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Characterization of a temperature-sensitive DNA ligase from *Escherichia coli*

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DNA ligases are essential enzymes in cells due to their ability to join DNA strand breaks formed during DNA replication. Several temperature-sensitive mutant strains of *Escherichia coli*, including strain GR501, have been described which can be complemented by functional DNA ligases. Here, it is shown that the *ligA251* mutation in *E. coli* GR501 strain is a cytosine to thymine transition at base 43, which results in a substitution of leucine by phenylalanine at residue 15. The protein product of this gene (LigA251) is accumulated to a similar level at permissive and non-permissive temperatures. Compared to wild-type LigA, at 20 °C purified LigA251 has 20-fold lower ligation activity *in vitro*, and its activity is reduced further at 42 °C, resulting in 60-fold lower ligation activity than wild-type LigA. It is proposed that the mutation in LigA251 affects the structure of the N-terminal region of LigA. The resulting decrease in DNA ligase activity at the non-permissive temperature is likely to occur as the result of a conformational change that reduces the rate of adenylation of the ligase.

INTRODUCTION

An essential component of DNA replication in all organisms is the joining of breaks in the phosphodiester backbone of DNA. This reaction occurs on the lagging strand of the replication fork and is performed by a ubiquitous class of enzymes known as DNA ligases (Doherty & Suh, 2000; Lehman, 1974; Timson *et al.*, 2000). If this end-joining reaction does not take place, the chromosomal DNA is totally degraded, and cells are no longer viable. Biochemical studies of DNA ligases purified from a variety of organisms and viruses have shown that the reaction mechanism can be broken down into three steps (Doherty & Suh, 2000; Lehman, 1974; Timson *et al.*, 2000). In the first step of the reaction, a covalent enzyme–adenylate intermediate is formed at a specific lysine in the catalytic motif of the enzyme (KXDG in motif I; see Fig. 3). The adenylate group can be provided either by ATP or by NAD^+, leading to the classification of the enzymes as ATP- or NAD^+-DNA ligases.

Within every bacterial genome sequenced to date are ORFs predicted to encode NAD^+-DNA ligases of similar size and extensive amino acid sequence homology (Wilkinson *et al.*, 2001). NAD^+-DNA ligases have been purified from many bacteria, and have similar biochemical structures and properties, but the paradigm for such enzymes is the *Escherichia coli* K-12 enzyme, which is a 671 amino acid, 74 kDa protein encoded by the essential gene *ligA* (Ishino *et al.*, 1986; Lehman, 1974). Microbiological and genetic analysis has confirmed that NAD^+-DNA ligases are also essential for *Bacillus subtilis* (Petit & Ehrlich, 2000), *Staphylococcus aureus* (Kaczmarek *et al.*, 2001) and *Mycobacterium tuberculosis* (Gong *et al.*, 2004; Sassetti *et al.*, 2003). Thus, NAD^+-DNA ligases are likely to be indispensable to all bacteria.

Due to the essential involvement of *ligA* in replication, its inactivation leads to the non-viability of most bacteria. However, several temperature-sensitive (ts) strains of *E. coli* were isolated during the 1970s and shown to have mutations in *ligA* (Dermody *et al.*, 1979; Gellert & Bullock, 1970; Karam *et al.*, 1979; Konrad *et al.*, 1973; Modrich & Lehman, 1971). *E. coli* GR501 is one of the most ligase-deficient of these strains, and initial characterization suggested that its ts phenotype was due to a mutation in *ligA*, resulting in a reduction in DNA replication at high temperatures (Dermody *et al.*, 1979). This conclusion has been reinforced by the observation that it can be complemented by DNA

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Abbreviation: ts, temperature sensitive.

ligases that participate in replication in other systems, including human DNA ligase I (Kodama *et al.*, 1991) and T7 DNA ligase (Doherty *et al.*, 1996). Although *E. coli* GR501 has been particularly useful for the analysis of DNA ligase function in bacteria, there has been little characterization of the nature and biochemical consequences of the mutation in this strain.

Recently, an NAD^+-DNA ligase was identified within the genome of Amsacta moorei entomopoxvirus (Sriskanda *et al.*, 2001), although functional NAD^+-DNA ligases have not been characterized in eukaryotic genomes. Thus, NAD^+-DNA ligases have been suggested as possible targets for broad-spectrum antibacterial compounds (Georlette *et al.*, 2003; Gong *et al.*, 2004; Kaczmarek *et al.*, 2001; Lee *et al.*, 2000; Singleton *et al.*, 1999; Sriskanda & Shuman, 2002). If substantial progress is to be made in the development of inhibitors that are specific to NAD^+-DNA ligases, it is vital that *in vivo* models are utilized to test the efficacy of such compounds. This has recently been demonstrated in elegant studies of the antibacterial nature of pyridochromanones, which are good inhibitors of the LigA from several bacteria (Brotz-Oesterhelt *et al.*, 2003; Gong *et al.*, 2004). Complementation experiments using *E. coli* GR501 demonstrated that these compounds do not act on human DNA ligase I, an ATP-DNA ligase (Brotz-Oesterhelt *et al.*, 2003). To understand the relationship of the mutation in *E. coli* GR501 to the structure and function of *E. coli* LigA, it is imperative that the molecular details of the mutation in this strain are identified. Here, we identify the mutation in the *ligA* gene of *E. coli* strain GR501, analyse its expression and overexpress the protein in a recombinant form. Biochemical analysis is used to pinpoint the molecular basis for the ts mutation in LigA from *E. coli* GR501.

METHODS

Growth of bacterial cultures. Strain and plasmid details are provided in Table 1. Growth of *E. coli* was monitored at a variety of temperatures on agar plates and in liquid cultures. In all cases, Luria broth (LB) was the nutrient medium. Antibiotics were added to media as required, with final concentrations of 100 μg ampicillin ml^{-1} and 50 μg chloramphenicol ml^{-1}. Stock cultures containing 25 % glycerol (v/v) were stored at −80 °C and streaked onto fresh LB agar plates as required. Cells were made competent for DNA transformation by chemical means, and stored in 200 μl aliquots at −80 °C (Sambrook & Russell, 2001).

