutants that were isolated in our laboratory based on the mutator phenotype that they confer. The mutator phenotype presumably reflects the proofreading deficiency they confer during DNA replication. Our analysis involves (i) measurement of the strength of the mutator phenotype, (ii) determination of the DNA sequence changes underlying the mutations, and (iii) assessment of the ability of the mutant proteins to interact with the α subunit via genetic complementation. In Figure 1, we compare the strengths of the *dnaQ* mutators along with two reference alleles, *mutD5* (36) and *dnaQ49* (37) which have been previously characterized (38–44). We used Rif^r as a mutation marker as this marker has been used previously to characterize mutators (9,38,41,43,45). Two obvious classes of mutator alleles were noted. Strains containing *dnaQ920* through *dnaQ933* exhibited mutant frequencies that were 6–20-fold above the wild-type level, while strains containing *dnaQ918* or *dnaQ942* through *dnaQ950* exhibited mutant frequencies 700–8000-fold above the wild-type level.

Further characterization of the *dnaQ* alleles was achieved by determination of the amino acid sequence changes responsible for each mutator phenotype. Each *dnaQ* gene was sequenced in its entirety and the observed base changes and resulting amino acid substitutions are presented in Table 1. All of the base changes involved G·C→A·T transitions, consistent with the mutational specificity of hydroxylamine, the mutagen used to create the alleles (31). Three of the mutations involved a tandem base change, but in each case only one amino acid substitution resulted. Amino acid substitutions were found in the putative Exo I and Exo II motifs (underlined) as well as in the region between these two motifs (Fig. 2). Amino acid substitutions were not found in the Exo III motif proposed by Blanco *et al*. (17), but several substitutions occurred in the alternative Exo IIIε motif (dashed underline) proposed by Barnes *et al*. (27). *dnaQ946* contains a base-pair substitution in the promoter region (G→A at 1087) in addition to the base-pair change in the coding region. The substitution in the promoter region is two bases removed from a potential RNA polymerase binding site identified by Cox and Horner (46). The substitution does not alter the amino acid sequence of the *rnh* gene, which is divergently transcribed with *dnaQ* and whose coding region partially overlaps with the *dnaQ* promoter (14,46). Five of the 15 mutations (D12N, E14K, D103N, A164V and D167N) were also seen in a recent study by Strauss *et al*. (29).

While the mutator activity of the *dnaQ* alleles is reflective of the proofreading deficiency that they confer, at least two different, although not exclusive, underlying mechanisms can be envisioned. The deficiency may be catalytic, reflecting the loss of exonuclease activity, or structural, reflecting the loss of binding affinity to the polymerase subunit. To possibly distinguish between these pathways, we performed genetic complementation experiments

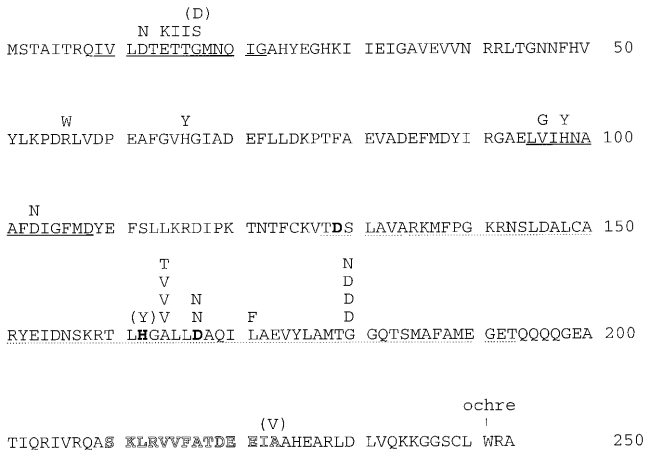

Figure 2. The amino acid sequence of ε subunit and changes observed in *dnaQ* mutator alleles. The amino acid sequence is as reported by Maki *et al*. (14). The one-letter amino acid code is used. The underlined letters represent amino acids in Exo motif I QIVLDTETTGMNQIG (residues 8–21) and Exo motif II LVIHNAFDIGFMD (residues 95–108) as proposed by Blanco *et al*. (17). Outlined letters (residues 209–223) represent the Exo III motif as proposed by Blanco *et al.* (17). The dashed line indicates the entire Exo IIIε motif (residues 128–192) as proposed by Barnes *et al*. (27), while bold letters represent highly conserved residues (Asp129, His162 and Asp167) within the extended motif. Letters above the sequence represent the substitutions found in the *dnaQ* mutators in this study. Other sequenced *dnaQ* mutations, not found in our study but observed by Strauss *et al*. (29), are indicated in parentheses above the sequence.

