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Molecular characterization of the *Salmonella typhimurium parE* gene

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ABSTRACT

The DNA sequence of the wild type *S. typhimurium parE* gene was determined. The predicted protein has 96.7% amino acid identity with the ParE protein of *E.coli*, but is 29 amino acids longer, due to an additional basepair in the 3′ end of the *S. typhimurium* gene. Subclones of the *S. typhimurium parE* gene localized the sites of four heat sensitive mutations within *parE*. The *parE206* and *parE374* mutations are identical (Val67-Met) and lie in a highly conserved region corresponding to the ATP binding pocket of GyrB. Two additional heat sensitive mutations were sequenced and predict the following amino acid substitutions: *parE377* (Gly399-Ser) and *parE493* (Thr583-Pro). All of the heat sensitive mutations lie in regions with strong amino acid homology to GyrB.

INTRODUCTION

Recently, a second type 2 topoisomerase (topoisomerase IV) was discovered in enteric bacteria (8,9,11). This enzyme is composed of two proteins, ParC and ParE, which have strong sequence similarity to GyrA and GyrB, the two subunits of DNA gyrase. Conditional mutations in the *parC* and *parE* genes cause a failure of proper nucleoid segregation (7,17), and of the decatenation of newly replicated small plasmids (2). These results have suggested that topoisomerase IV performs the decatenation events required during the replication of a circular DNA molecule, and that this decatenation is required to accomplish proper nucleoid segregation in bacteria.

Mutations in the linked *parECF* genes of *S. typhimurium* were identified within a large collection of conditional lethal mutants by the 'partitioning-defective' cytological phenotype of the mutants at the non-permissive temperature (17). Similar studies led to the characterization of the *parE* and *parC* genes in *E.coli* (7). The DNA sequences of the *parC* and *parE* genes of *E.coli*, and the *parC* and *parF* genes of *S. typhimurium* have been previously reported (8,11). The structures of the ParC and GyrA, and ParE and GyrB proteins are undoubtedly very similar, since both pairs of proteins have approximately 40% amino acid identity (8,11). Despite the structural similarity, DNA gyrase and topoisomerase IV must be functionally distinct, since conditional lethal mutants can be isolated in each of these genes.

This study describes the subcloning and sequencing of the wildtype *S. typhimurium parE* gene, as well as the mutational changes found in four of the *S. typhimurium parE* conditional lethal mutants. These results can be correlated with the crystal structure of an N-terminal fragment of the GyrB protein, which was recently reported (20).

MATERIALS AND METHODS

Strains

All *S. typhimurium* strains are derivatives of LT2. The temperature sensitive mutants of *S. typhimurium* are described in Schmid, et al. (16); the initial identification of the *parECF* mutations is described in (11,17). Strains were stored in 8% DMSO at −70°C.

Plasmids

Plasmids used in this study are shown in Figure 1. The complementing plasmid pMBS11 has been described (11) and was originally isolated from a SauIIIA partial digest library of *S. typhimurium* DNA in the pBR328 plasmid (4). Other plasmids were derived from these insert sequences by using the pBluescript II SK$^+$ and KS$^+$ vectors (Stratagene). Subclone pMBS115 was made by removing a HindIII fragment from pMBS11, leaving 4.7kb of DNA from the right-hand end of pMBS11. Subclone pMBS118 contains the 3.9kb PstI fragment of DNA from the same end of pMBS11, inserted into the PstI site of pBR328. Plasmids pMBS116 and pMBS117 were made by removing ClaI or EcoRV fragments, respectively, from pMBS11. The other subclones shown in Figure 1 were derived from pMBS118. Plasmids were moved into *S. typhimurium* strains by electroporation using the protocols of Bio-Rad, and then transferred between *S. typhimurium* strains by means of either electroporation or P22-mediated transduction, with selection for the plasmid-encoded drug resistance. The *E.coli* strain XL1-Blue (Stratagene) was used as the host for most recombinant DNA work involving the pBluescript II plasmids. XL1-Blue cells were transformed using a standard $CaCl_2$ competent cell protocol.