At the start of each experiment, cultures containing *E. coli* GR501 were confirmed to be temperature sensitive. Transformations with the required plasmids were performed from glycerol stocks of competent cells maintained at −80 °C (Sambrook & Russell, 2001) streaked onto fresh LB agar plates (with antibiotic as required) and grown overnight at 30 °C. To assay for complementation of the ts defect, single colonies were then streaked onto two fresh LB agar plates: the plate streaked first was incubated at 43 °C and the one streaked second was incubated at 30 °C. Note that complementation by proteins expressed from pTRC99A could be achieved without addition of IPTG, indicating that expression from the strong *lac*-derived promoter of this plasmid was not completely inhibited in *E. coli* GR501.

For liquid-culture growth, single colonies from plates grown at 30 °C were inoculated into 5 ml liquid medium and grown overnight at 30 °C. These cultures were diluted 100-fold into 5 ml fresh medium and incubated at the required temperature. Growth of bacteria was detected by monitoring OD_{600} every 30 min for the first 2 h and subsequently every 15 min for the remainder of the incubation period.

For analysis of the viability of the various strains harbouring different plasmids, the appropriate cultures were grown in LB (with antibiotic if required) at 30 °C overnight. Viable cell counts were determined by plating 200 μl of a 10^{-6} dilution of the overnight culture onto LB agar plates and counting the colonies after aerobic incubation at 30 °C or 43 °C for 24 h.

Table 1. Strains and plasmids used in this study

E. coli strain	Genotype	Source/reference
GR501	Hfr *LAM*$^-$ *ligA251 relA1 spoT1 thi-1 ptsI*$^+$	CGSC (Dermody *et al.*, 1979)
CHE30	Hfr *LAM*$^-$ *ptsI relA1 spoT1 bglR7 thi-1*	CGSC (Dermody *et al.*, 1979)
BL21pLysS	F$^-$ *ompT rB*$^-$ *mB*	Lab. stock (Sambrook & Russell, 2001)
DH5α	Δ*lacU169 recA1 endA1 gyrA96 relA1 hsdR17 thi-1 supE44*	Lab. stock (Sambrook & Russell, 2001)
Plasmid	**Description, antibiotic resistance**	**Source/reference**
pTRC99A	Expression vector derived from pKK233-2, Amp	Amersham Pharmacia
pET-16b	Expression vector designed for the purification of proteins, introduces a 10-His-tag at N-terminus of protein, Amp	Novagen
pRBL	pTRC99A containing gene for T4 DNA ligase, Amp	Ren *et al.*, 1997
pRJ345	pTRC99A expressing the colicin immunity protein Im9, Amp	Wallis *et al.*, 1995
pRB20	pET-16b expressing full-length *E. coli ligA* (including 10-His-tag), Amp	Wilkinson *et al.*, 2003
pRB158	pET-16b expressing full-length *E. coli ligA251* (including 10-His-tag), Amp	This work
pRB154	pTRC99A expressing full-length *E. coli ligA* (including 10-His-tag), Amp	Wilkinson *et al.*, 2003
pRB159	pTRC99A expressing full-length *E. coli ligA251* (including 10-His-tag), Amp	This work

Cloning of DNA ligases. Genomic DNAs were prepared from appropriate overnight cultures using a Wizard Genomic DNA purification kit (Promega). Cloning of *E. coli* K-12 NAD^+-DNA ligase (LigA; 671 amino acids) has been described previously (Wilkinson *et al.*, 2003). LigA from *E. coli* GR501 (LigA251) was prepared in a similar manner: amplified from genomic DNA isolated from *E. coli* GR501 by PCR with a proof-reading DNA polymerase. Note that the 5′ primer contained an *Nde*I site and that the 3′ primer contained a *Bam*HI site. PCR products were cloned using the Zero Blunt TOPO Cloning kit (Invitrogen) and sequenced to confirm that the recombinant gene was the same as that identified in *E. coli* GR501 genomic DNA. Fragments were excised from the TOPO vectors using the *Nde*I and *Bam*HI sites and cloned into pET16b (Novagen). Proteins overexpressed from this vector contain a 10-His tag within an extra 21 amino acids (2·5 kDa) at the N-terminus.

To allow overexpression of proteins in *E. coli* GR501, full-length ligases plus the His-tag were excised from pET-16b vectors using the *Nco*I and *Bam*HI sites and cloned into pTRC99A (Amersham Pharmacia). In control experiments analysing expression of proteins from pTRC99A, T4 DNA ligase was expressed from pRBL (Ren *et al.*, 1997) and Im9, an inhibitor of the colicin E9, was expressed from pRJ354 (Wallis *et al.*, 1995).

Protein purification. For protein expression, all *E. coli* cultures were grown at 37 °C in LB containing ampicillin and chloramphenicol. The pET16b derivatives were transformed into *E. coli* BL21 (DES) pLysS, and cells were plated on LB agar containing antibiotics and grown overnight. Single colonies were inoculated into 5 ml liquid medium, grown overnight and diluted 100-fold into fresh medium (50–500 ml). After growth to mid-exponential phase ($OD_{600}=0{\cdot}4$–$0{\cdot}6$), protein expression was induced by addition of IPTG to 0·4 mM. After 4 h further incubation, cells were harvested, sonicated and centrifuged to separate soluble and insoluble fractions. DNA ligases were purified from the soluble fraction using columns with affinity for the His-tag (HiTrap Chelating HP, Amersham Pharmacia). After concentration using Vivaspin 20 ml concentrators with a 5000 molecular weight cut-off PES membrane, the samples were loaded on to a HiLoad 16/60 Superdex 75 prep grade column. The column was run at 1 ml min^{-1} for 2 h in 20 mM Tris, pH 7·5, 200 mM NaCl. The OD_{280} was measured to find the peak fractions, which were collected, pooled and concentrated. Typically, full-length DNA ligases eluted after 45 min. Protein concentrations were determined by the Bradford method (Bio-Rad Protein Assay). In general, lower levels of expression were obtained for LigA251 than for LigA: standard amounts of pure protein obtained from each litre of induced culture were 100 mg for LigA and 15 mg for LigA251. The thermal stability of LigA and LigA251 was analysed by SDS-PAGE after incubation of 2 μg (30 pmol) of protein at temperatures between 4 and 37 °C for 21 h. For long-term storage at −80 °C, glycerol was added to a final concentration of 20 % (v/v).

