Mutational analysis of *Escherichia coli* DNA ligase identifies amino acids required for nick-ligation *in vitro* and for *in vivo* complementation of the growth of yeast cells deleted for *CDC9* and *LIG4*

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ABSTRACT

We report that the NAD-dependent *Escherichia coli* DNA ligase can support the growth of *Saccharomyces cerevisiae* strains deleted singly for *CDC9* or doubly for *CDC9* plus *LIG4*. Alanine-scanning mutagenesis of *E. coli* DNA ligase led to the identification of seven amino acids (Lys115, Asp117, Asp285, Lys314, Cys408, Cys411 and Cys432) that are essential for nick-joining *in vitro* and for *in vivo* complementation in yeast. The K314A mutation uniquely resulted in accumulation of the DNA–adenylate intermediate. Alanine substitutions at five other positions (Glu113, Tyr225, Gln318, Glu319 and Cys426) did not affect *in vivo* complementation and had either no effect or only a modest effect on nick-joining *in vitro*. The E113A and Y225A mutations increased the apparent Kₘ for NAD (to 45 and 76 µM, respectively) over that of the wild-type *E. coli* ligase (3 µM). These results are discussed in light of available structural data on the adenylylation domains of ATP- and NAD-dependent ligases. We observed that yeast cells containing only the 298-amino acid *Chlorella* virus DNA ligase (a ‘minimal’ eukaryotic ATP-dependent ligase consisting only of the catalytic core domain) are relatively proficient in the repair of DNA damage induced by UV irradiation or treatment with MMS, whereas cells containing only *E. coli* ligase are defective in DNA repair. This suggests that the structural domains unique to yeast Cdc9p are not essential for mitotic growth, but may facilitate DNA repair.

INTRODUCTION

DNA ligases catalyze the sealing of nicks containing 5′-phosphate and 3′-hydroxyl termini. Ligation entails three sequential nucleotidyl transfer reactions. In the first step, attack on the c- phosphorus of ATP or NAD by ligase results in release of pyrophosphate or NMN and formation of a covalent intermediate (ligase–adenylate) in which AMP is linked by a phosphoamide bond to the ε-amino group of a lysine. In the second step, the AMP is transferred to the 5′ end of the 5′-phosphate-terminated DNA strand to form DNA–adenylate (AppN). In the third step, ligase catalyzes attack by the 3′ OH of the nick on DNA–adenylate to join the two polynucleotides and release AMP.

DNA ligases are grouped into two families, ATP-dependent ligases and NAD-dependent ligases, according to the cofactor required for ligase–adenylate formation. The ATP-dependent DNA ligases are found in eubacteria, bacteriophages, archaea, bacteria, eukaryotes and eukaryotic viruses, whereas the NAD-dependent enzymes have been described only in eubacteria.

Genes encoding NAD-dependent DNA ligases have been identified and sequenced from at least 22 eubacterial species. The NAD-dependent ligases are of fairly uniform size (656–823 amino acids) and there is extensive amino acid sequence conservation throughout the entire lengths of the polypeptides. A conditional mutant of NAD-dependent *Escherichia coli* DNA ligase that results in growth arrest at the restrictive temperature can be complemented by human DNA ligase I (1). A lethal disruption of the DNA ligase gene in *Salmonella typhimurium* can be complemented fully by bacteriophage T4 DNA ligase (2). Thus, there is no essential functional distinction between NAD- and ATP-dependent ligases *in vivo* in bacteria.

Eukaryotic cells contain multiple ATP-dependent DNA ligases encoded by separate genes (3). DNA ligase I catalyzes the joining of Okazaki fragments during DNA replication and also plays a role in DNA repair. Mammalian DNA ligases IIIα (involved in DNA repair) and IIIβ (implicated in meiotic recombination) are the alternatively spliced products of a single gene; they differ in amino acid sequence only at their C-termini. Budding yeast does not encode a homolog of DNA ligase III, but vaccinia virus does. DNA ligase IV plays a role in the repair of double-strand DNA breaks during non-homologous end-joining.

Amino acid sequence comparisons suggest that a core catalytic domain common to all ATP-dependent ligases is embellished by isozyme-specific domains located at the N- or C-termini. It is thought that these flanking segments may target mammalian DNA ligases to sites where their action is required, most likely via the binding of ligases to other proteins involved in DNA replication, repair or recombination (3). For example, a 20-amino acid N-terminal segment of mammalian DNA ligase I targets...
the enzyme in vivo to intranuclear sites of DNA replication; the same peptide segment mediates the interaction in vitro of DNA ligase I with the DNA polymerase processivity factor PCNA (4–7). It was proposed that the recruitment of ligase I by residual PCNA on the lagging strand of the replication fork would uniquely enable ligase I to join adjacent Okazaki fragments (5).

The DNA ligases encoded by eukaryotic DNA viruses are smaller than their cellular counterparts. The Chlorella virus ligase, a 298-amino acid monomer, is the smallest eukaryotic DNA ligase known and consists solely of the catalytic core (8). We envisioned that a genetic assessment of the ability of Chlorella virus ligase to substitute for cellular ligases in vivo would provide insights into the biological roles of the domains flanking the catalytic core. We find that the Chlorella virus ligase can function in lieu of the more complex cellular ligase I (Cdc9p) in supporting the growth of CDC9 strains deleted singly for (Cdc9p) in vivo. Moreover, the NAD-dependent virus ligase is capable of complementing growth of the cdc9Δ and cdc9Δ lig4Δ strains. These findings suggest that nick-joining activity suffices for yeast cell growth and that macromolecular interactions imputed to domains flanking the catalytic core of DNA ligase I are not strictly essential for mitotic growth in S.cerevisiae. The yeast complementation assay was exploited, in conjunction with alanine scanning mutagenesis, to identify seven individual amino acids of E.coli DNA ligase that are essential for nick-joining in vitro and for ligase function in vivo.

