**A Primer-dependent Polymerase Function of *Pseudomonas aeruginosa* ATP-dependent DNA Ligase (LigD)**

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*Pseudomonas aeruginosa* encodes two putative DNA ligases: a classical NAD⁺-dependent DNA ligase (LigA) plus an ATP-dependent DNA ligase (LigD). LigD exemplifies a family of bacterial proteins that consist of a ligase domain fused to flanking domains that resemble nucleases and/or polymerases. Here we purify LigD and show that it possesses an intrinsic polymerase function resident within an autonomous C-terminal polymerase domain, LigD(533–840), that flanks an autonomous DNA ligase domain, LigD(188–527). Native LigD and the polymerase domain are both monomeric proteins. The polymerase activity is manifest in three ways: (i) non-templated nucleotide addition to a blunt-ended duplex DNA primer; (ii) non-templated addition to a single-stranded DNA primer; and (iii) templated extension of a 5′-tailed duplex DNA primer-template. The divergent catalytic cofactor requirement for non-templated and templated polymerase activity is satisfied by manganese or cobalt. rNTPs are preferred over dNTPs as substrates for non-templated blunt-end addition, which typically entails the incorporation of only 1 or 2 nucleotides at the primer terminus. Templated dNMP addition to a 5′-tailed substrate is efficient with respect to dNTP utilization; the primer is elongated to the end of the template strand and is then further extended with a non-templated nucleotide. The polymerase activity is abolished by alanine substitution for two aspartates (Asp-669 and Asp-671) within the putative metal-binding site. We speculate that polymerase activity is relevant to LigD function in nonhomologous end-joining.

DNA ligases are grouped into two families, ATP-dependent ligases and NAD⁺-dependent ligases, according to their nucleotide substrate requirement (1, 2). All known bacterial species have an NAD⁺-dependent DNA ligase, which is essential for growth, even in cases where the bacterium encodes additional NAD⁺-dependent or ATP-dependent DNA ligase enzymes (3, 4). ATP-dependent ligases have been characterized from several bacterial species, including *Haemophilus influenzae*, *Neisseria meningitidis*, and *Mycobacterium tuberculosis* (4–7). Mycobacterial proteomes contain the largest ensemble of ligase enzymes among known bacterial species. For example, *M. tuberculosis* encodes three distinct ATP-dependent ligases (LigB, LigC, and LigD) plus a classical NAD⁺-dependent ligase (LigA) (4). The nonpathogenic mycobacterium *M. smegmatis* encodes four ATP-dependent ligases (LigB, LigC1, LigC2, and LigD) plus LigA. Initial genetic deletion studies showed that none of the ATP-dependent mycobacterial ligases were essential for growth of *M. tuberculosis* or *Mycobacterium smegmatis* under standard laboratory conditions (4). Although the functions of mycobacterial LigB and LigC are unknown, recent studies (4, 7) implicate LigD in a pathway of bacterial DNA repair via non-homologous end-joining (NHEJ). In eukaryotes, the NHEJ pathway is responsible for rearrangement of T cell receptor and antibody gene segments, a process that is initiated by the generation of site-specific double-strand breaks. The proteins required for eukaryotic NHEJ include a DNA end-binding protein, Ku, and a specialized end-joining enzyme, ATP-dependent DNA ligase IV (LigIV) (reviewed in Refs. 9 and 10). Non-lethal loss-of-function mutations in eukaryal NHEJ proteins confer sensitivity to ionizing radiation and, in mammals, result in severe combined immunodeficiency. Until recently, double-strand break repair in bacteria was thought to occur exclusively through homologous recombination. However, the detection of putative homologs of Ku in several bacterial proteomes (e.g. *M. tuberculosis*, *M. smegmatis*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor*, *Xanthomonas axonopodis*, *Bordetella bronchiseptica*, *Agrobacterium tumefaciens*) suggested that NHEJ might be an alternative double-strand break repair pathway in some bacterial species (11, 12). This idea was underscored by the detection of homologs of mycobacterial LigD in many of the bacteria that encode Ku-like proteins (11, 13). By analogy to eukaryotic NHEJ, these bacterial Ku and LigD proteins were suggested to participate in a novel prokaryotic NHEJ pathway (11). We have shown that *M. tuberculosis* and *M. smegmatis* do indeed have a robust NHEJ pathway in vivo that depends on both LigD and Ku (4).