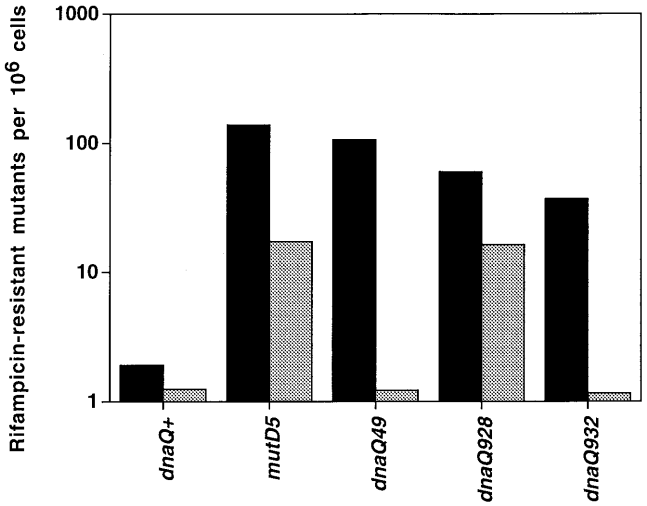

Figure 3. Complementation of *dnaQ* mutator alleles by *dnaQ*$^+$. The black bars indicate the level of Rif^r mutations in the strains without plasmid, while the gray bars indicate the level of Rif^r mutations in the same strain but containing plasmid pSTB101 (*dnaQ*$^+$). The data illustrate that *mutD5* and *dnaQ928* are dominant and that *dnaQ49* and *dnaQ932* are recessive. The results for the other dnaQ alleles are summarized in Table 1. The complementation experiments were performed in the mismatch-repair-defective background (Materials and Methods) to avoid indirect effects via restoration of mismatch repair. Mutant frequency determination was as described in Materials and Methods. Recessive alleles (R) are alleles for which the mutant frequency in the presence of pSTB101 (*dnaQ*$^+$) was equal to or less than the wild-type level. Dominant (D) alleles are those for which the mutant frequency in the presence of pSTB101 (*dnaQ*$^+$) fell ≤6-fold and remained >3-fold above the wild-type level. Mutants that did not satisfy both criteria for dominant alleles, but nevertheless remained significantly above the background, were designated partially dominant (PD). Formally, no ready distinction can be made between partially dominant and partially recessive mutants.

in which the *dnaQ* alleles were combined with a copy of the *dnaQ*$^+$ gene. In such experiments, catalytically-deficient mutants, due to their retained binding abilities, compete with the DnaQ$^+$ protein for insertion into the pol III core and therefore exert a mutator effect even in the presence of the wild-type gene. Such behavior in a complementation experiment is considered (semi)dominant. An example of a dominant *dnaQ* mutator is *mutD5* (38,40). In contrast, recessive *dnaQ* mutants are those that may still be catalytically proficient, but are impaired in their ability to bind to the α subunit and therefore do not compete effectively with the wild-type protein. An example of a recessive mutant is *dnaQ49* (40). A limitation of this assay is that it allows no straightforward distinction between partially dominant and partially recessive mutants.