Analysis of ligation activity. *In vitro* assays of ligation activity were performed using a double-stranded 40 bp DNA substrate carrying a single-strand nick between bases 18 and 19 (Timson & Wigley, 1999). This substrate was created in TBE buffer by annealing an 18-mer (5′-GTA AAA CGA CGG CCA GTG-3′) and a 22-mer (5′-AAT TCG AGC TCG GTA CCC GGG G-3′) to a complementary 40-mer (5′-CCC CGG GTA CCG AGC TCG AAT TCA CTG GCC GTC GTT TTA C-3′). At the 5′ end, the 18-mer contained a fluorescein molecule and the 22-mer was phosphorylated.

The nicked 40 bp substrate was used to assay the *in vitro* ligation activity of each enzyme. Time-course assays used 750 pmol DNA in 50 μl reactions and different amounts of LigA and LigA251, and were performed at various temperatures and times. Reactions were conducted in the presence of 26 μM NAD^+ in 30 mM Tris, pH 8·0, 4 mM $MgCl_2$, 1 mM DTT and 50 μg BSA ml^{-1}. At the end of the incubation, 5 μl samples were mixed with an equal volume of formamide loading buffer, heated to 95 °C, loaded onto a 15 % polyacrylamide/urea gel (10 × 10 cm) and run at 300 V for 1 h in 1× TBE. Reaction products on the gel were visualized and quantified using a Molecular Dynamics Storm phosphorimager.

To assay for ligation activity *in vivo*, we tested whether different plasmid constructs could complement *E. coli* GR501 at temperatures that are normally restrictive to growth. Following the guidelines for the use of *E. coli* GR501, cells were transformed with pTRC99A containing the gene for the relevant DNA ligase and grown on LB agar containing ampicillin at 30 °C or 43 °C.

Analysis of LigA expression. Bacterial cultures were grown at the required temperature and, at exactly $OD_{600}=0{\cdot}7$, 5 ml of culture was harvested and resuspended in 100 μl Tris/glycine SDS gel loading buffer (Sambrook & Russell, 2001). In some experiments, 0·4 mM IPTG was added at $OD_{600}=0{\cdot}2$ to induce overexpression of recombinant DNA ligases. Cell extracts were fractionated by SDS-PAGE, and LigA and LigA251 were detected by a standard protocol for Western blotting (Western Blue Express, Promega). Adenylated and deadenylated forms of LigA were resolved by electrophoresis at 100 V for 5 h on 15 % SDS-PAGE, whilst detection of His-tagged proteins was performed after electrophoresis at 150 V for 1 h on 8 % SDS-PAGE. The primary antibody to *E. coli* LigA (Davids Biotechnologie) was a rabbit polyclonal raised against purified LigA (containing His-tag), and the primary antibody to the His-tag was a mouse monoclonal (Amersham Pharmacia). Appropriate secondary antibodies were conjugated to alkaline phosphatase (Promega).

Sequence analysis and molecular modelling. Sequences of DNA ligases were identified from the NCBI database (http://www.ncbi.nlm.nih.gov/). Alignment of protein sequences was performed using the CLUSTAL W method (DNAStar LaserGene MegAlign). A molecular model for *E. coli* LigA was generated using the X-ray crystallographic structure of *Thermus filiformis* NAD^+-DNA ligase (Lee *et al.*, 2000) (Protein Database accession number 1DGT). The structure was automatically generated using SWISS-MODEL (http://www.expasy.ch/spdbv/) (Guex & Peitsch, 1997) and visualized using RasMol. Since the structure of *T. filiformis* DNA ligase does not contain high-resolution details of all amino acids (Lee *et al.*, 2000), only amino acids 1–586 are contained in our molecular model of *E. coli* LigA.

RESULTS

Growth analysis of *E. coli* GR501

Studies of the essential functions of LigA have been aided by several *E. coli* strains that carry temperature-sensitive (ts) mutations. Although many of these strains were isolated by the late 1970s, details about the genetic and biochemical basis of these ts mutations are incomplete. *E. coli* GR501 is viable at 30 °C, but is unable to grow at temperatures of 42 °C and above (Dermody *et al.*, 1979). Since this strain is becoming particularly useful for searches of potential inhibitors of NAD^+-DNA ligases (Brotz-Oesterhelt *et al.*, 2003), it is imperative that the genetic and biochemical basis of the ts mutation is identified. Note that the DNA ligase gene in *E. coli* GR501 is the *ligA251* allele derived from *E. coli* SG251 (Dermody *et al.*, 1979); we refer to the protein product of this gene as LigA251.

E. coli GR501 was obtained from the *E. coli* Genetic Stock Center (CGSC). To confirm that this strain had a

temperature-sensitive mutation in *ligA*, we tested for complementation of the strain with functional DNA ligases expressed from pTRC99A, which can be used to express genes in *E. coli* under the control of an IPTG-inducible strong promoter (Ren *et al.*, 1997). Previous studies have confirmed that expression of DNA ligases from this vector allows growth of *E. coli* GR501 at temperatures that are normally non-permissive (Brotz-Oesterhelt *et al.*, 2003; Kodama *et al.*, 1991; Ren *et al.*, 1997; Wilkinson *et al.*, 2003). Two controls were performed to confirm that complementation of growth at non-permissive temperatures was due to the expression of DNA ligases which are functional in *E. coli* GR501. Firstly, the thermosensitivity of cells harbouring the empty vector (pTRC99A) was assessed. Secondly, to confirm that the complementation was not due to non-specific protein expression, bacteria were transformed with pRJ345 (Wallis *et al.*, 1995), a pTRC99A-derived plasmid which expresses the colicin inhibitor Im9 and has no DNA ligase functions. *E. coli* GR501 strains harbouring these derivatives of pTRC99A were grown on plates at 30 or 43 °C. This assay was carried out without IPTG, as it had been shown that the vector expression system allowed a high level of protein synthesis even in the absence of the inducer (see below). As expected, growth was observed at 30 °C (Fig. 1a). However, cells lacking a wild-type DNA ligase grew more slowly than cells encoding such an enzyme from the vector. In contrast, only plasmids expressing *E. coli* LigA and T4 DNA ligase complemented the temperature-sensitive mutation and allowed *E. coli* GR501 to grow well on plates at 43 °C. Note that these observations confirm that mutations in the NAD^+-DNA ligase of *E. coli* can be complemented by overexpression of an ATP-DNA ligase (Doherty *et al.*, 1996; Kodama *et al.*, 1991).