The bacterial LigD proteins are putative multifunctional DNA-modifying enzymes composed of an ATP-dependent DNA ligase catalytic domain fused to a putative nuclease domain and a putative polymerase domain (11, 13). The domain order varies among bacterial LigD proteins, e.g. *M. tuberculosis* LigD consists of an N-terminal polymerase-like domain, a central nuclease-like domain, and a C-terminal ligase domain, whereas the LigD protein of *P. aeruginosa* is composed of an N-terminal nuclease-like domain, a central ligase domain, and a C-terminal polymerase-like domain (Fig. 1). Although genetic analysis showed that the LigD protein is required for efficient NHEJ in mycobacterium (4), it is not clear whether and how the putative nuclease and polymerase functions might contribute to the NHEJ mechanism. Indeed, it has not yet been demonstrated that bacterial

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LigD proteins actually have any catalytic functions other than DNA ligation (4, 7).

Here we produce and characterize *P. aeruginosa* LigD. We show that the LigD protein has an intrinsic polymerase function capable of templated and non-templated DNA primer-extension reactions. Initial mutational analysis highlights the essential role of conserved aspartates that comprise the metal-binding site of homologous polymerase/primase enzymes from FIG. 1. Primary structure of *P. aeruginosa* LigD and related proteins from other bacteria. The LigD polypeptide is depicted in schematic form with the N terminus on the left and the C terminus on the right. The amino acid sequence of *P. aeruginosa* LigD (Pae) is aligned to the sequences of homologous polypeptides encoded by *A. tumefaciens* (Atu) and *B. bronchosepticum* (Bbr). Gaps in the alignment are indicated by dashes. Conserved residues are indicated by ●. Ligase motifs I, III, IIIa, IV, V, and IV are highlighted in shaded boxes. The translation start sites and stop sites of the recombinant domain fragments are indicated by bent arrows above the sequence. The two conserved aspartates of the Pol domain that were changes to alanine are highlighted in boldface and indicated by vertical bars above the sequence.
Archaea and eukarya. We discuss the potential role of the LigD polymerase activity in light of new mechanistic studies of mycobacterial NHEJ.

EXPERIMENTAL PROCEDURES

P. aeruginosa LigD—The PA2138 gene encoding LigD was amplified by PCR with Herculase Enhanced DNA polymerase (Stratagene) using primers designed to introduce an NdeI restriction site at the start codon and a BamHI site at the 3’ end of the stop codon. The cosmid DNA template used for amplification was obtained from the Pseudomonas Genetic Stock Center, East Carolina University. The PCR product was digested with NdeI and BamHI and inserted into pET16b (Novagen) to generate expression plasmid pET-PaeLigD encoding the LigD polypeptide fused to an N-terminal His$_{10}$ tag. The insert was sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning.

The pET-PaeLigD plasmid was transformed into Escherichia coli BL21(DE3). A culture (1 liter) of E. coli BL21(DE3)/pET-PaeLigD was grown at 37 °C in Luria-Bertani medium containing 0.1 mg/ml ampicillin until the A$_{600}$ reached 0.6. The culture was adjusted to 0.5 mM isopropyl-b-thiogalactopyranoside and then incubated at 17 °C for 15 h with constant shaking. Cells were harvested by centrifugation, and the pellets were stored at −80 °C. All subsequent steps were performed at 4 °C. Thawed bacteria were resuspended in 50 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 0.5 mM NaCl, 10% sucrose, 15 mM imidazole). Lysis and sonication were performed to reduce viscosity, and insoluble material was removed by centrifugation. The supernatant was applied to a 2-ml column of Ni$_{2}$-nitrilotriacetic acid-agarose (Qia- gen, Chatsworth, CA) that had been equilibrated with lysis buffer. The polypeptide compositions of the gradient fractions were analyzed by SDS-PAGE. LigD was recovered predominantly in the 200 and 500 mM imidazole eluate fractions, which contained 2.6 mg of protein. The protein concentrations were determined by using the Bio-Rad dye reagent with bovine serum albumin as the standard. The recombinant LigD preparation was stored at −80 °C.