MATERIALS AND METHODS

Yeast CDC9 expression plasmids

A 4.5 kb NheII/SphI restriction fragment of yeast genomic DNA containing the CDC9 gene was subcloned into pUC18. A BamHI/SphI fragment was then transferred from pUC18 into yeast plasmids pSE-360 (CEN URA3) and pSE-358 (CEN TRP1) to generate CDC9 expression plasmids p360-Cdc9 and p358-Cdc9. The CDC9 insert extends from 989 bp upstream of the CDC9 start codon to 1277 bp downstream of the stop codon. Expression of CDC9 in p360-Cdc9 and p358-Cdc9 is under the control of its natural promoter. Restriction fragments NΔ65 and NA343 encoding truncated versions of Cdc9p that lack either 65 or 343 amino acids from the N-terminus were excised from p358-Cdc9 by digestion with either NcoI and HindIII (NΔ65) or NdeI and HindIII (NA343) and then cloned into yeast expression vector pYX132 (CEN TRP1) to generate plasmids pCdc9-NΔ65 and pCdc9-NA343. Expression of the truncated ligases in this context is under the control of the constitutive yeast TPI1 promoter. Plasmid pCdc9ΔNA99 was constructed by inserting an NcoI restriction site encoding residues 100–343 into the unique NcoI site of pCdc9-NA343 in an in-frame orientation. Additional N-terminal deletions NΔ178, NΔ214, NΔ225, NA243, NA252 and NA283 were constructed by PCR amplification of the gene with sense primers that introduced NcoI restriction sites at Met1179, Met215, Met226, Met244, Met253 and Met284. NcoI fragments of the PCR products were inserted in-frame into the NcoI site of pCdc9-NA343.

Yeast ligase deletion strains

Haploid strain YBSΔL1 (MATa ura3 ade2 trp1 his3 leu2 can1 cdc9::LEU2 p360-Cdc9) is deleted at the chromosomal CDC9 locus and contains a copy of CDC9 on a CEN URA3 plasmid. YBSΔL4 (MATa ura3 ade2 trp1 his3 leu2 can1 lig4::LEU2) is deleted at the chromosomal LIG4 locus. YBSΔL1ΔL4 (MATa ura3 ade2 trp1 his3 leu2 can1 lig4::LEU2 cdc9::KAN p360-Cdc9) is deleted at the chromosomal CDC9 and LIG4 loci. YBSΔL1, YBSΔL4 and YBSΔL1ΔL4 were derived by targeted gene disruptions in the diploid strain W303, followed by sporulation, tetrad dissection and genotyping of haploid progeny. CDC9/ cdc9 diploids were transformed with p360-Cdc9 prior to sporulation. Gene disruptions were confirmed by Southern blotting. In cdc9Δ strains, the entire CDC9 open reading frame was deleted and replaced by either LEU2 or KAN selectable markers. In the lig4Δ strains, the DNA segment coding for amino acids 304–818 was deleted and replaced with the LEU2 gene.

Yeast plasmids for expression of Chlorella virus ligase

NdeI–BamHI restriction fragments containing the wild-type Chlorella virus ligase gene (ChVLIG) and the mutant alleles T25A, K27A, D29A, G30A and R32A were excised from the respective pET16b-based plasmids (9) and inserted into a customized yeast expression vector pYX1-His, a derivative of pYX132 (CEN TRP1) in which six consecutive histidine codons and a unique NdeI site are inserted between the NcoI and BamHI sites of pYX132. In this vector, expression of ChVLIG is under the control of the yeast TPI1 promoter.

Expression and purification of recombinant E.coli DNA ligase

The coding sequence of E.coli DNA ligase was amplified by PCR from E.coli DH5α genomic DNA by Taq DNA polymerase using oligonucleotide primers that introduced an NdeI restriction site at the translation start codon and a BamHI site immediately downstream of the stop codon. The PCR product was digested with NdeI and BamHI and inserted into plasmid pET16b in-frame with an N-terminal leader peptide encoding 10 tandem histidines. The resulting plasmid, pET-EcoLIG, was transformed into E.coli BL21(DE3). A single ampicillin-resistant colony was inoculated into LB medium containing 0.1 mg/ml of ampicillin and grown at 37°C until the A600 reached 0.8. The culture (125 ml) was adjusted to 0.4 mM IPTG and incubation was continued for 3 h at 37°C. Cells were harvested by centrifugation and the pellet stored at −80°C. All subsequent procedures were performed at 4°C. Thawed bacteria were suspended in 10 ml of lysis buffer (50 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 10% sucrose) containing 0.1 mg/ml of lysozyme and 0.1% Triton X-100. After 30 min, the lysates were sonicated to reduce viscosity and then separated by centrifugation into soluble and insoluble fractions. The supernatant was mixed with 1 ml of Ni-NTA-agarose resin (Qiagen) for 30 min with constant rotation. The slurry was poured into a column and washed with IMAC buffer (50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 10% glycerol) containing 5 mM imidazole. The column was then eluted step-wise with IMAC buffer containing 50, 200 and 500 mM imidazole. The 200 mM imidazole eluate fraction containing the 76 kDa His-tagged E.coli ligase polypeptide was dialyzed overnight against buffer containing 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM DTT,
and 10% glycerol. The protein concentration was determined using the BioRad dye reagent with BSA as a standard. About 13 mg of purified wild-type ligase was recovered in the 200 mM imidazole fraction.

Mutational analysis of *E. coli* DNA ligase

N-terminal deletion mutants NAΔ38 and NAΔ78 were constructed by PCR amplification of the *E. coli* ligase gene with sense primers that introduced *NdeI* restriction sites at Met39 and Met79, respectively. The PCR product was digested with *NdeI* and *BamHI* and then inserted into pET16b. Single alanine-substitution mutations were introduced into the *E. coli* DNA ligase gene via the two-stage PCR overlap extension method. The second-stage PCR product was digested with *NdeI* and *BamHI* and then inserted into pET16b to generate a series of pET-EcoLIG-Ala expression plasmids. The entire ligase insert of these plasmids was sequenced to confirm the presence of the desired mutation and exclude the introduction of unwanted changes during amplification and cloning. pET-EcoLIG-NA and pET-EcoLIG-Ala plasmids were transformed into BL21(DE3). Expression and purification of the NA and Ala-substituted proteins was performed as described above for the wild-type ligase. The expression levels and solubilities of each of the Ala mutants (except C432A) were comparable to that of recombinant wild-type *E. coli* ligase. C432A was less soluble than the other recombinant polypeptides, but was purified to a comparable extent by Ni-agarose chromatography.

