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Escherichia coli DNA gyrase: genetic analysis of *gyrA* and *gyrB* mutations responsible for thermosensitive enzyme activity

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*Escherichia coli gyrA*43 and *gyrB*203 alleles conferring temperature-sensitive (ts) growth encoded Gly^{751}→Asp and Pro^{171}→Ser substitutions in the DNA gyrase A and B subunits, respectively. A plasmid-borne *gyrA*43 allele was genetically dominant over a chromosomal quinolone-resistant *gyrA* gene at 30°C but not at 42°C. These results and others confirm the ts phenotype of the mutation, the first to be identified in the C-terminal DNA binding/complex stabilizing domain of gyrase A protein. By contrast, the Pro^{171}→Ser mutation is located near the ATP-binding site of gyrase B protein and could interfere with energy coupling during DNA supercoiling. These data are discussed in regard to recently described *gyrA*(ts) mutations that affect the control of chromosome segregation.

DNA supercoiling; DNA gyrase; Temperature-sensitive mutation; Nalidixic acid resistance; *Escherichia coli*

1. INTRODUCTION

Escherichia coli DNA gyrase is an A_2B_2 tetramer encoded by *gyrA* and *gyrB* genes that catalyzes ATP-dependent DNA supercoiling and the formation and resolution of knotted and catenated DNA rings [1–3]. Studies of temperature-sensitive (ts) *gyrA* and *gyrB* strains have shown that gyrase is essential for bacterial growth, with roles in DNA replication, chromosome segregation and transcription [4–6]. The enzyme acts by passing a DNA segment through a transient double-stranded break in DNA [7]. The 97 kDa GyrA subunits mediate transient DNA breakage–reunion, a reaction interrupted by the 4-quinolone class of antimicrobial agents [8]. The 90 kDa GyrB subunits bind and hydrolyze ATP and are inhibited by coumarin antibiotics such as novobiocin and coumermycin [9]. Mutations conferring quinolone and coumarin drug resistance map predominantly in the *gyrA* and *gyrB* genes, respectively [8–14].

Several lines of evidence indicate that the *E. coli* GyrA and GyrB proteins are folded into domains with discrete functions [2,15–19]. A 50 kDa C-terminal GyrB fragment binds GyrA to reconstitute the DNA relaxation and cleavage activities of DNA gyrase but not DNA supercoiling [15]. The ATP binding site lies in the 43 kDa N-terminal GyrB fragment whose crystal structure was recently determined at high resolution [19]. Trypsin cleavage at residue 571 of the 875 residue GyrA protein generates two fragments: a 64 kDa N-terminal segment containing catalytic Tyr^{122} [20] and which carries the DNA breakage–reunion functions, and a 33 kDa C-terminal fragment that binds DNA and appears to stabilize the enzyme complex with DNA [16–18]. Studies of drug–gyrase interactions and identification of resistance mutations in gyrase genes have provided useful information, but this is restricted to those subunit domains involved in drug action. Given these limitations, we have sought to understand gyrase function by characterizing other novel mutations affecting enzyme activity. We have focussed on the thermosensitive *E. coli* strains, KNK453 and N4177, whose respective GyrA and GyrB proteins are active at 30°C but inactive at 42°C [4,21,22]. Here we report the sequence of the KNK453 *gyrA* and N4177 *gyrB* genes, identification of the ts mutations, and genetic studies of the *gyrA*43 allele.

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2. EXPERIMENTAL

2.1. *Polymerase chain reaction and DNA sequence analysis*

E. coli K-12 strains KNK453 [*gyrA*43(ts) F^- *polA thyA uvrA phx*] and N4177 [*strA gal gyrB*221 (cou^a) *gyrB*203 (ts)] were from K. Kreuzer and M. Gellert, respectively. The KNK453 *gyrA* gene was isolated in four segments by PCR using four pairs of oligonucleotides and KNK453 cells as the source of DNA. PCR primers and reaction conditions, restriction digestion of PCR products and subsequent cloning of fragments into M13mp18 and M13mp19 for sequence analysis were as described previously in the analysis of the DM750 *gyrA* gene [23]. For each fragment, recombinant clones derived from at least two independent PCR reactions were sequenced by the dideoxy chain-termination method [24] using the Sequenase version 2.0 protocol (United States Biochemicals). DNA sequences obtained for each complementary strand of the gene were in agreement.

