Many bacterial species have a nonhomologous end joining system of DNA repair driven by dedicated DNA ligases (LigD and LigC). LigD is a multifunctional enzyme composed of a ligase domain fused to two other catalytic modules: a polymerase that preferentially adds ribonucleotides to double-strand break ends and a phosphoesterase that trims 3′-oligoribonucleotide tracts until only a single 3′-ribonucleotide remains. LigD and LigC have a feeble capacity to seal 3′-OH/5′-PO₄ DNA nicks. Here, we report that nick sealing by LigD and LigC enzymes is stimulated by the presence of a single ribonucleotide at the broken 3′-OH end. The ribonucleotide effect on LigD and LigC is specific for the 3′-terminal nucleotide and is either diminished or abolished when additional vicinal ribonucleotides are present. No such 3′-ribonucleotide effect is observed for bacterial LigA or Chlorella virus ligase. We found that in vitro repair of a double-strand break by Pseudomonas LigD requires the polymerase module and results in incorporation of an alkali-labile ribonucleotide at the repair junction. These results illuminate an underlying logic for the domain organization of LigD, whereby the polymerase and phosphoesterase domains can heal the broken 3′-end to generate the monoribonucleotide terminus favored by the nonhomologous end joining ligases.

Direct evidence that bacteria catalyze DNA double-strand break (DSB)2 repair via nonhomologous end joining (NHEJ) emerged from studies of the repair of linear plasmid DNAs transfected into mycobacteria (1). NHEJ entails approximation of the broken DNA ends, aided by the bacterial end-binding protein Ku, followed by sealing of at least one of the broken strands by a specialized bacterial ATP-dependent DNA ligase, either LigD or LigC (1–9, 36). Unlike homologous recombination, which is generally error-free, NHEJ can be either faithful or mutagenic. The signature feature of NHEJ in mycobacteria is that half of the repair events at blunt or complementary 5′-overhang DSBs are unfaithful because the DSB ends are extended by polymerases or resected by nuclease to seal

By ligase (1, 8, 9, 36). Because NHEJ does not rely on a homologous DNA template, it can operate when only one chromosomal copy is available, e.g. during late stationary phase or after sporulation (10–13). Whereas mutagenic DSB repair might seem counterproductive, it can be advantageous, especially if the alternative is death of the quiescent bacterium or spore.

Biochemical, structural, and genetic studies of the NHEJ ligases and Ku proteins of Mycobacterium, Pseudomonas, Bacillus, and Agrobacterium are beginning to define a pathway with unique features and distinctive enzymatic components (1, 4–9, 14–17, 36). Ku and LigD are critical agents of the pathway. The efficiency of plasmid-based NHEJ of blunt and 5′-overhang DSBs in mycobacteria is reduced several hundredfold by deletion of Ku and by ~100-fold by deletion of LigD (1, 36). LigC provides an efficient backup sealing function in mycobacteria when LigD sealing activity is ablated by a mutation of the ligase active site (8, 36). LigD is a large multifunctional enzyme consisting of an ATP-dependent ligase (LIG) domain, a polymerase (POL) domain, and a 3′-phosphoesterase (PE) domain (1, 6, 14–16, 18). LigC is a minimal ligase with no auxiliary flanking domains (5, 16). The complexity of the bacterial NHEJ ligase menu ranges from the relatively simple state found in Pseudomonas aeruginosa (which has a single LigD and no LigC) to progressively more complex forms in Mycobacterium tuberculosis (single LigD and single LigC), Mycobacterium smegmatis (single LigD and two LigC enzymes), and Agrobacterium tumefaciens (two LigD and three LigC enzymes).

LigD and LigC are conspicuously feeble at sealing 3′-OH/5′-PO₄ DNA nicks in vitro (5, 16). This property distinguishes them from LigA, the essential “replicative” bacterial ligase (5). One explanation for the weak activity of LigD and LigC (although by no means the only one) is that 3′-OH/5′-PO₄ DNA breaks are not the optimal substrates for sealing during NHEJ. Indeed, the high frequency of end remodeling during NHEJ at blunt or 5′-overhang ends in vivo (1) and the substrate preferences in vitro of the POL and PE domains of LigD (1, 6, 14–17, 19–21) prompt the idea that ribonucleotides might be introduced at the break during NHEJ.