The *dnaQ* strains carrying the mutator allele on the chromosome were transformed with a low-copy (one or two per cell) mini-F plasmid, pSTB101, carrying the *dnaQ*$^+$ gene (Materials and Methods), and the Rif^r mutant frequencies were determined. These experiments were performed in the mismatch-repair-defective background to avoid the complications associated with the indirect effects that proofreading deficiencies have on the mismatch repair system through saturation (41). Figure 3 presents the results for two examples from the set of 20 alleles (*dnaQ928* and *dnaQ932*) along with the *mutD5* and *dnaQ49* controls. The data show that the mutant frequency of the *mutD5* strain, while being reduced several-fold, is still some 20-fold above the corresponding control, as expected based on its dominant character. The several-fold reduction is consistent with the presence of a (1–2) copy *dnaQ*$^+$ plasmid and a slightly anti-mutagenic effect of the pOF216 vector itself observed in some experiments (data not shown). In contrast, the mutability of the recessive *dnaQ49* allele is completely reduced to the background level. Figure 3 further shows that *dnaQ928* behaves as a dominant allele and *dnaQ932* as a recessive allele. The results in Table 1 indicate that of the 20 new alleles, six were classified as fully dominant (D), 13 as partially dominant/recessive (PD) and one as fully recessive (R) (see Fig. 3 legend for criteria). Partial dominance may result from alterations in catalytically important residues that also affect the structural properties of ε.

DISCUSSION

The current study represents a genetic approach to the analysis of ε, the 3→5 exonuclease editing subunit of DNA polymerase III. We attempt to correlate the mutator strength (a measure of proofreading loss) with the amino acid changes of each mutator *dnaQ* allele. We show that the mutator *dnaQ* alleles increase Rif^r mutant frequencies from 6- to 8000-fold, reflecting the range of proofreading deficiencies suffered by these strains during DNA replication. Interestingly, several of the mutator alleles exhibit mutant frequencies up to 3-fold above the *mutD5* level which has been generally considered the strongest known single mutator (39). These new strong mutators contain amino acid substitutions in the conserved Exo I, Exo II or Exo IIIε motifs (Figs 1 and 2; Table 1). The location of the residue changes is indicative of the critical importance of these residues (see below). We also noted that these strong mutators appeared less healthy (more heterogeneous colony morphology and decreased cell count at saturation) than the wild-type or weaker mutators. Previous studies of the *mutD5*