The N4177 *gyrB* gene was isolated similarly as a 1.2 kb PCR product using oligonucleotide primers 5' GATTAACCCAAGCTTAA ATGAGCG (nucleotide positions −34 to −11, an artificial *Hin*dIII site is underlined) [25] and 5' CGAGCGCACCTTTACGGCGG (1,195–1,176). DNA was digested with *Hin*dIII and *Cla*I, re-cut at a unique internal *Bgl*II site, and the fragments cloned into M13mp18 and M13mp19 for DNA sequence analysis.

2.2. *Construction of plasmid pMO43 bearing a gyrA(ts) gene*

Amp^R plasmid pRM386 containing a wild-type *gyrA* gene was kindly provided by Rolf Menzel [26]. The following procedure was used to construct pMO43 carrying the *gyrA*43(ts) allele. Plasmid pRM386 DNA lacking the *Kpn*I–*Sna*BI *gyrA* fragment was isolated by partial *Sna*BI digestion of *Kpn*I-cut plasmid and electrophoresis in 2% low gelling agarose. The DNA was ligated to the corresponding KNK453 *Kpn*I–*Sna*BI *gyrA* fragment obtained from an M13 replicative form clone of PRC product D [23] which had been fully sequenced and shown to encode the putative ts mutation. Recombinant products were transformed into *E. coli* DH5 (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1). Plasmids were isolated and characterized by restriction digestion with *Kpn*I, *Sna*BI, *Hin*dIII and *Bgl*I. One plasmid, pMO43, gave identical restriction digests to pRM386, suggesting assembly of a functional *gyrA*43 allele. This plasmid isolate was used to transform KNK453 to ampicillin resistance.

2.3. *Bacterial growth*

Bacterial cultures were grown in L-broth at 30°C. Cleared lysates were prepared identically by lysozyme treatment of bacteria grown to log phase (OD_{650} = 1.0) at 30°C as described previously [27]. Growth of KNK453 and its transformants was tested by spotting cultures (10 μl) on to H-plates containing 50 μg/ml ampicillin and incubating overnight at 30 or 42°C. For similar experiments involving DH5, H-plates containing 100 μg/ml nalidixic acid and 50 μg/ml ampicillin were used.

3. RESULTS

3.1. *Temperature-sensitive mutations in* E. coli gyrA *and* gyrB

The nucleotide sequence of the entire KNK453 *gyrA* gene and promoter was determined and found to be identical to that reported for the wild type *E. coli* K12 gene [28] except for one nucleotide change: a G→A transition at nucleotide 2,252 that produces a Gly^{751}→Asp substitution in the KNK453 GyrA protein. There were no other changes in the KNK453 gene or its promoter. The observed nucleotide transition is consistent with the 2-aminopurine mutagenesis protocol originally used in deriving KNK453 [5,29]. In contrast, N4177 *gyrB* exhibited two coding changes compared to the wild-type gene [25]: CGC→TGC and CCC→TCC changes at nucleotides 406 and 511, generating Arg^{136}→Cys and Pro^{171}→Ser substitutions in GyrB, respectively. The Arg^{136}→Cys substitution is known to confer coumarin resistance [11,12] implying that the thermosensitivity of N4177 GyrB arises from mutation of Pro^{171}. This analysis is consistent with the observation that coumermycin resistance and thermosensitivity of N4177 arise from two different but closely linked mutations in *gyrB* [21]. The same nucleotide changes in N4177 *gyrB* have recently been reported independently [12].

3.2. *Gly^{751}→Asp GyrA mutation alone confers ts phenotype*

To facilitate genetic analysis, the KNK453 *gyrA* gene was assembled by exchanging a *Kpn*I–*Sna*BI *gyrA* fragment bearing the *gyrA*43 mutation with its counterpart in the wild-type *gyrA* gene of plasmid pRM386 (Fig. 1) [10]. KNK453, transformed with the resulting plasmid pMO43, grew at 30 but not at 42°C, in contrast to KNK453 (pRM386) which grew at both temperatures (Fig. 2a). Only KNK453 (pRM386) over-expressed a 100 kDa protein (Fig. 2b), previously identified as GyrA by using a polyclonal anti-GyrA antiserum (not shown). The results suggested that the Gly^{751}→Asp GyrA protein is temperature sensitive and is unstable in KNK453 cells even at 30°C.