LigD Ligase Domain—Gene fragments encoding LigD-(1–527) [Nuc-Lig domain], LigD-(188–840) [Lig-Pol domain], and LigD-(188–527) [Lig domain] were amplified by PCR with sense strand primers that introduced an NdeI restriction site at the beginning of the open reading frame and antisense primers that introduced a BamHI site 3’ of the stop codon. The PCR products were digested with NdeI and BamHI and inserted into pET16b to generate expression plasmids encoding His$_{10}$-tagged versions of the putative Nuc-Lig, Lig, and Lig-Pol domains of PaeLigD. The plasmid inserts were sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning. The recombinant proteins were produced in E. coli BL21(DE3) and purified from soluble bacterial extracts by nickel-agarose chromatography as described above for full-length LigD.

LigD Polymerase Domain—A gene fragment encoding LigD-(533–840) was amplified by PCR from the pET-PaeLigD plasmid template with a sense-strand primer that introduced an NdeI restriction site and a methionine codon in lieu of the codon for Arg-532. The PCR product was digested with NdeI and BamHI and inserted into pET16b to generate expression plasmid pET-PaeLigD-(533–840) encoding a His$_{10}$-tagged version of the putative C-terminal polymerase domain of LigD. A double-alanine substitution mutation (D660A/D671A) was introduced into the pET-PaeLigD-(533–840) plasmid by PCR using the two-stage overlap extension method. The inserts of the wild-type and mutant pET-PaeLigD-(533–840) plasmids were sequenced completely to exclude the acquisition of unwanted changes during amplification and cloning. The recombinant wild-type and mutant pET-PaeLigD-(533–840) plasmids were transformed into E. coli BL21(DE3). Induction of protein expression with isopropyl 1-thio-D-galactopyranoside and preparation of soluble bacterial lysates were performed as described above for LigD. The supernatants containing the WT and mutant LigD-(533–840) polypeptides were applied to 4-ml columns of nickel-agarose that had been equilibrated with lysis buffer. The polypeptide compositions of the fractions were monitored by SDS-PAGE. LigD-(533–840) was recovered predominantly in the 200 and 500 mM imidazole eluate fractions, which contained 14 and 9 mg of protein, respectively.

Glycerol Gradient Sedimentation—Aliquots (40 µg) of the nickel-agarose preparation of LigD or the nickel-agarose preparation of the LigD-Pol domain were mixed with calf thymus DNA (30 µg), BSA (30 µg), and cytochrome c (30 µg) in 200 µl of buffer A. The mixtures were applied to 4.5-ml SW50.1 rotor at 50,000 rpm. Fractions (0.2 ml) were collected from the bottoms of the tubes. The polypeptide compositions of the gradient fractions were analyzed by SDS-PAGE. Aliquots of the fractions were assayed for activity as specified in the figure legends.

DNA Ligation Assay—A 24-µg DNA duplex containing a centrally placed 3’-OH/5’-PO$_{4}$ nick was formed by annealing a 5’-$^{32}$P-labeled 12-mer DNA strand and an unlabeled 12-mer 3’-OH strand to a complementary 24-mer DNA strand as described previously (14). Ligation reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MgCl$_{2}$, 1 pmol of $^{32}$P-labeled nicked DNA substrate, and ATP and LigD as specified were incubated for 20 min at 37 °C. The reactions were quenched by adjusting the mixtures to 10 mM EDTA and 48% formamide. The products were resolved by electrophoresis through a 15-cm 18% polyacrylamide gel con-
Polymerase Activity of Pseudomonas DNA Ligase