**Yeast plasmids for expression of *E. coli* DNA ligase**

*NdeI–BamHI* restriction fragments containing the wild-type *E. coli* DNA ligase gene (EcoLIG) and the Ala mutant alleles were excised from the respective pET16b-based plasmids and inserted into pYX1-His (CEN TRP1). The ligase gene with sense *I–* restriction sites at Met79, respectively. The PCR product was digested with *NdeI* and *BamHI* and then inserted into pET16b to generate a series of pET-EcoLIG-Ala expression plasmids. The entire ligase insert of these plasmids was sequenced to confirm the presence of the desired mutation and exclude the introduction of unwanted changes during amplification and cloning. pET-EcoLIG-NA and pET-EcoLIG-Ala plasmids were transformed into BL21(DE3). Expression and purification of the NA and Ala-substituted proteins was performed as described above for the wild-type ligase. The expression levels and solubilities of each of the Ala mutants (except C432A) were comparable to that of recombinant wild-type *E. coli* ligase. C432A was less soluble than the other recombinant polypeptides, but was purified to a comparable extent by Ni-agarose chromatography.

UV sensitivity

cdc9Δ lig4Δ cells containing the specified ligase genes were grown in YPD suspension cultures until the A600 reached 0.6. Aliquots (0.1 ml) of serial 10-fold dilutions were spread on YPD agar plates using glass beads. The plates were irradiated one at a time with the indicated UV dose using a Stratalinker 2400 254 nm UV light source. The plates were then incubated in the dark for 2 days at 30°C. The viable cell counts were determined and normalized to the cell number in a sample that was not exposed to UV (defined as 100%).

MMS sensitivity

cdc9Δ lig4Δ cells containing the specified ligase genes were grown in YPD suspension cultures until the A600 reached 0.5 and 1.0. The cells were harvested by centrifugation and resuspended in sterile water. The cells were centrifuged again and the cell pellets were resuspended in 70 mM sodium phosphate buffer (pH 7.0) containing 2% glucose at a cell density of ~2 x 10^7 cells/ml. Methyl methane sulfonate (MMS) was added to 0.5% final concentration and the cells were incubated at room temperature. Aliquots (0.1 ml) were withdrawn at the times specified and the MMS was immediately quenched by adding 0.1 ml of an ice-cold solution of 10% sodium thiosulphate. Aliquots (0.1 ml) of serial 10-fold dilutions were spread on YPD agar plates and the plates were incubated for 3 days at 30°C.

**RESULTS**

**Chlorella virus ligase complements a null mutation of yeast ligase I (Cdc9p)**

Prior work showed that human DNA ligase I and vaccinia virus DNA ligase could complement growth of a conditional *cdc9* mutant of *S. cerevisiae* at the restrictive temperature (10,11). These data proved the *in vivo* activity of the human and viral ligases, but did not address whether heterologous enzymes could fully replace Cdc9p. To address this issue, we tested by plasmid shuffle the ability of heterologous DNA ligases to complement growth of a yeast strain in which the chromosomal CDC9 gene was deleted. Viability of the cdc9Δ strain is contingent on maintenance of an extrachromosomal CDC9 gene on a *CEN URA3* plasmid. Hence, cdc9Δ cells cannot grow on medium containing 5-fluoroorotic acid (5-FOA) to select against the *URA3 CDC9* plasmid, but they can grow on 5-FOA if the cells have been transformed with a second copy of CDC9 on a *CEN TRP1* plasmid (Fig. 1A). We cloned the *Chlorella* virus DNA ligase gene (ChVLIG) into a *CEN TRP1* vector such that expression of the ligase was under the control of the constitutive yeast *TRP1* promoter. The instructive finding was that cdc9Δ cells bearing the ChVLIG plasmid grew...
on 5-FOA (Fig. 1A). ChVLIG cells grew as well as CDC9 cells on YPD plates at 25, 30 and 37°C (not shown). Thus, the 298-amino acid catalytic core domain of an ATP-dependent ligase was functional in vivo in lieu of the more complex yeast Cdc9p.

**Chlorella virus ligase complements a cdc9Δ lig4Δ double deletion**

Saccharomyces cerevisiae encodes a second DNA ligase homologous to mammalian ligase IV (12–14). Yeast LIG4 is not essential for cell growth, whereas CDC9 is essential. Although Lig4p is unable to perform the full repertoire of biological functions executed by Cdc9p, it is conceivable that ligase IV can perform some of the essential functions of ligase I, in which case the presence of Lig4p in cdc9Δ ChVLIG cells might compensate for some putative biological deficit of the minimal viral DNA ligase. In order to evaluate this scenario, we constructed a cdc9Δ lig4Δ strain carrying a CEN URA3 CDC9 plasmid. cdc9Δ lig4Δ cells transformed with a CEN TRP1 vector could not grow on 5-FOA, whereas cells transformed with CEN TRP1 CDC9 or CEN TRP1 ChVLIG plasmids were viable (Fig. 1B). The doubling times of CDC9 lig4Δ cells and ChVLIG lig4Δ cells grown in suspension culture in YPD medium at 30°C were 2.3 and 2.3 h, respectively (not shown). We conclude that yeast cells grow normally when *Chlorella* virus ligase is the only ligase present.

**Catalytic activity is required for complementation by Chlorella virus ligase**

Genetic evidence from studies of conditional ligase mutants suggests that the chief function of DNA ligase I in mitotic yeast cells is the sealing of Okazaki fragments generated during discontinuous lagging strand DNA replication (15,16). Formal proof that the catalytic activity of DNA ligase is essential in vivo emerges from the analysis of the effects of mutations in the ligase active site. The active site of ATP-dependent DNA ligases is composed of a set of conserved sequence motifs (I, IIIa, III, IV and V) that form the ATP binding pocket (17,18). Motif I (KxDGXR) includes the lysine that becomes covalently linked to AMP during ligation–adenylate formation (19). Alanine mutations in conserved residues Lys27, Asp29, Gly30 and Arg32 of motif I of *Chlorella* virus ligase abolished or severely decreased strand joining activity in vitro (9). The specific activities of the recombinant mutant proteins in nick-joining relative to the wild-type activity were: K27A (<0.01%), D29A (<0.01%), G30A (3%) and R32A (<0.01%). In contrast, a T25A mutant flanking motif I retained 64% of the wild-type ligation activity. These five mutant alleles of ChVLIG were inserted into yeast CEN TRP1 vectors under the control of the TPI1 promoter and tested by plasmid shuffle for complementation of cdc9Δ. The catalytically active T25A ligase supported cell growth, whereas the defective K27A, D29A, G30A and R32A mutants did not (not shown). These results indicate that the catalytic activity of the *Chlorella* virus ligase is required for in vivo complementation.