3.3. gyrA^R–gyrA*(ts) dominance studies: effects of copy number*

Conclusive evidence for the thermosensitivity of the recombinant KNK453 GyrA protein was obtained by exploiting the observation that (in meridiploid *E. coli* strains) quinolone-sensitive *gyrA* alleles are dominant over drug-resistant alleles [30]. This dominance is thought to arise because quinolones kill bacteria by trapping gyrase complexes on DNA [10]. In agreement with this idea, pRM386 blocked nalidixic acid-resistant growth of *E. coli* DH5 *gyrA*^{R} at both 30 and 42°C (Fig. 3a). However, transformation with pMO43 rendered DH5 drug sensitive at 30°C but drug resistant at 42°C. This behaviour is consistent with expression of the qui-

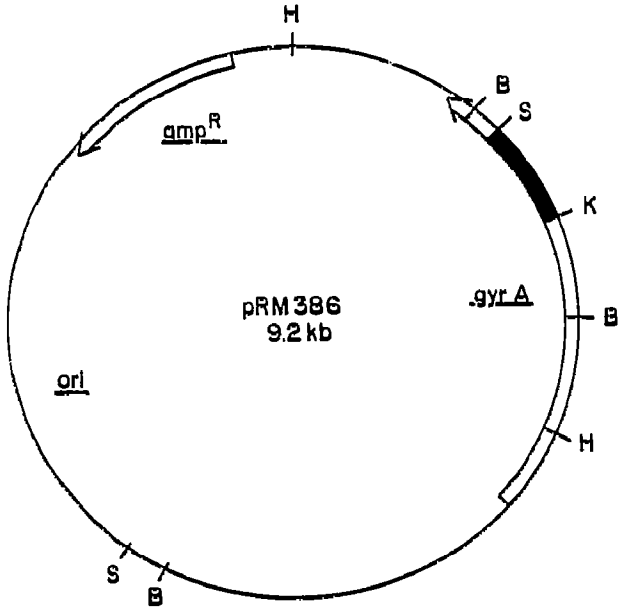

Fig. 1. Restriction map of *gyrA* plasmid pRM386 and its use in the expression of the KNK453 *gyrA* gene. B, H, K and S are sites for *Bgl*II, *Hin*dIII, *Kpn*I and *Sna*BI, respectively. Arrows, indicate *amp* and wild-type *gyrA* genes. Exchange of the *Kpn*I–*Sna*BI *gyrA* fragment (filled) with its KNK453 counterpart encoding a Gly^{751}→Asp GyrA mutation generated plasmid pMO43 which expressed the *gyrA*43 allele.