The LigD POL domain, which belongs to the archaean primase-polymerase family (9, 17), catalyzes either non-templated single-nucleotide additions to a blunt-ended duplex DNA or fill-in synthesis at a 5′-tailed duplex DNA (1, 6, 14, 16). These are the molecular signatures of mutagenic mycobacterial NHEJ in vivo at blunt-end and 5′-overhang DSBs, respectively (1). Pseudomonas LigD POL uses manganese as a cofactor and strongly prefers rNTPs over dNTPs as substrates for both non-templated blunt-end addition and templated fill-in synthesis.

The Journal of Biological Chemistry 2008, 283(13), 8331–8339; doi: 10.1074/jbc.M705476200

1. American Cancer Society Research Professor. To whom correspondence should be addressed: Molecular Biology Program, Sloan-Kettering Inst., 1275 York Ave., New York, NY 10021. E-mail: s-shuman@ski.mskcc.org.

2. The abbreviations used are: DSB, double-strand break; NHEJ, nonhomologous end joining; LigD, ligase domain; ATP-dependent ligase domain; POL domain, polymerase domain; PE domain, 3′-phosphoesterase domain; DTT, dithiothreitol; PaeLigD, Pseudomonas aeruginosa LigD; AtuLigD, Agrobacterium tumefaciens LigD.
Bacterial NHEJ Ligases

(14, 19). Template ribonucleotide addition is limited to about four cycles of rNMP incorporation because the primer-template is rendered progressively less active as ribonucleotides accumulate at the 3'-end (14). These properties suggest that the initial insertions preceding the sealing step of NHEJ might involve rNMP incorporation. Indeed, the ability of LigD to use rNTPs as substrates could be advantageous for break repair in quiescent cells because the dNTP pool might be limiting. Mycobacterium and Agrobacterium LigD POL proteins also prefer rNTPs to dNTPs (1, 16, 17).

LigD has an intrinsic manganese-dependent 3'-ribonuclease/3'-phosphatase activity, whereby it resects a short tract of 3'-ribonucleotides on a primer-template substrate to the point at which the primer strand has a single 3'-ribonucleotide remaining (15, 16, 20, 21). The failure to digest beyond this point reflects a requirement for a 2'-OH group on the penultimate nucleoside of the primer strand. The ribonucleotide resection activity resides within the PE domain of the Pseudomonas, Agrobacterium, and Mycobacterium LigD proteins and is the result of at least two steps: (i) the 3'-terminal nucleoside is first removed to yield a primer strand with a ribonucleoside 3'-PO4 terminus, and (ii) the 3'-PO4 is hydrolyzed to 3'-OH. Although LigD PE can remove more than one terminal ribonucleotide, it appears to do so via a sequential mechanism of mononucleoside removal rather than by cleaving a dinucleotide in a single step. The phosphodiesterase and phosphomonoesterase activities are both dependent on the presence and length of the 5'-single-strand tail of the primer-template substrate. These properties distinguish LigD PE from other 3'-end-processing enzymes. The 3'-ribonucleotide resection function of the PE domain dovetails nicely with the capacity of the LigD POL domain to add short ribonucleotide tracts to the 3'-end of a DNA primer-template. The expected effect of PE action would be to trim back the ribonucleotide-extended primer strand to the point that only a single ribonucleotide with a ligatable 3'-OH remains.

Here, we examine the influence of 3'-ribonucleotides on the sealing activity of five different NHEJ ligases: three LigD and two LigC enzymes. We found that strand sealing is stimulated by a single ribonucleotide at the 3'-OH terminus of the break. This 3'-ribonucleotide dependence is a signature property of NHEJ ligases (not shared with bacterial LigA or a prototypical eukaryotic ATP-dependent ligase) that rationalizes the bundling of auxiliary catalytic modules within LigD enzymes. We discuss the ribonucleotide requirement in light of recent crystal structures of DNA ligases bound to broken DNA.