**The N-terminal 99 amino acids of yeast DNA ligase I are dispensable for cell growth**

Motif I of yeast Cdc9p (KDYGER) is located at residues 419–424 of the 755-amino acid polypeptide. To test if the long N-terminal domain is required for Cdc9p function in vivo, we generated a series of N-terminal CDC9 deletion alleles and cloned them into a CEN TRP1 vector under the control of the TPI1 promoter (the same expression context used above for the *Chlorella* virus ligase). Plasmid shuffle assays were performed in both the cdc9Δ and the cdc9Δ lig4Δ yeast strains. Deletion of 65 or 99 amino acids from the N-terminus did not affect the ability of the truncated enzymes to complement either strain. However, more extensive deletions of 178, 214, 225, 283 or 343 amino acids from the N-terminus was lethal in both yeast strains (not shown). We conclude that the N-terminal 99 amino acids are dispensable for ligase function in vivo. This result is consistent with the report of Tomkinson et al. (20) that a 70 kDa C-terminal fragment of *S.cerevisiae* Cdc9p starting from residue Ser106 is active in strand joining in vitro.

**NAD-dependent E.coli ligase suffices for yeast cell growth**

The eubacterial DNA ligases have distinct NAD cofactor specificity and scant similarity in primary structure to the eukaryotic ATP-dependent DNA ligases. Can an NAD-dependent ligase function in the eukaryotic milieu? To answer this question, we constructed a CEN TRP1 plasmid encoding *E.coli* DNA ligase under the control of the yeast TPI1 promoter. We found that the *EcoLIG* plasmid supported the growth of cdc9Δ and cdc9Δ lig4Δ cells on 5-FOA (Fig. 1A and B). *EcoLIG* cells grew as well as wild-type cells on YPD plates at 25, 30 and 37°C (not shown). The doubling time of *EcoLig* lig4Δ cells grown in suspension culture in YPD medium at 30°C was 2.4 h (compared to 2.3 h for CDC9 lig4Δ cells). Thus, there is no essential functional difference between NAD-dependent and ATP-dependent ligases in vivo in yeast.

**Effects of mutations in motif I on the function of *E.coli* ligase in vitro and in vivo**

NAD-dependent DNA ligases contain the KxDG motif (motif I) that serves as the site of covalent nucleotidyl transfer in ATP-dependent ligases and GTP-dependent mRNA capping enzymes (17–19). Mutational analysis of the KxDG motif of the NAD-dependent *Thermus thermophilus* DNA ligase showed that the Lys and Asp side chains are essential for nick-joining activity (21). We were interested in determining if the structure–activity relationships established for the thermostable ligase applied to *E.coli* ligase. We constructed vectors that permitted induced bacterial expression of wild-type *E.coli* ligase and motif I mutants K115A and D117A. An N-terminal His-tag facilitated purification of the recombinant ligases from soluble bacterial extracts by adsorption to Ni-agarose and eluate fraction of WT ligase was nearly homogenous with the same expression context used above for the *Chlorella* virus ligase. Plasmid shuffle assays were performed in both the cdc9Δ and the cdc9Δ lig4Δ yeast strains. Deletion of 65 or 99 amino acids from the N-terminus did not affect the ability of the truncated enzymes to complement either strain. However, more extensive deletions of 178, 214, 225, 283 or 343 amino acids from the N-terminus was lethal in both yeast strains (not shown). We conclude that the N-terminal 99 amino acids are dispensable for ligase function in vivo. This result is consistent with the report of Tomkinson et al. (20) that a 70 kDa C-terminal fragment of *S.cerevisiae* Cdc9p starting from residue Ser106 is active in strand joining in vitro.

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into a yeast CEN TRP1 expression vector under the control of the TP11 promoter and tested for function in vivo by plasmid shuffle. Whereas the wild-type E.coli gene supported growth of yeast cde39 cells on 5-FOA, the two catalytically defective mutants K117A and D117A did not (not shown).

**Effects of cysteine to alanine mutations in the C-terminal domain of E.coli ligase**

Limited proteolysis of the NAD-dependent DNA ligase from *Bacillus stearothermophilus* suggested that the enzyme consists of two discrete functional domains (22). The N-terminal domain containing the KxDG motif is competent for reaction with NAD to form enzyme–adenylate, but is not effective in nick-joining. The C-terminal domain is implicated in DNA binding (22). Within the C-terminal domain are four cysteine residues, which are conserved among NAD-dependent ligases, that are suggested to form a zinc-finger (23). Analysis of the Bst ligase by atomic adsorption spectroscopy indicated that the native ligase and the C-terminal domain did indeed contain a bound zinc (22).

We mutated each cysteine of *E.coli* DNA ligase to alanine and tested the effects of these changes on ligase function in vitro and in vivo. *EcoLIG* alleles C408A, C411A and C432A were unable to support growth of cde39 cells on 5-FOA, whereas the C426A allele was viable (not shown). Recombinant C408A, C411A, C426A and C432A proteins were purified; SDS–PAGE analysis of the imidazole eluate fractions is shown in Figure 2A.

C408A, C411A and C432A were grossly catalytically defective. Their specific activities in nick-joining were gauged by enzyme titration and found to be <0.1% of the specific activity of wild-type *E.coli* ligase (Fig. 2B). The recombinant C426A enzyme was 7% as active as the wild-type ligase (Fig. 2B); this level of activity apparently sufficed for yeast mitotic growth.