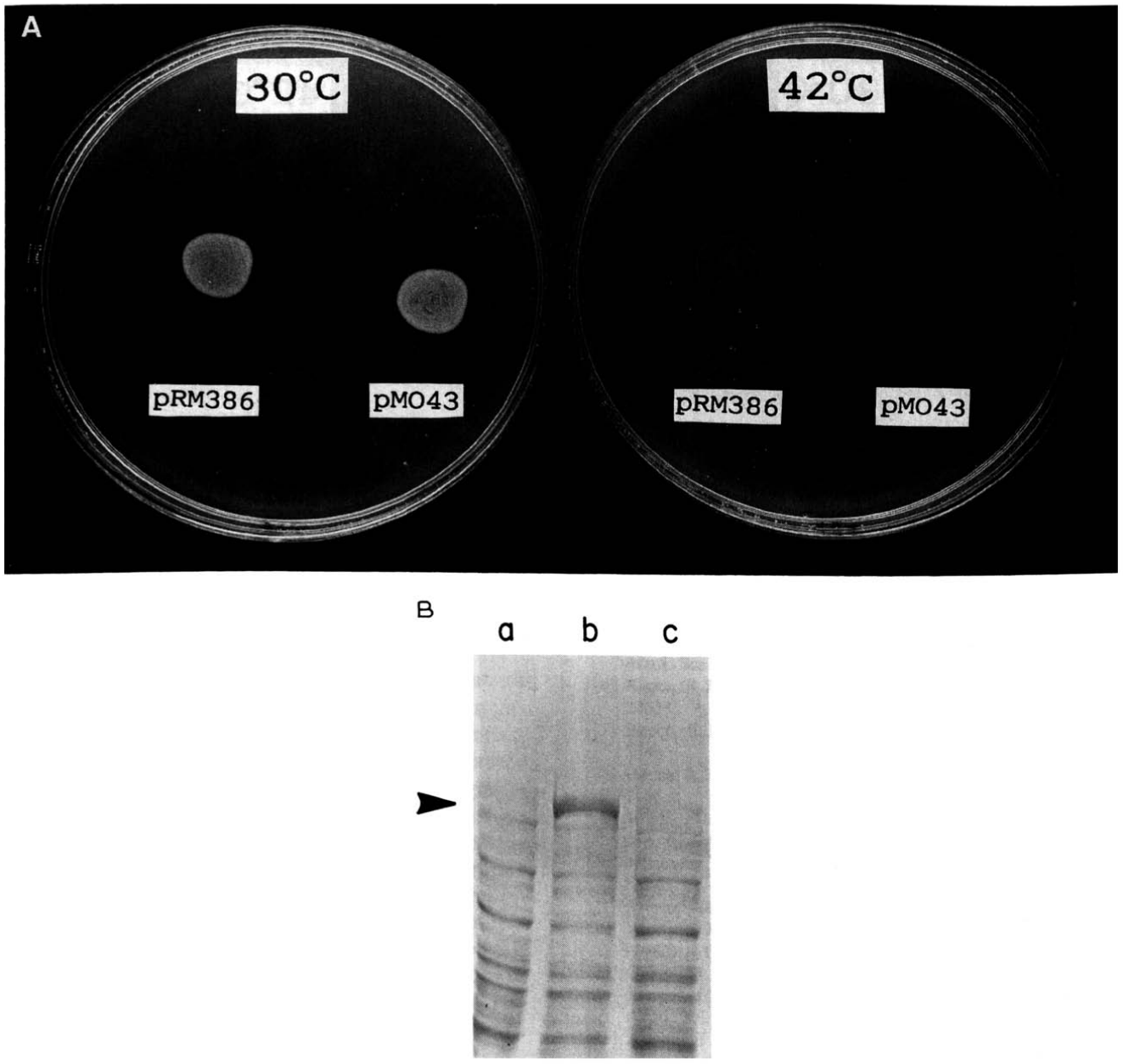

Fig. 2. Growth properties of *E. coli* KNK453 transformed with plasmid pRM386 or pMO43 bearing wild-type *gyrA* or *gyrA*43 alleles. (A) Temperature-sensitive growth of KNK453 at 42°C is rescued by transformation with pRM386 but not by pMO43. Bacteria were spotted on ampicillin plates and incubated overnight. (B) GyrA protein, indicated by the arrowhead, is over-expressed in KNK453 (pRM386) but not in KNK453 (pMO43) cells. Bacterial lysates (20 μg protein) from KNK453, KNK453 (pRM386) and KNK453 (pMO43) were run on a 7.5% SDS-polyacrylamide gel (lanes a–c, respectively).

nolone- and temperature-sensitive *gyrA* gene from pMO43, confirmed by analysis of protein extracts (Fig. 3b).

Previous studies have shown that plasmid-borne quinolone-resistant *gyrA* genes act dominantly over the chromosomal *gyrA*43 allele in KNK453 or the related strain KNK402 ([10,14] and refs. therein). Thus, the outcome of dominance tests between $gyrA^{+}$ and *gyrA*(ts) alleles appears to depend on their relative levels of expression.