**RESULTS**

**Purification and Nick Sealing Activity of *P. aeruginosa* LigD—**P. aeruginosa LigD is an 840-amino acid polypeptide consisting of a central ligase domain typical of ATP-dependent DNA ligases flanked by an N-terminal “nuclease” module that resembles the exonuclease III/apurinic endonuclease family of DNA repair enzymes and a C-terminal “polymerase” module that has similarity to the catalytic subunit of archaeal and eukaryal DNA primases (Fig. 1). LigD homologs with the same domain order are found in several other bacterial species, including *A. tumefaciens* and *B. bronchosepticum* (Fig. 1), as well as *Mezorhizobium loti*, *Desulfitobacterium hafniense*, *Novosphingobium aromaticivorans*, *X. axonopodis*, *Ralstonia eutropha*, and *Cytophaga hutchinsonii* (not shown). To evaluate the biochemical properties of PaeLigD, the protein was produced in *E. coli* as a His$_{10}$-LigD fusion and purified from a...
soluble E. coli extract by nickel-affinity chromatography followed by phosphocellulose chromatography. The 97-kDa LigD polypeptide was the predominant constituent of the phosphocellulose enzyme preparation (Fig. 2A).

Recombinant PaeLigD reacted with [α-32P]ATP in the presence of either magnesium or manganese to form an SDS-stable protein-32P-adenylate adduct (data not shown). LigD catalyzed sealing of a singly nicked duplex DNA substrate composed of a 5'-adenylated 12-mer strand and an unlabeled 12-mer 3′OH strand annealed to a complementary 24-mer (Fig. 3). In the absence of exogenous ATP, preformed LigD-adenylate catalyzed a single round of ligation, evinced by the conversion of the labeled 12-mer pDNA strand to a ligated 24-mer product (Fig. 3A). Only trace levels of the DNA-adenylate intermediate (AppDNA) were detectable in the absence of ATP. The yield of ligated DNA in the ATP-independent sealing reaction shown in Fig. 3A increased linearly as a function of input LigD (not shown). The slope of the titration curve indicated that 0.47 pmol of substrate was ligated per pmol of input LigD. Because ATP-dependent sealing is a single-turnover reaction, we thereby estimated that 47% of the LigD molecules had AMP bound covalently at the active site. Inclusion of 100 μM ATP resulted in a 2-fold stimulation of ligase activity (Fig. 3A). ATP addition also resulted in the accumulation of higher amounts of the AppDNA intermediate. Weak stimulation by ATP and ATP-dependent trapping of the AppDNA intermediate on a nicked duplex substrate is also characteristic of the LigD enzyme from M. tuberculosis (4).

The quaternary structure of PaeLigD was examined by zonal velocity sedimentation in a 15–30% glycerol gradient (Fig. 4). Marker proteins catalase (native size 248 kDa), BSA (66 kDa), and cytochrome c (12 kDa) were included as internal standards in the gradient. After centrifugation, the polypeptide compositions of the even-numbered gradient fractions were analyzed by SDS-PAGE. His10-LigD sedimented as a discrete peak (fractions 16–18) on the heavy side of the BSA peak (Fig. 4A). The nick sealing activity profile was coincident with that of the LigD polypeptide (Fig. 4B). An S value of 5.4 was determined for LigD by interpolation to the internal standard curve. These results are consistent with a monomeric structure for LigD.

An Autonomous Central Ligase Domain of PaeLigD—Fragments of the LigD polypeptide spanning amino acids 1–527 (a putative N-terminal nuclease-ligase domain), amino acids 188–840 (a putative C-terminal ligase-polymerase domain), and amino acids 188–527 (a putative central ligase domain) were produced in E. coli as His10 fusions and purified from soluble bacterial lysates by nickel-agarose chromatography (Fig. 2A). The purity of the 62-kDa Nuc-Lig domain, the 75-kDa Lig-Pol domain, and the 41-kDa Lig domain preparations was comparable with that of full-length LigD. The Nuc-Lig, Lig-Pol, and Lig domains were all capable of sealing the singly-nicked duplex DNA substrate in the presence of magnesium and ATP (Fig. 2B). AppDNA accumulated to an appreciable extent, no matter which domain fragment was included in the sealing reaction (Fig. 2B). We conclude that LigD(188–527) comprises an autonomous ligase domain.