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Media

Complex medium was LB (15g agar, 5g NaCl, 15g yeast extract, 10g Bacto-tryptone). Liquid complex medium was the same composition but without the agar. Tetracycline (20 μg/mL), ampicillin (25μg/mL), chloramphenicol (20μg/mL) were added to rich medium to select resistance to these drugs. 'High salt' plates were LB supplemented with 0.5M KCl.

Complementation and marker rescue

Plasmids were introduced into *S. typhimurium* strains selecting for inheritance of the plasmid drug resistance marker; subsequently, the ability of transductant or electroporant strains to grow at 44°C was tested. Colonies were tested by making 'patches' on plates incubated at the permissive and non-permissive temperatures. Plasmids that complemented a mutation yielded confluent growth in these patches. Plasmids that recombine with ('marker rescue') the mutation had indivdual colonies within patches, but not confluent growth. In general, the frequency of marker rescue was approximately 10^{-4}, which was quantitated by plating dilutions of strains on LB plates at permissive and non-permissive temperatures.

Enzymes

Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs. Calf intestine phosphatase was obtained from Boehringer-Mannheim.

DNA sequencing

The 3.9 kb PstI fragment from pMBS118 was inserted into the PstI site of pBluescript II KS$^+$ and pBluescript II SK$^+$ to create, respectively, pMBS198 and pMBS199. Subclones pMBS210 and pMBS213 were generated by removing ClaI fragments from pMBS199 and pMBS198, respectively. The plasmid pMBS211 contains a BssHII partial digest fragment containing 1.8 kb of the right hand end of the pMBS118 insert. pMBS212 was constructed by removing a small EcoRV fragment from pMBS198. These deletion plasmids were used for single stranded sequencing. The sequence of *parE* was completed by employing 18- and 20-nucleotide primers (made by the Princeton University Synthesis and Sequencing Facility) designed from sequence obtained from the above clones or from the *E.coli parE* gene (8). The names and DNA sequences of the oligonucleotide primers used to sequence DNA on the coding strand are: C1 (GACAGTGACCGAGGGTGG); C300 (GACGCGGGATG CCGGTGG); C600 (CTTCTTTGACAGCCCGCG); C800 (CGCTATTGTGGCTGCCGG); C1200 (CGTTGCCGGGGA AACTGG); C1600 (GCTACTCTGCTTTGCGCG). Primers used to sequenceDNA on the non-coding strand are: R600 (CA-GCACATGCGTCAGGCG); R900 (GCATCGCGTCCAACA-GGC); R1200 (CCAGTTTCCCCGGCAACG); R1950 (GCAGATCCAGATAAAGCGTG). Protocols and reagents for sequencing were from the Sequenase 2.0 kit (United States Biochemical). Sequencing was done using both dGTP and dITP, using [^{35}S]dATP (New England Nuclear, 12.5mCi/mL). The samples were then subjected to polyacrylamide gel electrophoresis, the gels were fixed and then dried under vacuum. Autoradiographs were prepared with Kodak XAR-5 film. Sequence homologies were determined using the Wisconsin Genetic Computer Group Programs (3).

Genbank accession number

The GenBank accession number of the *S. typhimurium parE* sequence reported in this work is L05544.

UV sensitivity

Log phase cultures were plated at appropriate dilutions on LB plates. The plates were then irradiated for 20 seconds with a hand-held UV lamp (Spectroline, short wavelength [254nm]) at a height of 12 cm. The number of colonies arising on irradiated plates after growth overnight at 30°C was recorded. Percent survival was measured by comparing these numbers to the numbers of colonies arising on plates that had not been irradiated.