To further assess the efficiency of complementation of *E. coli* GR501 by the recombinant DNA ligases, we compared strain viability at 30 and 43 °C on plates (Table 2). The effect of the *ligA251* allele was examined by comparison of growth with *E. coli* CHE30, which is isogenic to *E. coli* GR501 apart from the ts mutation (Dermody *et al.*, 1979). Generally, from each strain grown from a stationary-phase culture there were at least 10^9 c.f.u. ml^{-1} at 30 °C (Table 2), as expected for *E. coli* in good conditions for growth. Thus, overexpression of the T4 or *E. coli* DNA ligase had no significant effect on cell viability at 30 °C.

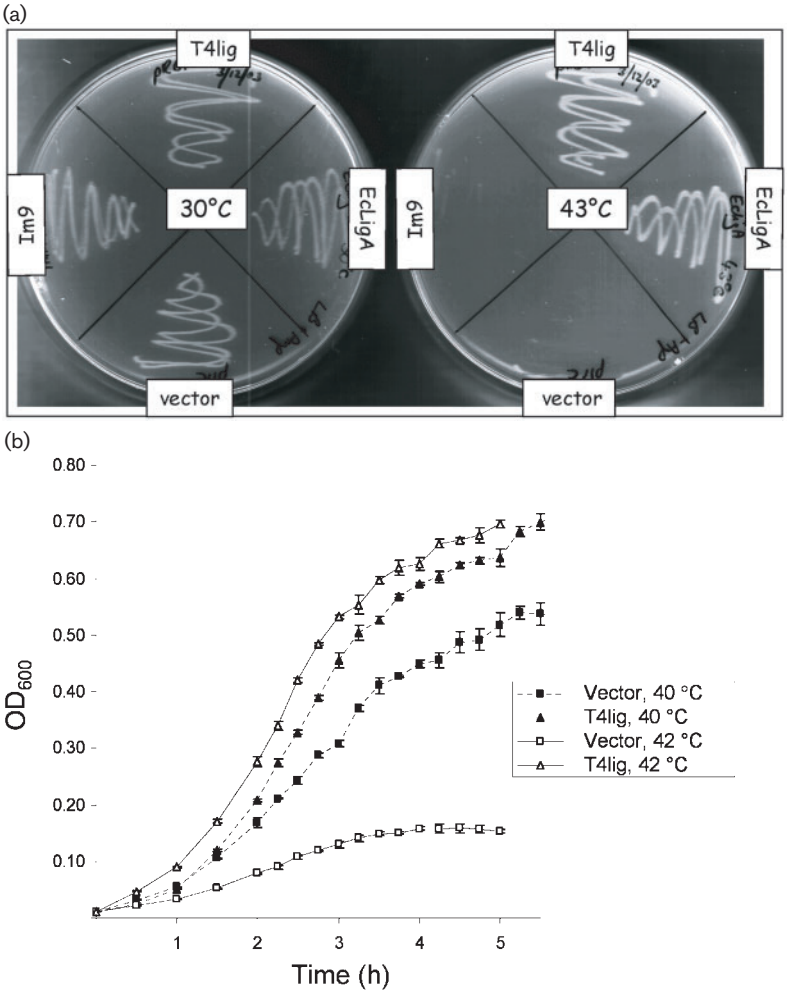

Fig. 1. Growth analysis and complementation of *E. coli* GR501. (a) *E. coli* GR501 transformed with vector pTRC99A expressing various proteins was streaked onto LB agar plates containing ampicillin and grown at 30 °C (left-hand plate) or 43 °C (right-hand plate). (b) Growth curves of *E. coli* GR501 with or without expression of T4 DNA ligase at 40 and 42 °C. Data represent the mean and SE of three experiments under the growth conditions indicated. 'Vector' and 'T4lig' refer to *E. coli* GR501 containing pTRC99A and pRBL, respectively.

Table 2. Strain viability at 30 °C and 43 °C

The left-hand column provides the plasmid name and its expressed protein product (see Table 1). Viability is expressed as the number of colony forming units per ml ($\times 10^9$) of the original culture. Results represent the mean ± SD of three independent experiments.

E. coli strain and plasmid	Viability at:	
	30 °C	43 °C
GR501	6·48 ± 0·98	< 0·005
GR501 + pTRC99A (vector alone)	3·06 ± 0·83	< 0·005
GR501 + pRBL (T4lig)	7·13 ± 1·01	4·54 ± 1·45
GR501 + pRB154 (*E. coli* LigA)	6·73 ± 0·15	5·36 ± 0·90
GR501 + pRB159 (*E. coli* LigA251)	5·36 ± 1·13	4·12 ± 1·04
CHE30	5·14 ± 0·76	2·67 ± 0·96
CHE30 + pTRC99A (vector alone)	3·08 ± 0·66	0·95 ± 0·32
CHE30 + pRBL (T4lig)	4·27 ± 1·33	0·74 ± 0·25
CHE30 + pRB154 (*E. coli* LigA)	4·10 ± 1·15	1·34 ± 0·36
CHE30 + pRB159 (*E. coli* LigA251)	4·48 ± 1·82	1·46 ± 1·21

Viable counts at 43 °C revealed the weak viability of *E. coli* GR501 alone or with the expression vector pTRC99A, and the good viability of the isogenic strains encoding the T4 or *E. coli* DNA ligase. Expression of recombinant DNA ligase had a small effect on the viability of the wild-type strain *E. coli* CHE30. This effect was observed consistently in independent experiments, although the cause was not clear. These experiments show that expression of recombinant DNA ligases restores the viability of *E. coli* GR501 to wild-type levels.

To better determine the ts phenotype of *ligA251*, growth of *E. coli* GR501 with or without expression of T4 DNA ligase was followed in liquid culture at various temperatures. At 30, 37 and 40 °C, both strains grew similarly, indicating that *E. coli* GR501 did not require expression of an additional wild-type DNA ligase at temperatures up to 40 °C (Fig. 1b and data not shown). At 42 °C, growth was dramatically reduced in the absence of the T4 DNA ligase, but not in its presence (Fig. 1b). Suppression of the growth defect was also observed with *E. coli* GR501 cells expressing *E. coli* LigA from pTRC99A (Fig. 5). Like most strains of *E. coli*, at 44 °C *E. coli* GR501 did not grow well in liquid culture even with expression of T4 DNA ligase from pRBL (data not shown). These experiments on solid and in liquid media confirm that *E. coli* GR501 carries a ts mutation that can be complemented by expression of a functional DNA ligase.