Our findings that three of the four conserved cysteines were essential for the function of *E.coli* DNA ligase in vivo and in vitro differ from those of Luo and Barany (21), who reported that three of the four cysteines of *Tth* ligase were non-essential for the function of the *Tth* enzyme in vivo and in vitro. Replacement of the proximal two cysteines of *Tth* ligase (Cys412 and Cys415) by alanine permitted supplementation of an *E.coli* lig–ts strain by the mutant *TthLIG* alleles and had almost no effect on nick closure activity by the mutant enzymes in vitro. The specific activities of the recombinant C412A and C415A enzymes were 41 and 79% of the wild-type *Tth* ligase activity, respectively (21). The mutational results at these two positions of *Tth* ligase are discordant with our *E.coli* ligase findings. Yet there are similar effects of alanine substitution for the third cysteine (preservation of function in vitro and in vivo) and the fourth cysteine (abrogation of function in vitro and in vivo) of the *Tth* and *E.coli* ligases. The available mutational data do not exclude the proposal that the conserved cysteines do indeed coordinate zinc, but the *Tth* ligase results raise the issue of whether a zinc finger is essential for the function of NAD-dependent ligases in general, i.e., because if that were the case one might expect activity to be abrogated by an alanine substitution at any one of the four cysteine side chains of *Tth* ligase. It is possible that the cysteines of *E.coli* ligase do coordinate zinc in a manner that requires only three of the four cysteines (i.e., essential positions Cys408, Cys411 and Cys432). Loss of a putative fourth coordinating side chain (Cys426) might be mitigated in the partially active C426A mutant enzyme by interaction of zinc with solvent or with an alternative side chain (e.g., a nearby histidine). High resolution structural analysis will be required to clarify this issue. Nonetheless, the mutational studies presented here and by Luo and Barany (21) do underscore the lesson that structure–activity relationships established for one NAD-dependent ligase cannot be presumed to apply to other members of the NAD-dependent ligase family.

**Alanine scanning mutagenesis of the *E.coli* DNA ligase identifies additional amino acids essential for nick-joining**

The nucleotide-binding pocket of ATP-dependent DNA ligases is composed of five collinear motifs: I, III, IIIa, IV and V (Fig. 3). These same motifs comprise the nucleotide-binding pocket of ATP-dependent DNA ligases. The available mutational data do not exclude the proposal that the conserved cysteines do indeed coordinate zinc, but the *Tth* ligase results raise the issue of whether a zinc finger is essential for the function of NAD-dependent ligases in general, i.e., because if that were the case one might expect activity to be abrogated by an alanine substitution at any one of the four cysteine side chains of *Tth* ligase. It is possible that the cysteines of *E.coli* ligase do coordinate zinc in a manner that requires only three of the four cysteines (i.e., essential positions Cys408, Cys411 and Cys432). Loss of a putative fourth coordinating side chain (Cys426) might be mitigated in the partially active C426A mutant enzyme by interaction of zinc with solvent or with an alternative side chain (e.g., a nearby histidine). High resolution structural analysis will be required to clarify this issue. Nonetheless, the mutational studies presented here and by Luo and Barany (21) do underscore the lesson that structure–activity relationships established for one NAD-dependent ligase cannot be presumed to apply to other members of the NAD-dependent ligase family.
protein resembled that of the equivalent segment of ATP-dependent DNA ligase of bacteriophage T7 (25). A structure-based alignment of the NAD-dependent and ATP-dependent enzymes showed conservation in the Bst ligase of several of the key residues in what would correspond to nucleotidyl transferase motifs III, IIIa, IV and V of the ATP-dependent ligase/GTP-dependent capping enzyme superfamily (17,25) (Fig. 3). Although the structure of the Bst ligase domain was solved in the absence of NAD, inferences can be drawn as to the possible functions of some of the conserved residues by comparison with the crystal structures of T7 DNA ligase with bound ATP and the Chlorella virus capping enzyme–GTP cocrystal, which reveal specific contacts between the conserved side chains and the nucleotide substrate (18,24,25). Here we tested the effects of introducing alanine substitutions for some of these amino acids in the E.coli DNA ligase.

Six mutant enzymes with alanine substitutions within the putative nucleotidyl transferase motifs (E113A, Y225A, D285A, K314A, Q318A and E319A) were expressed in bacteria and purified from soluble lysates by Ni-agarose chromatography. SDS–PAGE analysis of the purity of the imidazole eluate fractions is shown in Figure 4A. The preparations were tested for nick-joining activity in reaction mixtures containing 20 µM NAD and 1 pmol of a singly-nicked hairpin duplex DNA substrate (Fig. 4B). The specific activities of the mutant proteins were calculated from the slopes of the titration curves and normalized to the specific activity of the wild-type ligase. The EcoLIG-Ala alleles were cloned into yeast CEN TRP1 expression vectors and tested for function in vivo by plasmid shuffle. The results are summarized in Figure 4C.

Eschcherichia coli ligase mutation D285A in motif IV abrogated nick-joining activity in vitro (Fig. 4B) and abolished in vivo complementation of the cdc9 Δ strain (Fig. 4C). These findings are in accord with mutational data demonstrating the essentiality of the motif IV aspartate of yeast capping enzyme (26) and the motif IV glutamate of vaccinia DNA ligase (27). The acidic side chain in motif IV interacts with the motif I lysine nucleophile in the Chlorella virus capping enzyme structure (24) and it has been suggested that this side chain may serve as a general base during attack of the lysine on the α-phosphorus (26).

Mutation K314A in motif V reduced ligase specific activity by three orders of magnitude and also resulted in loss of function in vivo (Fig. 4C). Analysis of the products of the in vitro reaction of K314A with nicked DNA under conditions of enzyme excess revealed only trace formation of the ligated 60mer, but substantial accumulation of the DNA–adenylate intermediate (Fig. 4B). DNA–adenylate is barely detectable during the reaction of wild-type ligase with nicked DNA. A kinetic analysis of the reaction of wild-type and K314A ligase–adenylate with nicked DNA under single-turnover conditions (enzyme excess, no added NAD) is shown in Figure 5A. Wild-type ligase catalyzed rapid nick-joining with no detectable DNA–adenylate formation; the extent of ligation at 30 s (the earliest time point taken) was 83% of the endpoint value. In contrast, K314A catalyzed a rapid burst of DNA–adenylate formation, with 44–55% of the input 5′-phosphate strand at the nick becoming adenylated in

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Figure 3. Conserved nucleotidyl transferase motifs in DNA ligases and capping enzymes. Five collinear sequence elements, designated nucleotidyl transferase motifs I, III, IIIa, IV and V, are conserved in ATP-dependent DNA ligases and mRNA capping enzymes (17). These motifs were detected in NAD-dependent DNA ligases by visual inspection and by reference to Singleton et al. (25). The amino acid sequences are aligned for four subgroups of nucleotidyl transferases: (i) the NAD-dependent DNA ligases of E.coli (Eco), B.stearothermophilus (Bst) and Tithermophilus (Tth); (ii) ATP-dependent DNA ligases of vaccinia virus (Vac), Schizosaccharomyces pombe (Spo), S.cerevisiae (Sce), human ligase I (Hu1), human ligase 3 (Hu3), Chlorella virus (ChV) and bacteriophage T7; (iii) GTP-dependent capping enzymes (CE) of S.cerevisiae (Sce), Shope fibroma virus (SFV) and molluscum contagiosum virus (MVC). The number of amino acid residues separating the motifs is indicated by (–). The residues in the E.coli DNA ligase that were subjected to mutational analysis in the present study are indicated by dots.