Polymerase chain reaction (PCR) amplification of genomic DNA

DNA fragments containing *parE377* and *parE493* mutations were amplified using a polymerase chain reaction protocol developed by Perkin-Elmer Cetus, and modified by F. Russo (15). Template DNA for amplification was obtained from a colony of the appropriate *S. typhimurium* strain. The colony was dispersed into a 0.5 mL eppendorf tube containing oligonucleotide primers, nucleoside triphosphates and PCR buffer solution (Perkin-Elmer Cetus) with 2.5 mM $MgCl_2$. This tube was then boiled for three minutes and spun briefly before addition of enzyme (2.5 U Ampli-Taq polymerase; Perkin-Elmer Cetus) and overlayed with mineral oil to prevent evaporation before PCR amplification. The *parE377* and *parE493* mutations were amplified from chromosomal DNA using the C800 and R1950 oligonucleotide primers.

Sequencing PCR-amplified DNA

Approximately 70−100 ng of the DNA produced by PCR amplification was purified from primers and non-specific products by electrophoresis in a 1% Nu-Sieve low melting point agarose gel (FMC). The desired band was cut out of the gel and melted.

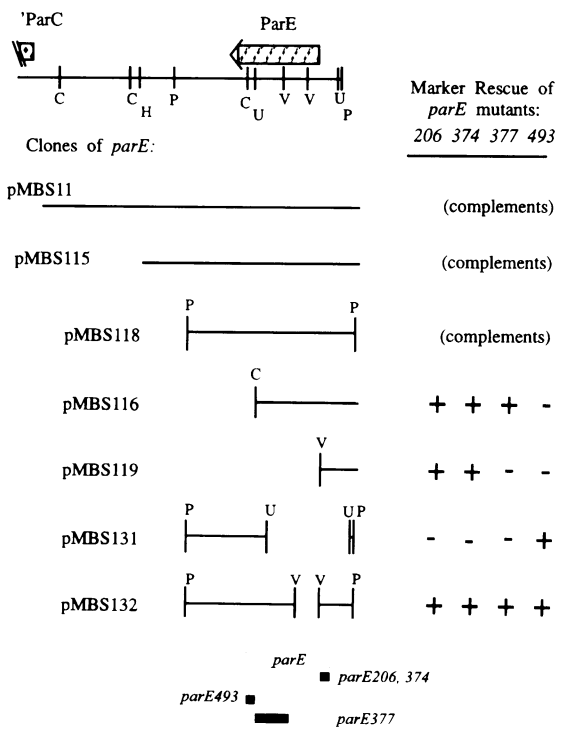

Figure 1. Physical map of the parE region showing clones and marker rescue results. Inserts are cloned into pBR328. Abbreviations: C=ClaI, H=HindIII, P=PstI, U=PvuII, V=EcoRV. Localization of *parE* mutations within the *parE* gene based on the marker rescue results is indicated in the lower part of the figure.

This mixture was used directly for sequencing (15). Approximately 40–70 ng of DNA-agarose was added to 30ng oligonucleotide primer (total volume 9μL) and this mixture was boiled for two minutes. Annealing was performed by incubating the mixture at 55°C for five minutes. This DNA was then kept at 37°C and sequencing reactions were performed using the Sequenase 2.0 protocol (United States Biochemical) described above except that the labelling reaction was performed at 37°C (10). Each mutation was identified by sequencing PCR-amplified DNA from each of two independently isolated colonies. The *parE377* mutation was sequenced by reading from the C800 oligonucleotide primer (see Table 1), *parE493* was sequenced by priming either direction in the region from C1600 and R1950 primers.