Identification of the mutation in *E. coli* GR501

Temperature-sensitive phenotypes can be caused by different phenomena, including altered expression or function of a protein. To determine if the ts mutation of *E. coli* GR501 was due to reduced expression of LigA251, we analysed the amount of protein present in cell extracts prepared from

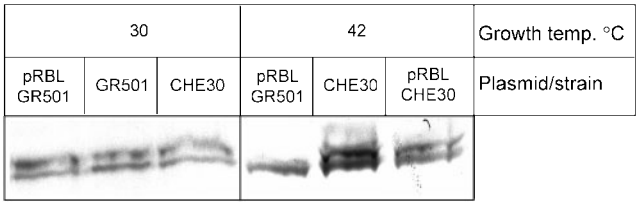

Fig. 2. Western blot analysis of ligase expression from *E. coli* GR501. *E. coli* GR501 and CHE30, with or without expression of the T4 ligase from pRBL, were grown at 30 and 42 °C. Total protein samples from cultures grown to $OD_{600} = 0·7$ were separated by 15 % SDS-PAGE and transferred to a nitrocellulose membrane. The ligase expressed from the *E. coli* chromosome was detected using a polyclonal antibody. The upper and lower bands are likely to indicate the adenylated and non-adenylated forms of LigA, respectively.

cultures grown at permissive and non-permissive temperatures. Previously, we had prepared a rabbit polyclonal antibody to *E. coli* K-12 LigA (Wilkinson *et al.*, 2003) which showed cross-reactivity to LigA251 from *E. coli* GR501. As a positive control for wild-type *ligA*, we used *E. coli* CHE30 (Dermody *et al.*, 1979). Bacterial cultures were grown at various temperatures to $OD_{600} = 0·7$, and equal quantities of cells were analysed by SDS-PAGE and Western blotting using the antibody to *E. coli* LigA (Fig. 2). In these experiments we detected two forms of LigA with slightly altered mobility during PAGE. These two forms have been shown to be equivalent to adenylated and non-adenylated protein for several DNA ligases, including that from *Thermus thermophilus* (Barany & Gelfand, 1991). Note that adenylated LigA migrates more slowly (equivalent to higher molecular mass). At 30 °C, the level of expression of DNA ligase was similar in *E. coli* GR501 and *E. coli* CHE30, with approximately equal amounts of adenylated and non-adenylated enzyme. Thus, the ts mutation does not alter the level of expression of LigA251 under conditions where the strain is normally viable.

In liquid culture, we showed that 42 °C was the optimal temperature for detecting complementation of *E. coli* GR501 by T4 DNA ligase (see above). To assess expression of the chromosomal form of LigA251 at 42 °C (where *E. coli* GR501 is normally non-viable), we analysed cells complemented with T4 DNA ligase. The T4 enzyme did not cross-react with the antibody to *E. coli* LigA (data not shown), and in any case had a different mobility to that of LigA on SDS-PAGE. Inclusion of pRBL, which expresses T4 DNA ligase, did not significantly affect the expression of the ligase in *E. coli* GR501 at 30 °C or *E. coli* CHE30 at 42 °C (Fig. 2). Furthermore, the Western blot analysis showed no major changes in ligase concentration in any of the strains grown at 30 and 42 °C (Fig. 2). However, this analysis showed that, for E. coli GR501 at 42 °C, most LigA251 was present in a non-adenylated form. These data suggest that, although LigA251 is expressed in *E. coli* GR501

at all temperatures monitored, at the higher temperatures most of the protein is non-adenylated and is therefore not competent for DNA end-joining. Thus, at non-permissive temperatures, the ts mutation has no major effect on the level of expression of DNA ligase, but reduces the level of active adenylated enzyme.

Since these data suggest that LigA251 is expressed at 42 °C in *E. coli* GR501, we assessed whether the sequence of the protein could explain the ts mutation. Genomic DNA was prepared from *E. coli* GR501, and sequencing of its *ligA251* revealed a single base substitution, compared to *ligA* in *E. coli* K-12, of cytosine to thymine at base pair 43. This mutation results in a change from Leu to Phe at position 15 of the protein (Fig. 3). Although this residue is not absolutely conserved in all NAD^+-DNA ligases, it is always a hydrophobic amino acid. In the NAD^+-DNA ligases, this amino acid resides within the bi-helix that is close to the N-terminus. Since this region of LigA is important for binding of NAD^+ (Sriskanda & Shuman, 2002), the ts mutation in *E. coli* GR501 is likely to affect binding of NAD^+ and the subsequent adenylation of LigA.

Biochemical analysis of LigA from *E. coli* GR501

Previously, we have purified an active recombinant form of LigA from *E. coli* K-12 with a 10-His-tag at the N-terminus (Wilkinson *et al.*, 2003). Using a similar strategy, *ligA251* from *E. coli* GR501 was amplified from genomic DNA using a proof-reading polymerase and cloned into the expression vector pET-16b. DNA sequencing confirmed that the only mutation in the cloned gene was the cytosine to thymine transition at base pair 43, which produces a change from Leu to Phe at amino acid 15 of the protein. The protein product (LigA251) was overexpressed and purified by affinity chromatography, with further purification by gel-filtration chromatography. Analysis by SDS-PAGE detected no proteins other than full-length DNA ligase (data not shown).