Experimental Procedures

Enzymes—P. aeruginosa LigD; A. tumefaciens LigD1, LigD2, LigC2, and LigC3; Escherichia coli LigA; and Chlorella virus DNA ligase were produced in E. coli as His10-tagged fusions and then purified from soluble bacterial lysates as described previously (14, 16, 22, 23). The autonomous Lig domain and bifunctional PE-LIG and LIG-POL domains of P. aeruginosa LigD were produced in E. coli as His10-tagged fusions and purified from soluble bacterial lysates as described (14). Protein concentrations were determined using Bio-Rad dye reagent with bovine serum albumin as the standard.

DNA Ligase Assay—A 24-bp duplex nucleic acid containing a centrally placed 3'-OH/5'-PO4 nick was formed by annealing a 5'-32P-labeled 12-mer DNA strand and an unlabeled 12-mer 3'-OH strand to a complementary 24-mer DNA strand as described previously (24). Ligation reaction mixtures (20 μl) containing 50 mM Tris buffer as specified, 5 mM dithiothreitol (DTT), 5 mM MnCl2, 1 pmol of 32P-labeled nicked DNA substrate, 250 μM...
ATP, and ligases 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 containing 7M urea in 90 mM Tris borate and 2.5 mM EDTA. The products were visualized by autoradiography and quantified by scanning the gel with a Fuji BAS-2500 imaging apparatus.

**RESULTS**

*Pseudomonas* LigD Preferentially Seals a 3’-Monoribonucleotide-containing Nick—DNA ligases seal 3’-OH/5’-PO₄ breaks via three nucleotidyl transfer reactions. In the first step, ligase reacts with ATP or NAD⁺ to form a covalent ligase-adenylate intermediate. In the second step, the AMP is transferred to the 5’-end of the 5’-phosphate-terminated DNA strand to form a DNA-adenylate intermediate. In the third step, ligase catalyzes attack by the 3’-OH on DNA-adenylate to join the two polynucleotides and liberate AMP (25).

To gauge whether LigD activity was affected by ribonucleotides on the 3’-OH strand, we compared a series of singly nicked 24-bp duplexes (5’-32P-labeled at the nick) in which the unlabeled 12-mer 3’-OH strand consisted of 12 deoxynucleotides (D₁₂), 11 deoxynucleotides and 1 ribonucleotide (D₁₁R₁), 10 deoxynucleotides and 2 ribonucleotides (D₁₀R₂), or 9 deoxynucleotides and 3 ribonucleotides (D₉R₃).

*P. aeruginosa* LigD (PaeLigD) displayed weak activity on the D₁₂ substrate (Fig. 1). Although the yield of the radiolabeled 24-mer ligation product increased with PaeLigD concentration, only ~1.3 fmol of sealed strand was produced at the highest enzyme concentration.
Bacterial NHEJ Ligases

formed per fmol of input ligase with the D12 substrate during the 20-min reaction. Moreover, there was evident accumulation of the DNA-adenylate intermediate, which migrated just above the $^{32}$P-labeled 12-mer substrate strand. The instructive finding was that the D11R1 substrate was sealed much more effectively by PaeLigD than was the D12 substrate (Fig. 1). PaeLigC2 and LigC3 sealing activities were both suppressed by the introduction of one or two additional ribonucleotides in the D10R2 and D9R3 substrates (Fig. 2, F and G).

AtuLigD2 specific activity was 7-fold higher in sealing the D11R1 substrate versus the all-DNA nick (Fig. 2, B and E). AtuLigD2 was less sensitive to the penultimate ribonucleotide of D10R2 than were the other NHEJ ligases analyzed (Fig. 2E).