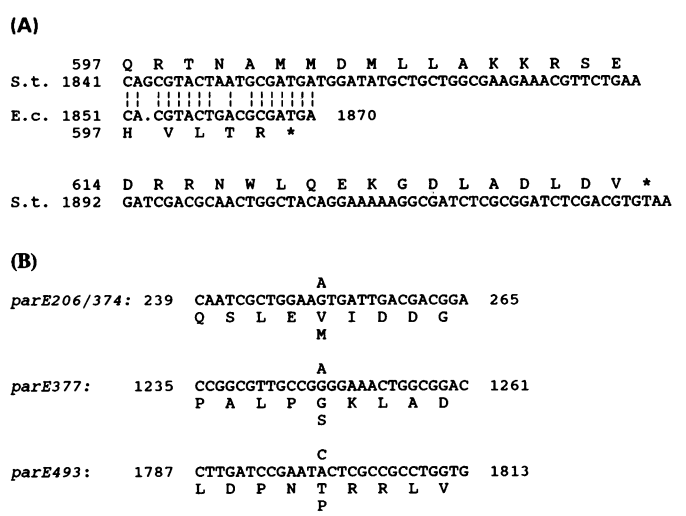

Figure 2. (**A**) The difference between the 3'-end of the *S. typhimurium parE* gene (top line) and the *E.coli parE* gene (bottom line). The extra base in the *S. typhimurium* sequence causes the translational reading frameshift, predicting the extra amino acids shown in the *S. typhimurium* ParE protein. (**B**) The sequences surrounding each of the temperature sensitive mutations are given. Above the wild type sequence is the base change found in the mutant; below the wild type translation is the amino acid change predicted for the mutant protein.

Cloning of *parE206* and *parE374*

The marker rescue phenomenon described above suggests that a very small number of cells in a culture of pMBS118/*parE206* should carry the *parE206* mutation on the plasmid as well as in the chromosome. Such strains should show heat-sensitive growth and remain drug resistant. To find these rare homogenotes, an ampicillin enrichment technique was employed to enrich for strains carrying a *parE206*-containing plasmid (13). Although this technique did yield pMBS201 (1 clone out of 10,150 colonies screened), it was too laborious to enable a rapid screen for more clones. Thus, another genetic screen was employed for isolation of clones carrying *parE206* and *parE374* mutations. This screen exploits the ability of deletions in a sequence to be repaired by marker rescue, and the salt correctable phenotype of the *parE206/374* mutations. Strains with *parE206* or *parE374* in the chromosome were transformed with pMBS131, which carries an internal deletion of *parE* that removes the *parE206/374* mutation site (see Figure 1). P22 lysates grown on these two plasmid-containing strains were used to transduce a *parE377* strain (SE7763) to chloramphenicol resistance. Only transductants that inherited recombined plasmids carrying the *parE206* or *parE374* mutations should grow at high temperature in the presence of 0.5M KCl. Plasmid DNAs were restriction mapped to demonstrate that the internal deletion of pMBS131 had been repaired, yielding plasmids with restriction maps identical to pMBS118. Mutation-bearing plasmids were obtained at a frequency of 1×10^{-5} (1/99,000 colonies screened) for *parE206* (pMBS206) and 1.3×10^{-5} (2/150,000 colonies screened) for *parE374* (pMBS202, pMBS207).