In vitro analysis of purified protein was used to test whether or not the ts phenotype of *E. coli* GR501 was due to reduced protein stability at higher temperatures. SDS-PAGE was used to analyse if there was a difference in the

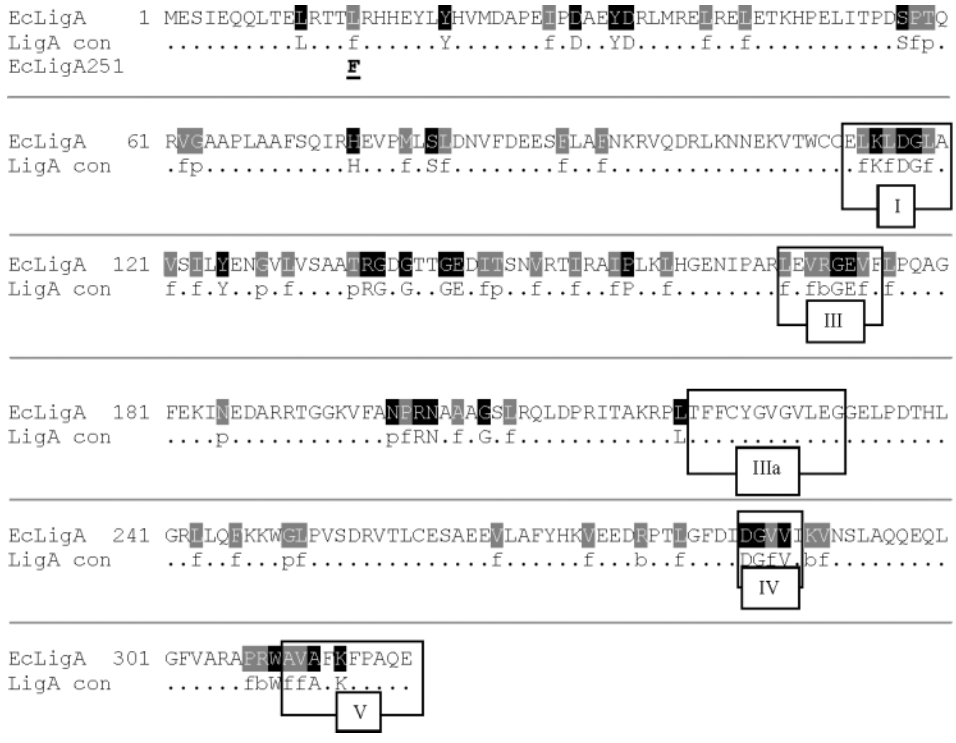

Fig. 3. Amino acid sequence of LigA251. The sequence shows only the first 319 amino acids (out of 671) of *E. coli* LigA and the mutation identified in LigA251 (Leu to Phe at amino acid 15). All other residues are identical in LigA and LigA251. Boxes indicate five sequence motifs (motifs I, III, IIIa, IV and V) conserved in DNA ligases and mRNA capping enzymes (Wilkinson *et al.*, 2001). On the *E. coli* LigA sequence, highlighting indicates residues which are identical (black) or have similar functionality (grey) in more than 90 % of NAD^+-dependent DNA ligase sequences in the NCBI database. For this set of NCBI sequences, 'LigA con' shows the identical amino acids (capitals) and conserved functional groups (lower case). Standard functional groups of amino acids are used as follows: a (acidic) = DE; b (basic) = HKR; f (hydrophobic) = A, F, I, L, M, P, V, W; p (polar) = C, G, N, Q, S, T, Y.

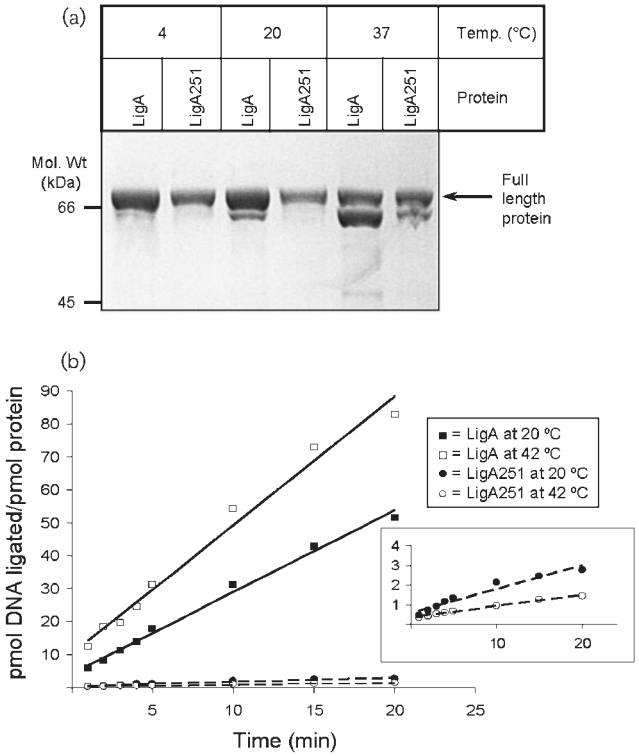

Fig. 4. *In vitro* analysis of LigA251 activity. (a) Approximately 30 pmol of recombinant LigA251 or LigA were incubated for 21 h at the temperatures indicated. Samples were analysed by 10% SDS-PAGE and protein was detected by Coomassie Brilliant Blue staining. Sizes (kDa) are shown for molecular weight markers included on the same gel. (b) *In vitro* ligation assays with recombinant LigA251 or LigA were performed for various times at 20 °C (filled symbols) or 42 °C (open symbols). DNA substrate (750 pmol) was incubated with 1·6 pmol LigA or 70 pmol LigA251. Data are plotted as the extent of cumulative ligation over time. Reactions with LigA251 are indicated by circles (dashed line) and reactions with LigA are indicated by squares (solid line). Data are the mean of two experiments and the standard error was within the symbol area. To allow improved evaluation of reactions with LigA251, the inset shows the same data on an expanded *y* axis.

stability of wild-type and ts LigA when incubated at different temperatures for extended periods. Some fragmentation of proteins was observed during extended incubation (21 h) at 42 °C, but the effects were no worse for LigA251 than for LigA (Fig. 4a). Thus, *in vitro* analysis suggests that the mutation does not decrease the overall stability of the protein at high temperature.