4. DISCUSSION

We have identified two mutations in ts gyrase genes, one in *gyrA* and one in *gyrB*. These mutations result in Gly^{751}→Asp and Pro^{171}→Ser changes in the respective GyrA and GyrB proteins. The ts phenotype of the Gly^{751}→Asp mutation was confirmed directly by examining the genetic properties of the *gyrA*43 allele expressed from a high copy plasmid. As expected, the plasmid borne is allele was dominant over a quinolone-

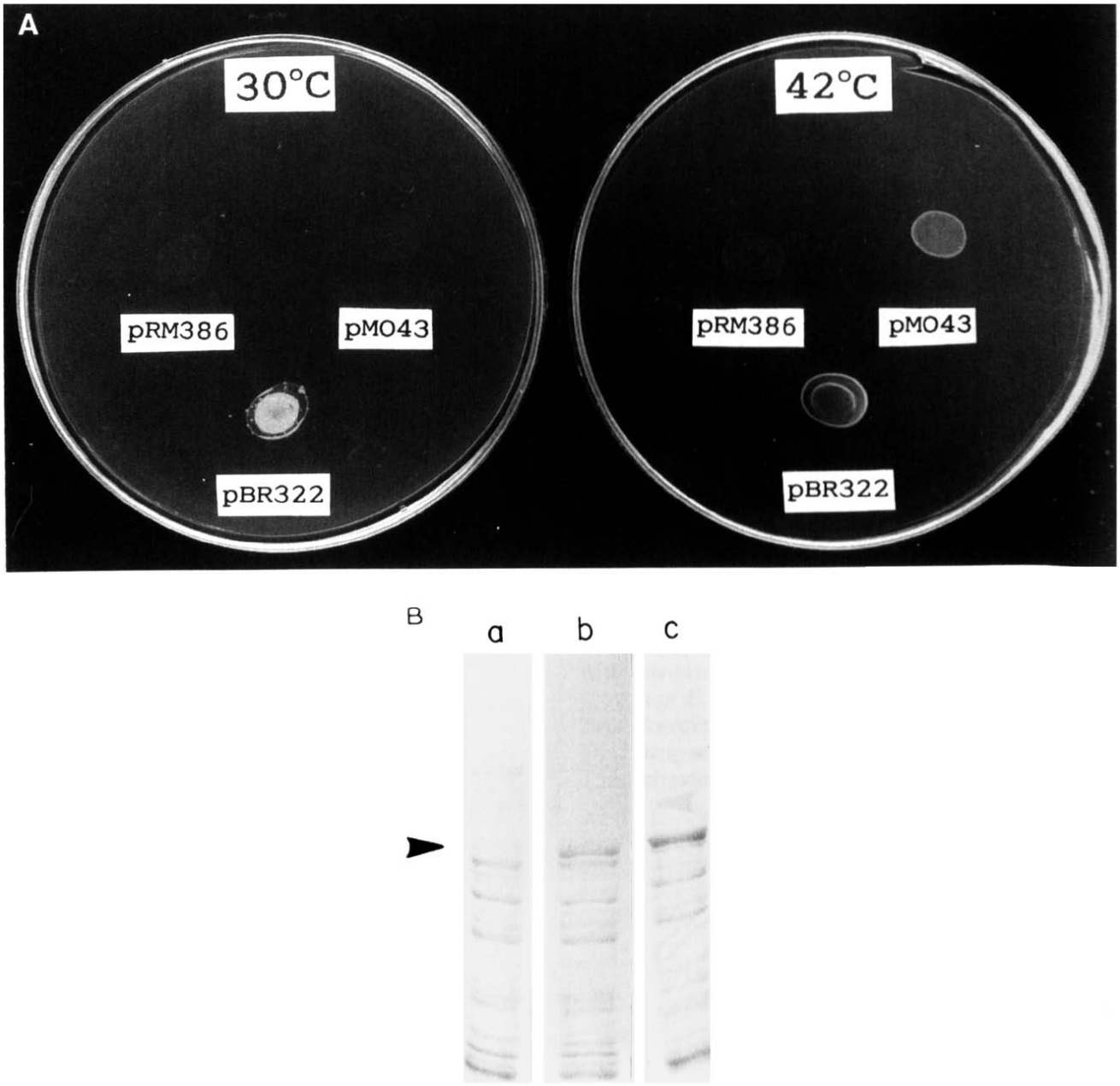

Fig. 3. The *gyrA*43 allele over-expressed in quinolone-resistant *E. coli* DH5 exerts a dominant quinolone-sensitive phenotype. (A) pMO43 confers nalidixic acid sensitivity on DH5 at 30 but not at 42°C. The wild-type *gyrA* gene on pRM386 blocked DH5 growth at both temperatures. DH5 cells transformed with the *amp* plasmid, pBR322, lacking a *gyrA* gene were resistant to nalidixic acid. Plates contained ampicillin and nalidixic acid. (B) GyrA protein is over-expressed from plasmid pMO43 in *E. coli* DH5. Bacterial lysates of DH5, DH5(pMO43) and DH5 (pRM386) were run on a 7.5% SDS-polyacrylamide gel (lanes a–c, respectively).