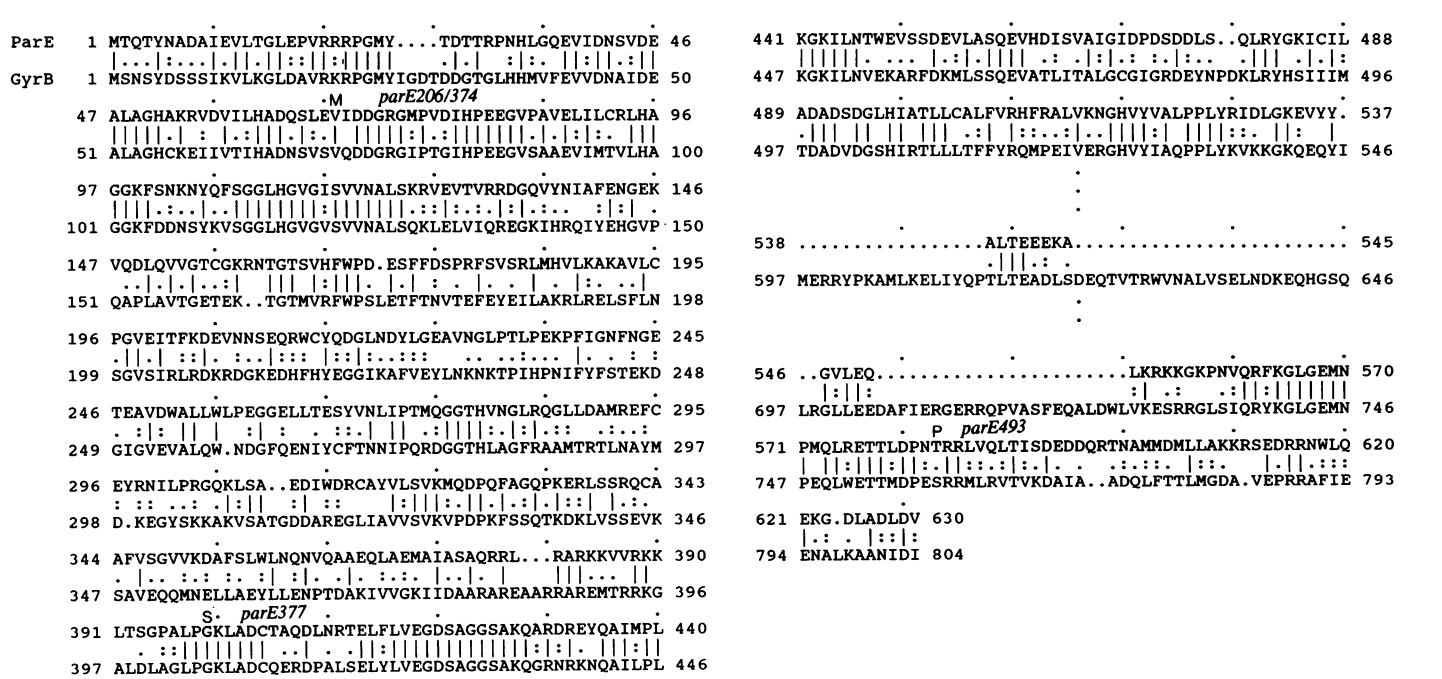

Figure 3. Comparison of the *S. typhimurium* ParE protein and the *E.coli* GyrB protein. Regions of much lower than average homology are found between amino acids 174–248 (16.2% identity); 340–39 (21.4% identity); and 590–630 (20% identity).

RESULTS

DNA sequence of *S. typhimurium parE*

The *S. typhimurium parE* gene was sequenced as described in Materials and Methods. The gene is 1892 basepairs in length and shows 86.5% identity to the *E.coli parE* DNA sequence. The *S. typhimurium parE* sequence has an additional basepair at position 1843 that causes a frameshift in the protein sequence relative to that predicted by the *E.coli* DNA sequence (see Figure 2). Because of this, the predicted *S. typhimurium* ParE amino acid sequence differs beyond amino acid 597 and is 29 amino acids longer than the predicted *E.coli* ParE protein. Otherwise, the *S. typhimurium* ParE protein is 96.7% identical to the *E.coli* ParE protein and 41.1% identical to the *E.coli* GyrB protein. The homologies with GyrB occur throughout the ParE protein, as shown in Figure 3.

Localization of *parE* mutations

Four temperature sensitive mutants with defects in *parE* have been identified from the collection of *S. typhimurium* temperature sensitive strains (11). These mutations: *parE206, parE232, parE374* and *parE377*, are complemented by the 3.9 kb insert found in pMBS118 (Figure 1). One additional allele, *parE493*, was identified in this study. Non-complementing subclones of pMBS118 were prepared and used to localize the sites of these mutations. Homologous recombination between a plasmid insert (which carries wild type DNA) and the chromosome can repair the chromosomal temperature-sensitive defect. These recombinants are observed as temperature-resistant papillae at a frequency much higher than the spontaneous reversion frequency (11). From these experiments, the *parE206* and *parE374* mutations fall in the promoter proximal portion of the gene, while the *parE493* and *parE377* mutations lie in the promoter distal half of the gene. These results are summarized in Figure 1.