For cell extracts from an *E. coli* strain with the same ts mutation as GR501, it was observed that DNA end-joining activity was reduced by almost 500-fold at 40 °C compared to 25 °C (Dermody *et al.*, 1979). By contrast, there was little difference in the DNA ligation activity of an isogenic wild-type strain at these two temperatures. We wished to analyse whether a similar relationship in DNA ligation activity occurred in our recombinant proteins. DNA ligase activity of LigA251 and LigA was analysed using a standard *in vitro* assay for DNA ligation (Timson & Wigley, 1999; Wilkinson *et al.*, 2003). To compare the DNA ligase activity of LigA251 with that of LigA, we performed time-course reactions at different temperatures (Fig. 4b). The rate of DNA nick ligation by wild-type LigA was approximately 5 mol per mol protein per min at 20 °C. This rate is comparable to that reported for other recombinant NAD^+-DNA ligases (e.g. see Kaczmarek *et al.*, 2001; Sriskanda & Shuman, 2002; Tong *et al.*, 2000). At 20 °C, the rate of DNA ligation by LigA251 was about 20-fold lower than that of LigA. Raising the temperature to 42 °C increased slightly the activity of LigA, but the activity of LigA251 decreased; at this temperature, the difference in rate of ligation between LigA251 and LigA was about 60-fold. These observations are in broad agreement with earlier measurements of the effects of ts mutations on DNA ligation activity in cell extracts (Dermody *et al.*, 1979; Lehman, 1974), though the difference is more pronounced with crude extracts.

Effect of overexpression of LigA251 on *E. coli* GR501 viability

The above data show that LigA251 retained low levels of DNA ligation activity at 42 °C (Fig. 4). Since the non-viability of cultures at higher temperatures is likely to be due to insufficient DNA end-joining activity, we reasoned that overexpression of LigA251 might complement and allow growth of the ts strain. Following procedures outlined above for other DNA ligases, LigA251 was cloned into pTRC99A and transformed into *E. coli* GR501. These bacteria were able to grow well on plates lacking IPTG at 42 °C (Table 2), indicating that overexpression of LigA251 from pTRC99A could complement the ts mutation. To confirm this observation, *E. coli* GR501 overexpressing either LigA or LigA251 was grown in liquid culture at various temperatures. In all cases, complementation of *E. coli* GR501 by expression of LigA251 or LigA resulted in similar growth patterns, as indicated at 40 and 42 °C in Fig. 5a. Thus, overexpression of LigA251 was able to overcome the growth problems that normally result from the reduced activity of this mutated LigA. Levels of expression of the recombinant proteins at 42 °C were analysed by Western blot using a primary antibody to the His-tag (Fig. 5b). Similar levels of expression were observed at 30 °C (data not shown). Note that significant levels of recombinant protein were detected in the absence of IPTG, indicating that expression from the strong promoter of pTRC99A was not effectively inhibited in *E. coli* GR501. There was some regulation at the *lac*-based promoter, since addition of 0·4 mM IPTG increased expression of the recombinant proteins by a further five to tenfold (Fig. 5b). Comparison with known amounts of purified recombinant LigA allowed estimation of the expression levels of LigA251 from the chromosome of *E. coli* GR501 and from pTRC99A in the absence of IPTG (data not shown). This analysis identified that the amount of DNA ligase expressed from

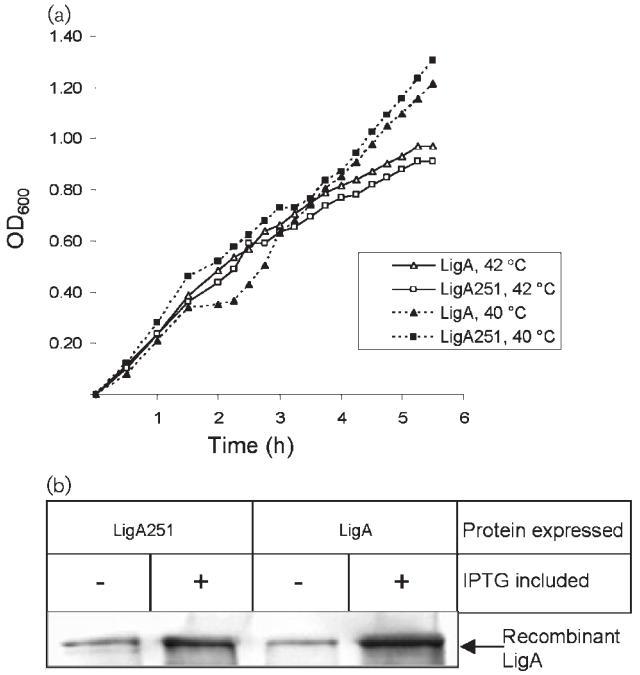

Fig. 5. Growth complementation of *E. coli* GR501 by increased expression of its host DNA ligase. (a) *E. coli* GR501 expressing *E. coli* LigA or LigA251 was grown at 40 or 42 °C in LB containing ampicillin. (b) *E. coli* GR501 expressing *E. coli* LigA or LigA251 was grown at 42 °C. In some cases, 0·4 mM IPTG was added at $OD_{600}=0·2$. Total protein samples from cultures grown to $OD_{600}=0·7$ were separated on 8 % SDS-PAGE and transferred to a nitrocellulose membrane. Recombinant LigA was detected using a monoclonal antibody to the His-tag. Note that these electrophoresis conditions did not resolve the adenylated and non-adenylated forms of LigA.

the vector pTRC99A was 20- to 50-fold higher than that expressed from the chromosomal gene.

DISCUSSION

NAD^+-DNA ligase (LigA) is an essential enzyme in *E. coli*, and it is likely that homologous proteins are essential for all bacteria (Wilkinson *et al.*, 2001). Bacterial strains with temperature-sensitive (ts) mutations in such essential genes are required to study their role(s) *in vivo*. Several ts strains of *E. coli* are thought to have mutations in *ligA* (Dermody *et al.*, 1979; Gellert & Bullock, 1970; Karam *et al.*, 1979; Konrad *et al.*, 1973; Modrich & Lehman, 1971). Although such strains have been available for many years, their mutations have not been well characterized at the molecular level. Using *in vitro* and *in vivo* experiments, we have filled this gap for *E. coli* GR501 and have determined the mutation leading to its ts activity.

E. coli GR501 is one of the most ligase-deficient strains identified, and the ts phenotype is a consequence of problems with the completion of DNA replication at elevated temperature (Dermody *et al.*, 1979). The mutation can be complemented by a variety of DNA ligases, indicating that the ts mutation is related to the function of *E. coli ligA*. Interestingly, the strain can be complemented by both NAD^+- and ATP-DNA ligases (Brotz-Oesterhelt *et al.*, 2003; Doherty *et al.*, 1996; Kodama *et al.*, 1991; Wilkinson *et al.*, 2003). This reinforces the conclusion that the mutation is related to effects on replication, and supports the use of this strain in studies that aim to identify inhibitors that are specific to the NAD^+-versions of the DNA ligases (Brotz-Oesterhelt *et al.*, 2003).