Metal Cofactor Requirement for Ligase Activity—Nick sealing by LigD in the presence of 100 μM ATP required a divalent cation cofactor (Fig. 3B). Magnesium supported the conversion of the 12-mer pDNA strand to a discrete 24-mer ligation product. An additional minor species corresponding to AppDNA was also produced. Manganese supported nick sealing activity, but the product distribution was strikingly different, insofar as the ligated products consisted of a triplet of 24-, 25-, and 26-mer species (Fig. 3B). In addition, manganese prompted the appearance of discrete radiolabeled 13- and 14-mer species. Note that the electrophoretic mobility of the 13-mer formed in the presence of manganese was different from that of the 5'-adenylated 12-mer generated in the magnesium-containing reaction. The novel products seen in the manganese-containing reaction reflect ATP-dependent addition of nucleotides to the 3′ end of the 12-mer pDNA substrate and the 3′ end of the 24-mer ligation product (see below).

Nick joining occurred in the presence of cobalt, although
most of the ligated 24-mer that was formed was elongated to 25- and 26-mer products (Fig. 3B). The 12-mer pDNA strand was also extended by 1 or 2 nucleotides in the presence of cobalt. Cadmium, copper, and zinc were ineffective as cofactors for the nick-joining reaction (Fig. 3B). Calcium did not support the overall ligation reaction but did allow the conversion of the 12-mer pDNA to a discrete product of the size of AppDNA (Fig. 3B).

Analysis of the divalent cofactor requirements for nick sealing by the isolated Lig domain, LigD-(188–527), showed that only the 24-mer ligation product was formed in manganese- and cobalt-containing reactions (Fig. 3B). Thus, the manganese and cobalt effects on the outcomes of the nick-sealing reactions of native LigD versus the isolated Lig domain highlight the presence of a polymerase activity in the full-length LigD enzyme preparation (see below). The Lig domain, like LigD, was unable to seal nicks using cadmium, copper, or zinc. Calcium supported the adenylylation of the 5′ pDNA strand (step 2 of the ligase pathway) but apparently did not suffice for the step of phosphodiester bond formation (step 3).

Non-templated Polymerase Activity Associated with LigD—LigD is implicated in a recently discovered bacterial NHEJ pathway (4). Initial studies of the NHEJ mechanism in M. smegmatis revealed that blunt-ended double-strand breaks are sealed efficiently, albeit with a high frequency of single-nucleotide insertions at the repair junction.1 Deletion of M. smegmatis LigD reduced the efficiency of blunt-end NHEJ in vivo by a factor of 50, implicating LigD as an agent of the mutagenic end-joining pathway. To test whether LigD might be responsible for the end addition that accompanied NHEJ in vivo, and to further explore the apparent association of a polymerase-like activity with full-length Pseudomonas LigD, we tested the ability of recombinant PaeLigD to add non-templated nucleotides to a 5′ 32P-labeled 18-mer primer DNA strand annealed at a blunt duplex end (Fig. 5). We found that incubation of LigD with the blunt-ended 18-mer primer in the presence of manganese and ATP resulted in elongation of the primer by a single nucleotide step; a minor fraction of the product was elongated by two nucleotide steps (Fig. 5B). The yield of the elongated product was proportional to the amount of LigD added to the reaction. The sedimentation profile of the non-templated AMP addition activity in the recombinant LigD preparation coincided with that of the LigD polypeptide (Fig. 4B).

An Autonomous C-terminal Polymerase Domain—To localize the end-addition function within the LigD protein, we produced and purified a His6-tagged C-terminal fragment, LigD-(533–840), that starts 17 amino acids downstream of nucleotidyltransferase motif IV of the ligase domain (indicated by an arrow in Fig. 1) and that embraces the segment of LigD that displays primary structure similarity to archaeal/eukaryal DNA primases. The recombinant Pol domain of LigD was purified from a soluble bacterial extract by nickel-agarose chromatography, yielding a preparation that was highly enriched...
with respect to the 37-kDa His$_{10}$-LigD-(533–840) polypeptide (Fig. 5A). The isolated Pol domain catalyzed the same AMP addition reaction at the blunt DNA primer terminus that was observed for full-length LigD (Fig. 5B). Here, too, the major product was elongated by a single nucleotide step, with a minor fraction elongated by two nucleotides.