Phenotypic characteristics of the *parE206* and *parE374* mutations

The *parE206* and *parE374* mutations are genetically and physiologically distinct from other *parE* alleles. First, cultures of strains carrying either of these mutant alleles contain a large proportion of non-viable cells after a 2 hour incubation at the non-permissive temperature. In contrast, cultures of strains carrying either *parE232* or *parE374* have only slightly reduced viabilities after the same non-permissive temperature incubation (17). Second, *parE206* and *parE374* are both salt-correctable: they do not show a temperature-sensitive defect when grown at 44°C on complex medium supplemented with 0.5M KCl (see Table 1). Salt-correctability was not associated with any of the other *parE* alleles, nor with any *parC* or *parF* alleles (data not shown). Third, the *parE206* and *parE374* mutations confer a slight UV sensitivity in the mutant strain (see Table 1). This phenotype was not observed in strains carrying other *parE* alleles.

Sequence of the *parE206* and *parE374* mutations

Two strategies for moving the *parE206* and *parE374* mutations onto the cloned fragment were employed and are described in Materials and Methods. Each of the resulting plasmids (pMBS201,202,206,207) should express the mutant ParE proteins. Strains carrying these plasmids as well as a chromosomal *parE206* or *parE374* mutation remain temperature sensitive. In addition, the ParE(ts) plasmids do not cause temperature sensitivity in a wild type background, demonstrating that the *parE206* and *parE374* mutations are completely recessive to a single wild type copy of the *parE* gene.

Table 1. Salt-correctability and UV sensitivity of *parE* alleles.

Strain[a]	Growth at 44°C on 0.5M KCl[b]	Percent survival after UV[c]
recA1	NT[d]	10^{-3}%
parE493	−	70
parE232	−	70
parE377	−	70
parE374	+	10
parE206	10	
LT2	+	80
leu-485	+	90

[a]The parE strains used were derivatives of *leu-485* (SE5017). The *parE* mutations were moved into this background by transduction. Strains used were: SE7008, SE8466, SE7778, SE7763, SE7985, SE8041, SE5003, SE5017, respectively.
[b]Strains were streaked onto LB + 0.5 M KCl plates and incubated two days at 44°C.
[c]Log phase cultures were plated at appropriate dilutions on LB plates, and then irradiated for 20 seconds with a hand-held UV lamp. Irradiated plates were grown overnight in the dark at 30°C.
[d]NT = Not tested.

Two independently isolated clones were obtained for each mutation, and derivatives of each of these clones were sequenced. Double-stranded sequencing of the inserts in pMBS203, pMBS204, pMBS206 and pMBS209 revealed that *parE206* and *parE374* mutations contain the same basepair change, a G-C to A-T change at base 251 of the gene. This results in a valine-methionine change at amino acid 67. Plasmids containing *parE206* were unable to marker rescue the *parE374* mutation on the chromosome, and vice versa, further confirming the identity of these two mutations.

Sequence of the *parE377* and *parE493* mutations

The *parE377* and *parE493* mutations were sequenced from genomic DNA amplified by PCR as described in Materials and Methods. The mutation *parE377* causes a G-C to A-T change at basepair 1247 resulting in a glycine to serine amino acid change at position 399 (Figure 2). The *parE493* mutation is an A-T to C-G change at basepair 1799, resulting in a threonine to proline amino acid substitution at position 583 (Figure 2).

DISCUSSION

The DNA sequence of the *S. typhimurium parE* gene predicts a protein of 630 amino acids, which is 29 amino acids longer than the predicted *E.coli* ParE protein; otherwise, the proteins are 96.7% identical. The difference in size of the proteins results from an additional G-C basepair in the *S. typhimurium* sequence. There are several reasons that we believe that the longer protein predicted by the *S. typhimurium* sequence is correct for both *S. typhimurium* and *E.coli*. Most importantly, the DNA homology is retained after this frameshift, removing the possibility that a chromosome rearrangement had occurred, and suggesting that the reported *E.coli parE* sequence contains a simple frameshift error. Secondly, the *S. typhimurium parE* gene predicts a UAA termination codon, the stop codon most commonly used in prokaryotic genes (19); the *E.coli* protein terminates with a UG-A triplet. Moreover, active ParE protein could not be purified

from *E.coli* clones that express only the protein sequence reported by Kato, et al. (8) (H. Peng and K. Marians, personal communication). In addition, these truncated clones would not complement the *parE* temperature sensitive mutations used in this study (data not shown). In contrast, active protein and complementing activity were obtained from clones generated from the longer *S. typhimurium parE* sequence reported here (data not shown, and H. Peng & K. Marians, personal communication). These observations suggest that the ParE proteins of both *E.coli* and *S. typhimurium* extend for 630 amino acids.