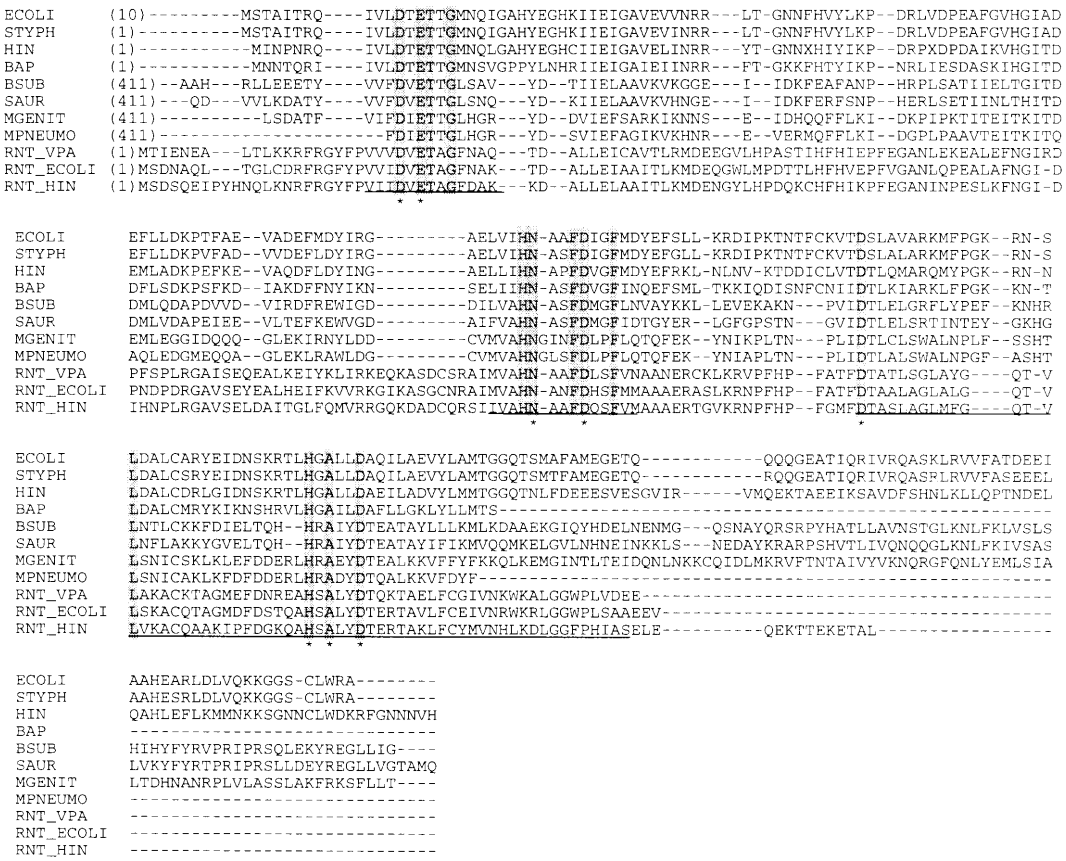

Figure 4. Comparison of *E.coli* ε with the ε subunits of other gram-negative organisms, the DNA polymerase III-associated 3′→5′ exonucleases of gram-positive organisms and the RNases T from several organisms. Exo motifs I, II and extended IIIε are underlined. Highly conserved residues are shown shaded. Those marked with an asterisk (*) are residues proposed to serve as metal ligands (30). The number in parentheses before the start of the alignment refers to the GenBank number for the first residue. Protein sequences were downloaded from GenBank and aligned by the ClustalW 1.7 program (Baylor College of Medicine at http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). ECOLI, *E.coli* ε; STYPH, *Salmonella typhimurium* ε; HIN, *Haemophilus influenzae* ε; BAP, *Buchnera aphidicola* ε; BSUB, *B.subtilis* DNA polymerase III; SAUR, *Staphylococcus aureus* DNA polymerase III; MGENIT, *Mycoplasma genitalium* DNA polymerase III; MPNEUMO, *Mycoplasma pneumoniae* DNA polymerase III; RNT_VPA, Rnase T of *Vibrio parahaemolyticus*; RNT_ECOLI, Rnase T of *E.coli*; RNT_HIN, Rnase T of *H.influenzae*. A more extensive alignment containing 148 additional proteins from more diverse lineages can be found in Moser *et al*. (30).

and *dnaQ926* alleles (the latter is a D12A, E14A double mutant created by site-specific mutagenesis) revealed somewhat reduced viability of *mutD5* and, in fact, complete inviability of *dnaQ926* (28). These viability problems have been directly related to the high level of mutation production in these strains, as they lead to frequent mutations in essential genes (28). In turn, the very high mutability of proofreading-defective strains results from the fact that the elevated level of replication errors overwhelms, either completely or partially, the capacity of the mismatch repair system, thereby amplifying the observed mutator effect by one or two orders of magnitude (41,43,48,49). In the extreme case, the phenomenon has been referred to as error catastrophe, where excessive mutations lead to cell death (28). The growth impairment of the new, stronger *dnaQ* mutators is similar to or exceeding that observed for *mutD5* and is consistent with this error catastrophe model.

The mutant frequency data also suggest that there are two distinct groups of mutants, one group exhibiting mutant frequencies 700–8000-fold above the background level; the other group exhibiting 6–20-fold increases in mutant frequency. Within the context of the error catastrophe model, our observation of these two groups of mutators suggests that in the one group the increase in replication errors exceeds the threshold for mismatch repair saturation, whereas in the second group it does not.

The combined results provide several important insights into the functional regions of ε. Below, we discuss these data in relation to the proposed Exo motifs and an alignment (Fig. 4) of the ε subunit from four gram-negative bacteria, along with the 3′ exonuclease of several gram-positive organisms and a set of RNases T, which have been found to share the Exo I, II and IIIε motifs (30,50,51). A much more extensive alignment has shown these motifs to be present in 148 different proteins (30). In the Exo I motif there are five observed mutants, including the *mutD5* mutation. Four (D12N, E14K, T15I and T16I) are very strong, while one (G17S) is in the moderate category. Two of the mutants, D12N and E14K, were seen previously (29) along with a G17D mutant (29). All of the five mutants are dominant (or partially dominant) suggesting that these residues may serve a role in catalysis. The five residues at which they reside are strongly conserved in the DNA polymerase III-associated 3′ exonucleases of gram-negative and gram-positive organisms as well as in the RNases T (Fig. 4), indicating an important role in the function of

these enzymes. The analogous residues in the *E.coli* DNA polymerase I Klenow fragment (D355 and E357) are postulated to be involved in binding and coordination of the divalent metal ions (24,52) which are essential for catalysis. Our data are consistent with the Exo I motif being of critical importance in catalysis by ε.