The native size of the Pol domain was examined by glycerol gradient sedimentation. His$_{10}$-LigD-(533–840) sedimented as a discrete peak (fractions 19–23) between BSA and cytochrome c (Fig. 6). An S value of 2.9 was determined by interpolation to the internal standard curve. These results are consistent with a monomeric structure for the C-terminal Pol domain. The sedimentation profile of the non-templated AMP addition activity in the recombinant Pol domain preparation was coincident with that of the LigD-(533–840) polypeptide (Fig. 6). The findings that end-addition activity cosedimented with both LigD and the truncated Pol domain, and that the apparent size of the end-addition activity shifted as a consequence of the protein truncation, provide strong evidence that the end-addition reaction is intrinsic to the recombinant Pseudomonas proteins.

Characterization of the Non-templated End-addition Reaction—Extension of the blunt-ended 18-mer primer required an exogenous NTP (Fig. 5C). Any of the four ribonucleoside triphosphates could serve as the substrate for blunt-end addition catalyzed by either LigD or the isolated Pol domain (Fig. 5C). Subtle differences in the electrophoretic mobility of the extended primers produced in reactions containing ATP, CTP, GTP, or UTP suggested that the respective NMPs had been incorporated at the 3' end of the 18-mer DNA. Deoxynucleoside triphosphates were less effective in supporting primer extension than the equivalent concentrations of the corresponding rNTPs (Fig. 5C). Whereas extension in the presence of ATP, CTP, or GTP resulted mainly in the addition of a single nucleotide, the UTP- and dTTP-dependent reactions yielded a higher fraction of products that were extended by two or more nucleotides (Fig. 5C).

Non-templated extension of the blunt-ended primer by the Pol domain in the presence of ATP was optimal at pH 7.5–8.5 (not shown). Non-templated extension required a divalent cation cofactor (Fig. 7A). A survey of various divalent metals at 5 mM concentration showed that manganese and cobalt best satisfied the requirement. Zinc was less active, and other divalent cations (calcium, copper, cadmium, and magnesium) were ineffective (Fig. 7A). Cofactor titrations in primer extension reactions containing 50 µM ATP indicated that activity was optimal at 0.8 mM manganese, 0.4–0.8 mM cobalt, or 0.4 mM zinc (Fig. 7B). Little or no activity was detected at 25, 50, or 100 µM concentrations of either manganese, cobalt, or zinc (Fig. 7B), suggesting that the metal is required in excess of the input NTP. Activity at 6.2 mM manganese or cobalt was 80–90% of the peak value. Magnesium failed to support non-templated addition at concentrations ranging from 25 µM to 25 mM (Fig. 7B and data not shown).

The effects of nucleotide concentration on the outcomes of the blunt-end addition reaction are shown in Fig. 8. The reactions were effectively saturated at 10 µM of each of the rNTPs and dNTPs. The efficiency of the reaction, expressed as the percent of the input primer extended, was higher for each rNTP than for its corresponding dNTP. Thus, the apparent preference of LigD for addition of a ribonucleotide versus a deoxynucleotide to a blunt DNA end is not a function of a gross differential affinity for rNTPs versus dNTPs.

Extension of a Single-stranded DNA Primer—Reaction mixtures (20 µl) containing 50 mM Tris-HCl (pH 7.5), 5 mM DTT, 5 mM MnCl$_2$, 1 pmol of 32P-labeled 18-mer single-stranded DNA substrate, 1.5 µg of LigD-(533–840), and nucleotide (µM) as specified above the lanes were incubated at 37 °C for 20 min. The extent of the end-addition reactions, expressed as the percent of the input 18-mer primer that was elongated, is indicated in italics below the lanes.