resistant chromosomal *gyrA* allele in a quinolone sensitivity assay at 30°C but not at 42°C (Fig. 3a). Surprisingly, when the plasmid and chromosomal locations of the two alleles are reversed, then the plasmid $gyrA^R$ gene becomes dominant over *gyrA*43 even at the permissive temperature [10,14]. Under the latter conditions, it would seem that a large excess of resistant GyrA subunits produced by the high copy plasmid is able to sequester the limited amount of available GyrB protein yielding predominantly drug-resistant gyrase complexes. Another contributory factor could be the lower basal level of host GyrA protein observed in KNK453 cells [4], perhaps reflecting the apparent instability of the Gly^{751}→Asp protein even at 30°C (Figs. 2b, 3b). These results highlight the need to consider copy number effects in $gyrA^R$–*gyrA*(ts) dominance studies.

How do ts mutations in gyrase result in thermolabile enzyme activity? Studies of ts mutations in other systems have identified a variety of structural alterations. Recent work has shown that thermolability can often

ensue from substitution of a proline residue located in a turn motif [31]. Consistent with this observation, we note from the crystal structure of the 43 kDa N-terminal GyrB fragment, that the Pro^{171}→Ser mutation in N4177 GyrB lies at a turn of the polypeptide chain at the C-terminal end of a β strand [19]. Thus, although the full *gyrB* sequence was not determined, these results strongly suggest that the mutation at Pro^{171} confers the ts phenotype of N4177. Residue 171 is adjacent to, but not part of, the ATP binding site and its mutation to Ser could act by disrupting ATP utilization by gyrase at the non-permissive temperature.

The ts mutation in KNK453 GyrA substitutes Gly^{751} in a highly conserved sequence (Thr-Glu-Asn-Gly-Tyr-Gly-Lys-Arg-Thr) which is predicted to form a β turn (not shown). The mutation is the first to be reported in the 33 kDa C-terminal segment of GyrA protein, a DNA binding domain that stabilizes the gyrase complex on DNA [18]. Substitution of a flexible glycine with the negatively charged aspartate residue could affect DNA binding by the complex. These ideas are speculative in the absence of high resolution X-ray data for GyrA protein. However, the KNK453 GyrA mutation should provide a novel probe with which to investigate the functions of the DNA binding GyrA domain.

Finally, ts mutations in gyrase genes have attracted recent attention in the context of chromosomal segregation. Miki et al. [32] have identified several *E. coli* mutations which suppress growth inhibition by the sex factor F *letD* gene, the protein product of which inhibits partitioning of chromosomal DNA and host cell division. One mutation, *tldC*15, specified a Gly^{214}→Glu substitution in GyrA and conferred a dual phenotype: tolerance to *letD* product growth inhibition in a host carrying an F*letA* mutant, and ts growth at 42°C. The *tldC* mutation (like *gyrA*43) involves substitution of a glycine with an acidic residue, but lies in the DNA breakage–reunion domain of the A protein. Surprisingly, the *gyrA*42(ts) mutation (isolated with *gyrA*43 and *gyrA*45 by a completely different proceudre [12]), but not a *gyrB*(ts) mutation, had the same phenotype as *tldC*15. These results suggested that GyrA is the direct (or indirect) target of the *letD* protein, and that certain (ts) mutations in GyrA, e.g. *gyrA*42, are able to suppress the biological effects of this interaction. The availability of gyrase ts alleles, particularly those such as *gyrA*43 for which the mutational basis is known, may prove useful in further defining the gyrase–letD protein interaction and the role of gyrase in chromosome partitioning.

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