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![Figure 3](image-url)
Figure 4. Alanine-scanning and N-terminal truncation of E. coli DNA ligase. (A) Aliquots (4 µg) of the 200 mM imidazole eluate fractions of the indicated E. coli ligase mutants proteins were analyzed by SDS–PAGE. Polypeptides were visualized by staining with Coomassie blue dye. The positions and sizes (in kDa) of marker polypeptides are indicated on the left. (B) Ligation reaction mixtures containing 20 µM NAD, 1 pmol of nicked hairpin duplex DNA and 2 pmol of the indicated ligase preparation were incubated for 10 min at 22°C. The products were analyzed by PAGE. An autoradiograph of the gel is shown. The positions of the 5′-labeled 18mer oligonucleotide substrate (pDNA), the DNA–adenylate intermediate (AppDNA) and the 60mer ligation product are indicated on the right. (C) Mutational effects on ligase function in vitro and in vivo. The Ni-agarose preparations of wild-type and mutant E. coli ligase were titrated for nick-joining activity on the nicked hairpin duplex substrate. Specific activities were determined in the linear range of enzyme dependence and are expressed as percent values relative to that of wild-type ligase. The mutant ligase alleles were tested for in vivo complementation of cdc9Δ yeast cells by plasmid shuffle. Lethal mutations were those that formed no colonies on 5-FOA. ++ Indicates colony size indistinguishable from strains bearing wild-type ligase.

Figure 5. Escherichia coli ligase mutant K314A accumulates DNA–adenylate intermediate and mutants E113A and Y225A have reduced affinity for NAD. (A) Reaction mixtures containing (per 20 µl) 50 mM Tris–HCl pH 7.5, 5 mM DTT, 5 mM MgCl₂, 10 mM (NH₄)₂SO₄, 500 fmol of radiolabeled nicked hairpin duplex DNA and 6 pmol of WT or K314A ligase were incubated at 22°C. Aliquots (20 µl) were withdrawn at the times indicated and quenched immediately with EDTA and formamide. Reaction products were resolved by PAGE and the distribution of ³²P-label as ligated DNA, DNA–adenylate (AppDNA), and residual 18mer substrate was determined by scanning the gel with a Phosphorimager. The levels of ligated product and DNA–adenylate are plotted as a function of reaction time. (B) Reaction mixtures containing 50 mM Tris–HCl pH 7.5, 5 mM DTT, 5 mM MgCl₂, 10 mM (NH₄)₂SO₄, 20 µM NAD, 1 pmol of radiolabeled nicked hairpin duplex DNA, either 25 fmol of WT E. coli ligase, 50 fmol of E113A or 50 fmol of Y225A, and NAD as specified were incubated for 10 min at 22°C. The extent of ligation is plotted as a function of NAD concentration.
0.5–2 min (Fig. 5A). Ligated product accumulated linearly from 2 to 30 min concomitant with a decline in DNA–adenylate. These results indicate that the K314A mutation severely and selectively impairs the third step of the ligation pathway (the attack of the 3′ OH on DNA–adenylate to form a phosphodiester) relative to step 2 (transfer of AMP from ligase–adenylate to the nick) to the point that step 3 is rendered rate-limiting. The corresponding motif V lysine in the ligase and capping enzyme crystals interacts directly with the α-phosphorus of the nucleotide (18,24).

*Escherichia coli* ligase mutations Q318A and E319A in motif V had little effect on strand joining *in vitro* and yeast *cdc9Δ* cells expressing these mutant enzymes were viable on 5-FOA (Fig. 4C). The Gln and Glu positions are conserved (as Glu-Glu) in the NAD-dependent enzymes from *B. stearothermophilus* and *T. thermophilus* and are structurally aligned to a Glu-Ala dipeptide within motif V of the T7 ligase (25), but are not otherwise conserved among ligases or capping enzymes (Fig. 3).

*Escherichia coli* ligase mutation E113A elicited a modest decrement in strand joining activity *in vitro* (to 40% of wild-type specific activity under standard reaction conditions), but did not affect complementation of *cdc9Δ* (Fig. 4C). The glutamate side chain is located 2 amino acids upstream of the lysine nucleophile and is strongly conserved in NAD-dependent and ATP-dependent ligases (except the *Chlorella* virus ligase). This position is conspicuously not glutamate in any of the GTP-dependent capping enzymes. The crystal structure of the T7 ligase reveals a hydrogen bond between this glutamate and the 6-amino moiety of the adenine ring; this led Subramanya et al. (18) to suggest that the glutamate–adenine interaction contributes to the specificity of the ligases for adenine nucleotides. Therefore it was remarkable that replacement of this glutamate by alanine in the *E. coli* DNA ligase had so little effect on enzyme activity. Taken together with earlier findings that a T27A mutation of *Chlorella* virus ligase also had little effect on strand joining activity *in vitro* (9), we surmise that hydrogen bonding interactions of the amino acid side chain in this position are not generally required for ligation chemistry or for discrimination of the adenine nucleotide substrate. To test if the glutamate might contribute to the affinity of *E. coli* ligase for NAD, we gauged the dependence of strand joining on NAD concentration (Fig. 5B). There was a clear shift to the right in the NAD titration curve for E113A compared to that of the wild-type ligase. The wild-type *E. coli* ligase displayed an apparent *Kₘ* for NAD of 3 µM, whereas the *Kₘ* of E113A was increased to 45 µM.

The Y225A mutation of *E. coli* ligase reduced strand joining activity *in vitro* to one-quarter of the wild-type value and *EcoLIG*-Y225A still complemented the *cdc9Δ* mutation in yeast (Fig. 4C). An aromatic side chain is conserved at this position in ATP-dependent ligases, GTP-dependent capping enzymes and NAD-dependent ligases (except for *Tth* ligase, which has a leucine) (Fig. 3). The crystal structures of the T7 ligase and *Chlorella* virus capping enzyme show that the conserved aromatic side chain of motif IIa stacks on the purine base (18,24). This aromatic residue aligns structurally to Tyr221 in *Bst* ligase (25) and, by sequence comparisons, to Tyr225 of the *E. coli* enzyme. The finding that replacement of this tyrosine by alanine in the *E. coli* DNA ligase did not eliminate enzyme activity implies that the base stacking interaction is not strictly required for ligation or for recognition of the adenine nucleotide substrate. Nonetheless, loss of the aromatic moiety significantly diminished the affinity of the Y225A mutant for NAD (*Kₘ* = 76 µM) (Fig. 5B).