Two mutants were found between Exo motifs I and II (R56W and H66Y). Their mutator strength is only moderate. Interestingly, both R56 and H66 are conserved among the four gram-negative organisms (Fig. 4) but not in the gram-positive organisms. R56W is dominant and H66Y is partially dominant, indicating that the stretch of amino acids between the Exo I and II motifs may be important for full catalytic efficiency. R56 and H66 may be involved in catalysis directly via participation in the catalytic reaction or indirectly by maintaining the overall global structure necessary for a functional active site.

Two mutants were found in the Exo II motif, H98Y and D103N. H98Y is a moderate mutator while D103N is very strong. Both are (partially) dominant mutators. The location and properties of these mutants are consistent with the Exo II motif playing an important role in catalysis. H98 and D103 are well conserved among the gram-negative and gram-positive organisms, implying an important role for these residues in the functioning of these 3′ exonucleases and RNases T. Based on studies on the corresponding aspartate in pol I Klenow fragment (24,52), D103 is probably another ligand for the divalent metal involved in catalysis. Evidence for an additional structural role of certain Exo II residues is suggested by the strong, but recessive, *dnaQ49* mutator (V96G) which also resides in this motif. The recessive nature of *dnaQ49* probably suggests that the valine residue is important in maintaining the overall structure of the ε protein. This valine residue is well conserved among the gram-positive and gram-negative organisms and the RNases T.

A total of 11 mutants were discovered within the limits of the extended Exo IIIε motif (27). However, all our mutants were found in a 17 amino acid stretch (residues 164–180). No mutants were discovered of the conserved residue D129 (nor in any other study), although site-specific mutagenesis of this residue in *B.subtilis* pol III caused a near-complete loss of exonuclease activity (27). It is possible that a larger collection of mutants would have yielded mutants in this residue, although at this time the importance of this residue in ε remains to be determined. The changes at A164 and D167, which are both highly conserved (Fig. 4), yielded very strong mutators that again were dominant. These data are in strong support of the proposed Exo IIIε playing a critical role in catalysis, with D167 being a possible metal-coordinating ligand. Alterations of the nearby H162 were not found in this study, although a H162Y change was found in another study (29). The combined data confirm the importance of the HxAxxD sequence (Fig. 4) within the Exo IIIε motif for this group of exonucleases. This contrasts to the YxxxD sequence that constitutes the core of the Exo III motif present in most other exonucleases (17,30,51). Three other nearby mutations were found (L171F, G180D and G180N). These were among the moderate mutators. Nevertheless, they were dominant or partially dominant. This suggests that these residues affect the exonuclease activity, but perhaps in an indirect manner through structural changes affecting the active site.

We obtained one mutant in the C-terminal portion of ε (W241ochre), which leads to an ε subunit lacking the three C-terminal residues. Complementation experiments revealed that this mutant (*dnaQ932*) was fully recessive. Strains containing this allele exhibited Rif^r mutant frequencies 15–18-fold above the wild-type level. We suggest, based on the recessive character of the mutation, that this mutator phenotype results from reduced binding of ε to the α (polymerase) subunit. If correct, this suggests that one, two, or all three of the C-terminal amino acids are important for proper interaction of the α and ε subunits. That the C-terminal region of ε may represent a separate domain is further suggested by the presence of the Q_4 stretch at residues 194–197. Glutamine-rich stretches have been observed at linker regions between domains and are thought to serve as a hinge between domains (54–56). Specifically, the region of DnaQ, residues 190–212, that includes the four glutamine residues has been proposed as a member of the Q-linker family of interdomain linkers (57). The generally good homology between ε and the 3′ exonuclease of the gram-positive DNA polymerase IIIs ends at the Q_4 stretch (Fig. 4; 27). This divergence is consistent with a difference in function of the C-terminal regions between the two organisms (52): in *E.coli* the α and ε subunits are separate subunits, while in *B.subtilis* the exonuclease and polymerase are part of the same polypeptide.

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