The *S. typhimurium* ParE protein shows 41.1% amino acid identity to the *E.coli* GyrB protein. This amino acid homology extends throughout the GyrB-ParE proteins, but there are four regions that appear to distinguish the two proteins. First, and most noticeably, the *E.coli* GyrB protein has an 'insert' of approximately 180 amino acids found between amino acids 546–726 (1,6,21). The predicted GyrB proteins of two other gram-negative eubacteria also carry this insert (*Pseudomonas putida (14), Neisseria gonorrhoeae* (18)). In contrast, three bacteria in the gram-positive branch (*Mycoplasma pneumoniae, Bacillus subtilis, Staphylococcus aureus (12)*) and one archaebacterial species *(Haloferax (5))* lack the insert. This insert is found in neither the *E.coli* nor *S. typhimurium* ParE proteins. In addition, there are three regions that have much lower than average amino acid identity between GyrB and ParE but that are highly conserved between eubacterial GyrB sequences. These regions are: amino acids 174–248 (16% identity); amino acids 340–396 (21% identity); amino acids 590–630 (20.4% identity) (see Figure 3). (Amino acid numbers are those of the *S. typhimurium* ParE protein). These regions may contribute to the functional differences between the gyrase and topoisomerase IV proteins. The crystal structure of an N-terminal fragment of the E.coli GyrB protein has been solved to 2.5 Å resolution (20), and includes GyrB residues 2–392. Two of the three non-conserved regions are within the crystallized portion of GyrB. Amino acids 175–247 include a segment that forms a surface region adjacent to the putative DNA 'gateway' created by the interaction of two GyrB subunits (20). The second non-conserved region is the long α-helix that forms the base of the proposed gateway. These comparisons suggest that one source of difference between gyrase and topoisomerase IV will lie in the putative 'DNA gateway', where a strand of DNA has been hypothesized to pass through the GyrB protein. Additionally, the ParC protein must contribute to the functional distinctions between these two enzymes, since the C-terminal third of the ParC protein has only 20% identity to the GyrA protein (11).

The temperature sensitive mutations all lie in regions that are highly conserved between ParE and GyrB. The valine residue altered in *parE206/374* (V67M) is conserved in all GyrB and ParE sequences. The glycine changed by *parE377* (G399S) is also a completely conserved residue. The residue altered in *parE493* (T583P) is not conserved among the sequenced GyrB proteins nor between GyrB and ParE, but it lies within a stretch of 18/25 amino acids that are conserved between *E.coli* GyrB and ParE (72% identity). All of the temperature sensitive mutations alter residues that are identical in the *S. typhimurium* ParE and *E.coli* ParE proteins.

The residue affected by the *parE206/374* mutation (V67M) lies within the highly conserved nucleotide binding pocket, seen in the GyrB crystal structure. The alteration may impose a steric hinderance to binding of ATP at any temperature. However, we imagine that the mutant's temperature sensitive phenotype must arise primarily by destabilizing the β-sheet structure that forms the nucleotide binding pocket.

The genes encoding DNA gyrase and topoisomerase IV provide a unique evolutionary example. These genes probably arose by an ancient gene duplication, with subsequent functional specialization and fixation. During the evolution leading to the Enterobacteriaceae, two dramatic evolutionary events occurred: the *gyrA* and *gyrB* genes became unlinked and the *gyrA* gene acquired an insertion in the coding region. Comparisons of these genes in other bacteria will undoubtedly provide insight into mechanisms of bacterial evolution.

Note added in proof

An extra 'G' residue was found at basepair 1853 of the *E.coli parE* DNA sequence by P.M. Brotherton and P.T. Barth (Zeneca Pharmaceuticals, Cheshire, England).

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