**Effects of N-terminal truncations of *E. coli* DNA ligase**

The crystal structure of the adenyllylation domain of the NAD-dependent *Bst* ligase revealed an 80-amino acid N-terminal segment, composed of four α-helices, that has no counterpart in the ATP-dependent T7 ligase (25). To assess whether the unique N-terminus of the bacterial ligase is functionally relevant, we engineered two N-terminal deletion mutants—*EcoLIG*(79–671) and *EcoLIG*(39–671)—which we refer to as NA78 and NA38. The NA78 deletion eliminates the N-terminal segment corresponding to the segment of *Bst* ligases from positions 1–80. The starting methionine of NA78 aligns with the first residue of the T7 ligase (25). The NA38 deletion eliminates the equivalent of the first α-helix and the proximal part of the second helix in the *Bst* ligase structure. The NA78 and NA38 mutants were expressed in soluble form in *E. coli* and were purified by affinity chromatography (Fig. 4A). Both recombinant proteins were severely defective in nick-joining *in vitro* (<0.01% of wild-type specific activity) and neither deletion mutant could complement the yeast *cdc9Δ* strain *in vivo* (Fig. 4B and C).

**UV and MMS sensitivity**

Conditional *cdc9* mutants are hypersensitive to UV or MMS when the cells are transiently held at restrictive temperature immediately post-irradiation or MMS treatment before plating them at permissive temperature (15,28). To assess the capacity of heterologous ligases to repair DNA damage in yeast, we examined the UV and MMS sensitivities of *CDC9, CDC9-ΔN65, ChVLIG* and *EcoLIG* cells. In each case, the indicated ligase gene on a *CEN* plasmid is the sole source of DNA ligase activity. In the *CDC9* cells, expression of the full-sized yeast *Cdc9p* is under the control of its natural promoter, whereas the expression of truncated yeast *Cdc9-ΔN65p, ChLIG* and *EcoLIG* is under the control of the *TP11* promotor. Semilog plots of cell survival versus UV dose are shown in Figure 6A. Replacement of *CDC9* by *CDC9-ΔN65* or *ChVLIG* had little impact on UV survival up to 100 J/m²; at the higher dose of 150 J/m², the *CDC9-ΔN65* or *ChVLIG* strains were between 4- and 9-fold more sensitive than *CDC9* cells. It is conceivable that this difference reflects the fact that *Cdc9p* expression from its natural promoter is induced by UV damage (29). The mild effects of deletion of the N-terminus of *Cdc9p* or replacement by *Chlorella* virus ligase on UV repair are in stark contrast to the effects of a *rad6* deletion, which serves as a true gauge of a full-blown UV repair defect. The survival of the *rad6* strain is reduced by more than three orders of magnitude by 10 J/m², a dose that has no impact on the *LIG1-ΔN65* or *ChVLIG* cells. Replacement of endogenous yeast ligase by *E. coli* ligase does result in a UV repair phenotype, with ~25-fold enhanced sensitivity at 100 J/m² relative to *LIG1* cells.

Semilog plots of cell survival versus the duration of exposure to 0.5% MMS are shown in Figure 6B. Replacement of *CDC9* by *CDC9-ΔN65* had essentially no effect on survival, i.e., the MMS kill curves overlapped within experimental error. The difference between *CDC9* and *CDC9-ΔN65* cell sensitivity to MMS was at most 2-fold, which may again reflect the induction of
Cdc9p expression from its natural promoter by MMS treatment (29). The ChVLIG cells were 3-fold more sensitive than CDC9-NA65 cells up to 20 min of MMS exposure and 10-fold more sensitive after 40 min. The mild effects of replacement of Cdc9p by Chlorella virus ligase on MMS repair are quite different from the severe effects of a rad6 deletion. Replacement of endogenous yeast ligase by E.coli ligase resulted in ~100-fold enhanced sensitivity to a 20-min exposure to MMS.

**DISCUSSION**

**A minimal DNA ligase suffices for yeast cell growth**

Unique biological functions in DNA replication have been imputed to DNA ligase I. For mammalian ligase I, it has been proposed that biological specificity and/or targeting of the enzyme is contributed (at least in part) by protein segments outside the catalytic domain, particularly the very N-terminal segment. Consistent with this model, a deletion mutant of mammalian ligase I lacking the N-terminal 231 amino acids (NA231) was unable to rescue the lethality of a homozygous lig1 null mutation in embryonic stem cells, even though the mammalian NA231 ligase was functional in budding yeast (30). Mammalian ligase I binds to PCNA through its N-terminal 20-amino acid peptide, which includes a conserved motif found in other PCNA-binding proteins (4, 7). A related sequence is present in the N-terminal segment of Cdc9p (residues 33–54).

Here we found that the N-terminal 65 or 99 amino acids of *S.cerevisiae* Cdc9p are dispensable for yeast cell growth. Thus, it may be that (i) other segments of the yeast ligase serve an essential targeting role or (ii) a replication-targeting region is not strictly essential in yeast. Consistent with the latter view, we find that Chlorella virus DNA ligase, which consists of the minimal catalytic domain of an ATP-dependent ligase, is fully capable of sustaining mitotic growth of budding yeast when the viral enzyme is the only ligase present *in vivo*. Moreover, Chlorella virus ligase was active *in vivo* when expressed from a single-copy CEN plasmid. In previous studies, the growth of *cdc9*-ts strains at restrictive temperature was complemented by expressing either the NA231 mutant of mammalian ligase I (a 688-amino acid polypeptide) or the full-length vaccinia virus ligase (a 552-amino acid polypeptide) at high gene dosage using multicopy 2µ plasmids (11, 30). The 298-amino acid Chlorella virus ligase (with only 28 amino acids upstream of the lysine nucleophile and no polypeptide segment distal to motif VI) is about half the size of the other ‘viable’ heterologous ligases. The inference drawn by others that a ‘minimal catalytic domain’ of an ATP-dependent DNA ligase can support yeast growth (30) remains valid, but our results show clearly that the minimal functional domain *in vivo* is actually much smaller than what had been surmised previously.