Temperature-sensitive strains contain conditional-lethal mutations: they result in lethality under restrictive conditions, but retain normal function under permissive conditions. Such ts mutations can arise via different processes, producing effects at the level of expression or activity of the ts gene at the non-permissive temperature. We have confirmed that LigA251 is expressed at similar levels at all temperatures tested. *In vitro* ligation assays have confirmed that the ts DNA ligase is active, but has reduced activity, at higher temperatures. Compared to wild-type LigA, LigA251 has reduced activity at all temperatures, but the difference in activity is exaggerated at temperatures that are non-permissive for growth.

These observations suggest that *E. coli* GR501 is temperature sensitive because its DNA ligase activity is insufficient to sustain growth at non-permissive temperatures. Our observed 60-fold difference in the rates of ligation by LigA and LigA251 at 42 °C is less than the effect of ts mutations on the DNA ligation activity of *E. coli* cell extracts (Dermody *et al.*, 1979; Lehman, 1974). These differences may indicate that another cofactor influences the ligation activity of crude extracts, or they may reflect limitations in the accuracy of the different assays of DNA ligation. Interestingly, we observed that a 20- to 50-fold overexpression of LigA251 overcomes the ts phenotype, which is in reasonable agreement with the effect of the mutation on the *in vitro* ligation activity of the protein at 42 °C. However, moving from 30 to 42 °C has only a small effect on the activity of LigA251, and it is unlikely that this is sufficient to explain the pronounced consequences for viability. Rather, the overall effect is likely due to a combination of factors affecting the bacteria. Although *E. coli* GR501 has good viability at temperatures up to 40 °C, it grows quite slowly on LB agar plates, suggesting that the growth rate of the ts strain may be compromised even at low temperatures. Switching growth to higher temperatures applies additional stresses to the bacteria, which may mean that DNA ligase has a more important role to play due to the fact that more replication forks are likely to be active, or perhaps the enzyme is required to carry out more DNA repair events. Thus, the relatively small change in biochemical activity of the mutated DNA ligase may be linked to a large physiological effect.

For *E. coli* GR501, the level of adenylated LigA251 appeared to be reduced at 42 °C compared to 30 °C and

also in comparison to wild-type strains at the same temperature. Preliminary *in vitro* experiments confirmed that LigA251 was less readily adenylated by NAD^+ than LigA (unpublished data). A thorough *in vitro* biochemical analysis is required to understand the full implications of the ts mutation on the reaction mechanism.

Sequencing of genomic and cloned DNA established that the mutation in *ligA251* of *E. coli* GR501 is a cytosine to thymine transition at base 43, which leads to the substitution of Phe for Leu at residue 15 of the protein (LigA251). The effects of mutations in this position of *E. coli* LigA have not been studied before, but the N-terminal 38 residues are required for adenylation of the protein (Sriskanda *et al.*, 1999). Amino acids in the homologous position to Leu15 are conserved as hydrophobic residues in all NAD^+-DNA ligases (Fig. 3), suggesting that it has an important function. The fact that both Leu and Phe are hydrophobic may explain why LigA251 retains some DNA ligase activity. However, the introduction of this change clearly has some effect on protein activity. To aid evaluation of the effect of this mutation on LigA function, we used the X-ray crystallographic structure of LigA from *Thermus filiformis* (Lee *et al.*, 2000) to generate a molecular model of *E. coli* LigA. Fig. 6 shows the backbone structure of the molecular model, with highlighting of the side-chains of Leu15 and the active site lysine (Lys115). Clearly, no direct interactions between Leu15 and the active site are predicted from this model. The main difference in the structure of Leu and Phe is the bulky benzene ring in the side chain of Phe. Although the relationship of the amino acid mutation to enzymic activity or structure has not been directly demonstrated at this time, the molecular model suggests that the alteration of amino acid 15 from Leu to Phe may alter the packing involving α-helix A, which is close to the N-terminus. This bi-helix is implicated in the binding of NAD^+ to LigA (Georlette *et al.*, 2003; Sriskanda & Shuman, 2002), supporting our proposal that the temperature sensitivity of LigA251 is a consequence of factors altering the rate of adenylation of the protein.

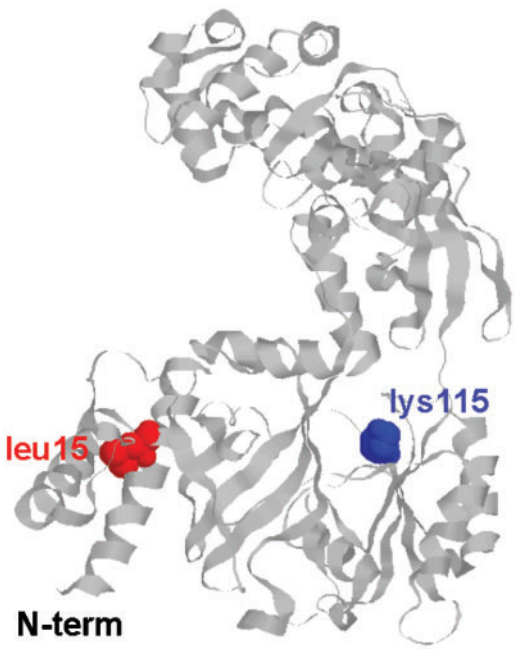

Fig. 6. Putative molecular structure of *E. coli* DNA ligase. A molecular model covering amino acids 1–586 of *E. coli* LigA was generated by the SWISS-MODEL program based on the crystallographic structure of the *T. filiformis* ligase and the polypeptide backbone was visualized using RasMol. Residues highlighted in blue and red indicate the active site lysine (Lys115) and the site of mutation in LigA251 (Leu15), respectively.

For many ts mutations, the affected proteins may have a relatively normal function at permissive temperatures, but at the non-permissive temperature the mutation gives rise to structural changes that are significant enough to disrupt the activity of the protein. We suggest that the mutation in LigA251 gives rise to such a phenomenon. Further biochemical and biophysical investigations are required to provide a full appreciation of how the temperature-sensitive mutation affects the molecular structure and biochemical activity of LigA.

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