The pyrimidine rNTPs and dNTPs were more effective substrates than the purine rNTPs and dNTPs for extension of the single-stranded 18-mer primer.

Templated DNA Primer Extension—The LigD Pol domain was capable of template-directed DNA synthesis, as gauged by its ability to extend a 5' 32P-labeled 18-mer DNA strand annealed to a complementary 36-mer strand to form the 5'-tailed molecule shown in Fig. 10. When provided with a mixture of the four dNTPs, the Pol domain catalyzed time-dependent extension of the primer strand to yield a limit product consisting of the substrate (Fig. 9).
of a mixture of $^{32}$P-labeled 36- and 37-mer strands (Fig. 10A). The sizes of the products are indicative of template-directed DNA elongation to form a 36-mer blunt-ended product, followed by non-templated addition of an extra dNMP to the blunt end. The product distribution after a 1-min reaction showed that the majority of the input primer strands had been extended by less than 10 nucleotides. Fully extended products accumulated between 2 and 5 min (Fig. 10A).

Templated DNA synthesis was strictly dependent on exogenous dNTPs (Fig. 10B, lane 0). The efficiency of primer extension (i.e., the fraction of input 18-mer primers that were elongated by at least 1 step) and the size distribution of the extended primers both increased with increasing dNTP concentration in the range of 0.01 to 0.31 mM of each dNTP (Fig. 10B). The reaction was effectively saturated at 1.25 mM of each dNTP. Given that the concentration of the DNA primer was 0.05 mM, these results indicate that the DNA synthesis reaction was efficient with respect to scavenging a limiting nucleotide pool.

Templated DNA synthesis by the Pol domain required a divalent cation cofactor, which could be either cobalt or manganese (Fig. 10C). Calcium, cadmium, copper, magnesium, and zinc were ineffective (Fig. 10C).

Further evidence that LigD catalyzes template-directed synthesis emerged from an analysis of the effect of varying the length of the 5'-tail of the primer-template construct (Fig. 11A). Although the primer extension reaction with a substrate containing a 36-mer template strand yielded a mixture of 36- and 37-mer products, a 30-mer template strand programmed synthesis of a mixture of 30- and 31-mer products. Further shortening the template strand to 24 nucleotides elicited a concomitant reduction in the size of the major primer-extension products to a mixture of 24- and 25-mer strands (Fig. 11A). Thus, the Pol domain will extend the primer to the end of the template strand and then incorporate a non-templated nucleotide. Kinetic analysis of the extension reactions programmed by the 30- and 24-mer template strands indicates the following: (i) the onset of primer
The effects of dNTP omission on the outcome of the templated DNA polymerase reactions of LigD and the isolated Pol domain are shown in Fig. 12. The 18-mer primer was extended by a single step when 1 μM dATP was the only nucleotide present; this result is consistent with the presence of a deoxythymidine at position n + 1 in the template strand. However, when the reaction mixtures contained dATP plus dTTP (both at 1 μM concentration), the major extension product was a 22-mer, rather than the 21-mer predicted from the sequence of the template strand. Apparently, the LigD polymerase is capable of misincorporating a nucleotide opposite the deoxyguanosine at the n + 4 position on the template strand. (Alternatively, the dNTPs might contain trace levels of contaminating dCTP that are scavenged by the polymerase.) When the reaction mixtures included dATP, dTTP, and dCTP (1 μM each), we observed the predicted 23-mer as a major product, but we also noted the presence of 24-, 25-, 26-, and 27-mer species, resulting perhaps from misincorporation opposite the n + 6 deoxycytidine nucleoside on the template strand. Inclusion of all four dNTPs (1 μM each) permitted further extension to yield a mixture of longer products ranging from 26 nucleotides to the fully extended 36-mer (Fig. 12).