**NAD-dependent bacterial ligase suffices for yeast cell growth**

A notable finding was that the NAD-dependent DNA ligase from *E.coli* sufficed for mitotic growth of *S.cerevisiae*. To our knowledge, this is the first demonstration that an NAD-dependent enzyme is biologically active in a eukaryotic organism. To date, no NAD-dependent DNA ligase has been characterized from a eukaryotic source. However, a recent report of the genomic DNA sequence of an insect poxvirus— *Melanoplus sanguinipes* entomopoxvirus—described an open reading frame that encodes a 522-amino acid polypeptide with similarity to eubacterial NAD-dependent DNA ligases (31). Although it remains to been proved that the insect virus protein has NAD-dependent DNA ligase activity, the entomopoxvirus finding, together with the present data on bacterial ligase function in yeast, raises the prospect that other NAD-dependent ligases will be found in the eukarya and their viruses. It is remarkable that the different genera of poxviruses encode either an ATP-dependent ligase resembling mammalian ligase III (vaccinia,
Shope fibroma virus and fowlpox virus), an NAD-dependent ligase homolog (entomopoxvirus) or no DNA ligase at all (molluscum contagiosum virus). Given the likelihood that cytoplasmic poxviruses acquire new genes from host cell cDNAs, it will be of great interest to see if any NAD-dependent ligases are encoded by arthropod organisms.

**Structure–function studies of E.coli DNA ligase highlight similarities to ATP-dependent ligases and capping enzymes**

The in vivo activity of bacterial ligase in yeast and the results of mutational analysis of the bacterial enzyme underscore functionally the lessons from crystallography that the catalytic sites of NAD- and ATP-dependent enzymes are more similar than had been previously thought. The motif I lysine and aspartate side chains have been found to be critical for nick-joining activity by eukaryotic cellular and viral ATP-dependent ligases (1,9,27,32) and by NAD-dependent ligases of enteric and thermophilic bacteria (this study and ref. 21). Here we showed that other residues within the N-terminal domain of E.coli ligase that have conserved counterparts in ATP-dependent ligases and capping enzymes are either required for ligation activity or else contribute to affinity for the nucleotide substrate. The structural alignment of Singleton et al. (25) indicates that Asp285 and Lys314 in the E.coli ligase are the counterparts of essential side chains in motifs IV and V of capping enzyme and/or T7 ligase that interact with the lysine nucleophile and the α-phosphate, respectively. The extreme inactivation of E.coli ligase by the D285A and K314A mutations is consistent with a direct involvement of these side chains in the chemistry of nucleotidyl transfer.

Our findings that E.coli ligase residue Glu13 contributes to NAD binding are consistent with the existence of a hydrogen bond between the adenine base of the nucleotide substrate and the homologous glutamate of T7 ligase (25). Nonetheless, the alanine-scanning data for the E.coli and Chlorella virus ligases indicate that hydrogen bonding interactions of the side chain 2 amino acids proximal to the motif I lysine are not essential for ligase function. Therefore, the observation of Kodama et al. (1) that human ligase I was inactivated by replacement of the corresponding glutamate with a lysine may reflect the adverse effects of charge inversion rather than a specific role for the glutamate.

That E.coli ligase residue Tyr225 also contributes to NAD binding is in accord with the stacking interaction between the nucleotide base and the homologous aromatic amino acid of T7 ligase (18). Nonetheless, it appears that an aromatic group in this position is not crucial for NAD-dependent ligase function because (i) the Y225A mutant of E.coli ligase retains considerable function, and (ii) the NAD-dependent Tth ligase has a leucine in this position (Fig. 4). The effects of mutating the corresponding motif IIIa aromatic residue of an ATP-dependent ligase are not known. However, mutation of the motif IIIa phenylalanine of yeast capping enzyme to either alanine or leucine resulted in loss of activity in vivo, whereas the conservative change to tyrosine was well tolerated (26). It would appear then that while certain conserved residues make similar contacts to the nucleotide substrate in polynucleotide ligases and capping enzymes, individual enzymes may rely to a greater or lesser extent on a given side chain to carry out nick-joining or capping. Structure–function analysis of several members of the NAD- and ATP-dependent ligase families will better illuminate the structural basis for reaction chemistry and substrate specificity.

**Heterologous ligases and DNA repair**

Genetic experiments implicate Cdc9p in the repair of DNA damage caused by UV and MMS (15,28) and Cdc9p catalyzes DNA ligation during excision repair in yeast cell free extracts (33). Although heterologous ligases can clearly substitute for Cdc9p during mitotic growth (where the principal function is the sealing of processed Okazaki fragments), different heterologous ligases vary in their ability to contend with DNA damage. The growth rate of EcoLIG yeast cells did not differ from that of isogenic CDC9 cells, yet the EcoLIG cells were defective in repair of UV and MMS damage compared to CDC9 and ChVLIG cells. It is not clear if this finding reflects an inherent difference in the repair-proficiency of an NAD-dependent bacterial ligase versus ATP-dependent ligases in the yeast milieu. Although the yeast CDC9-NA65, Chlorella virus and E.coli ligases were expressed under the control of the same yeast promoter on a single-copy vector, it is possible that the EcoLIG cells were UV- and MMS-sensitive because the bacterial ligase (i) accumulated to steady-state levels that were sufficient for normal cell growth, but were not adequate to fully cope with incurred UV or MMS damage; (ii) did not accumulate in sufficient amounts within the nuclear compartment to contend with the damage; or (iii) was less efficiently localized within the nucleus to sites of DNA damage, e.g., as might occur if the bacterial ligase lacks the capacity to interact with other DNA repair proteins in a eukaryotic cell. Although mammalian ligase I does interact with mammalian replication/repair proteins, including PCNA, it is not known if yeast Cdc9p engages in such interactions or if such contacts are functionally relevant to repair in yeast. The finding that yeast cells containing Chlorella virus ligase are relatively repair-proficient suggests that the catalytic domain of an ATP-dependent ligase can function in repair pathways in yeast, but leaves open the possibility that the large protein domains of Cdc9p that have no counterpart in the Chlorella virus protein do make some contribution (up to several fold) to the efficiency of the UV and MMS repair pathways.

**REFERENCES**