**DISCUSSION**

*Pseudomonas* LigD has a complex domain architecture composed of putative nuclease, ligase, and polymerase modules. It is homologous to mycobacterial LigD, which plays a critical role in mycobacterial NHEJ (4). Here we produced PaeLigD and its component domains, and we characterized the associated ligase and polymerase activities. PaeLigD, like MtuLigD (4), is a weak nick-joining enzyme that accumulates AppDNA in the presence of ATP. The central portion of PaeLigD from residues 188 to 527 comprises an autonomous ligase catalytic domain that embraces the six defining nucleotidyltransferase motifs of the ATP-dependent DNA ligase family (19–21). The margins of the ligase catalytic domain extend only 50 amino acids upstream of the lysine adenylylation site in motif I and only 12 amino acids downstream of motif VI in the OB-fold portion of the ligase domain. Thus, the Lig domain of PaeLigD is similar in size to naturally minimized versions of an ATP-dependent DNA ligase, exemplified by the bacteriophage T7 and *Chlorella* virus enzymes (20, 21).
The polymerase activity of LigD is manifest in three ways: (i) non-templated nucleotide addition to a blunt-ended duplex DNA primer; (ii) non-templated addition to a single-stranded DNA primer; and (iii) templated extension of a 5′-tailed duplex DNA. Any or all of these reactions are potentially relevant to LigD function in bacterial NHEJ. In particular, the non-templated addition of single nucleotides to blunt DNA ends is immediately pertinent to the mechanism of LigD-dependent sealing of blunt double-strand DNA breaks in vivo in M. smegmatis, a process that results in a high incidence of single-nucleotide insertions at the repaired DNA ends. The ability of LigD to perform templated fill-in DNA synthesis at a 5′-overhanging end is similarly pertinent to the finding that such ends are frequently filled in during the in vivo LigD-dependent repair of double-strand breaks with 4-nucleotide 5′-overhangs in M. smegmatis.

That Pseudomonas LigD contains within a single polypeptide at least two (a ligase and a polymerase) and perhaps three (a putative nuclease) catalytic functions associated with NHEJ in eukarya (9, 10) underscores the likelihood that LigD is dedicated to an NHEJ pathway in P. aeruginosa, akin to that of LigD in mycobacteria. The demarcation of individual functional domains of LigD and the inactivation of a single activity by targeted mutation of the active site provide molecular tools for an eventual genetic assessment of the roles of the various LigD activities during NHEJ in vivo.

To our knowledge, there has been no prior characterization of a polymerase activity resident in the same polypeptide as a DNA ligase. The Pol domain of LigD has structural and functional similarities to archaeal/eukaryal primase enzymes. Eukaryal primase has been characterized as having exceptionally low fidelity (8). The nucleotide omission experiment shown here in Fig. 12 hints that LigD is also prone to misincorporate when it is deprived of the correct templated nucleotide substrate. Inactivation of the LigD polymerase function by the D669A/D671A mutation echoes the effects of analogous mutations on archival primase-polymerase (18). The active sites of archival/eukaryal primases resemble those of the Pol X family of nucleic acid polymerases (16). In yeast, a Pol X family member, Pol4, has been implicated as a catalyst of fill-in synthesis during NHEJ (22). Pol4 interacts physically and functionally in trans with the yeast NHEJ-specific DNA ligase, Lig4 (23). Our studies suggest that bacterial LigD achieves a similar functional coupling of DNA extension and DNA sealing by fusing both activities in the same protein.

The ability of LigD to catalyze templated fill-in DNA synthesis and non-templated end addition with fairly similar efficiency suggests that it is a functional hybrid of two classes of eukaryal Pol X family members as follows: (i) yeast Pol4 and mammalian Polβ, which catalyze templated fill-in reactions; and (ii) mammalian terminal transferase, which catalyzes non-templated end addition during immunoglobulin gene recombination. The non-templated polymerase activity of PaeLigD at a blunt DNA end is certainly not an exceptional property, insofar as many DNA polymerases from diverse sources are capable of such reactions (24–26). PaeLigD is remarkable as follows: (i) it is adept at adding either ribonucleotides or deoxynucleotides to a blunt DNA end; and (ii) its polymerase activity (non-templated and templated) is specifically dependent on manganese or cobalt as the divalent cation cofactor.
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