To verify the specificity of the ribonucleotide effect on NHEJ sealing, AtuLigD1 was extremely feeble at sealing the D12 substrate (~0.09 fmol of ends sealed per fmol of input ligase in a 20-min reaction), but was stimulated 140-fold by the 3'-monoribonucleotide of the D11R1 nick (Fig. 2, A and D). The additional flankng ribonucleotides of the D10R2 and D9R3 substrates were frankly inhibitory to sealing by AtuLigD1 (Fig. 2D).

AtuLigC3 was also very poor at sealing the D12 nick, and its activity was 97-fold higher on the D11R1 substrate (Fig. 2, C and F). AtuLigC2 displayed a similarly strong preference for sealing the 3'-monoribonucleotide substrate (Fig. 2G). LigC2 and LigC3 sealing activities were both suppressed by the introduction of one or two additional ribonucleotides in the D10R2 and D9R3 substrates (Fig. 2, F and G).

To verify the specificity of the ribonucleotide effect on NHEJ sealing, the substrates in parallel with Chlorella virus DNA ligase, which is the smallest euakaryotic DNA ligase known and which has been extensively characterized, biochemically and structurally (26, 27). Chlorella virus ligase was equally adept at sealing the D11R1 and D12 nicks (Fig. 2H).

Positional Specificity of Monoribonucleotide Stimulation of Sealing—To evaluate whether the gain of function elicited by a monoribonucleotide in the 3'-OH strand was specific to the terminal nucleotide, we tested singly ribonucleotide-substituted nicked duplexes in which the ribonucleotide was phased leftward to the penultimate position (see the D10RD substrate in Fig. 3F) or recessed from the nick by two nucleotides (see the D9RD2 substrate). The phased ribonucleotide-containing nicks were assayed in parallel with the D11R1 and D12 nicks for each of the four Agrobacterium NHEJ ligases and for the isolated LIG domain of Pseudomonas LigD. The product analyses are shown in Fig. 3 (A–E). Three themes emerged from the experiment. First, the 3'-terminal monoribonucleotide was uniquely stimulatory compared with the all-DNA nick. Second, the penultimate monoribonucleotide was overtly deleterious to sealing by each of the four NHEJ ligases and for the iso-

strates and ligases in D–G. AtuLigD1 was extremely feeble at sealing the D12 substrate (~0.09 fmol of ends sealed per fmol of input ligase in a 20-min reaction), but was stimulated 140-fold by the 3'-monoribonucleotide of the D11R1 nick (Fig. 2, A and D). The additional flankng ribonucleotides of the D10R2 and D9R3 substrates were frankly inhibitory to sealing by AtuLigD1 (Fig. 2D).

AtuLigC3 was also very poor at sealing the D12 nick, and its activity was 97-fold higher on the D11R1 substrate (Fig. 2, C and F). AtuLigC2 displayed a similarly strong preference for sealing the 3'-monoribonucleotide substrate (Fig. 2G). LigC2 and LigC3 sealing activities were both suppressed by the introduction of one or two additional ribonucleotides in the D10R2 and D9R3 substrates (Fig. 2, F and G).

AtuLigD2 specific activity was 7-fold higher in sealing the D11R1 substrate versus the all-DNA nick (Fig. 2, B and E). AtuLigD2 was less sensitive to the penultimate ribonucleotide of D10R2 than were the other NHEJ ligases analyzed (Fig. 2E).

To verify the specificity of the ribonucleotide effect on NHEJ sealing, AtuLigD1 was extremely feeble at sealing the D12 substrate (~0.09 fmol of ends sealed per fmol of input ligase in a 20-min reaction), but was stimulated 140-fold by the 3'-monoribonucleotide of the D11R1 nick (Fig. 2, A and D). The additional flankng ribonucleotides of the D10R2 and D9R3 substrates were frankly inhibitory to sealing by AtuLigD1 (Fig. 2D).

AtuLigC3 was also very poor at sealing the D12 nick, and its activity was 97-fold higher on the D11R1 substrate (Fig. 2, C and F). AtuLigC2 displayed a similarly strong preference for sealing the 3'-monoribonucleotide substrate (Fig. 2G). LigC2 and LigC3 sealing activities were both suppressed by the introduction of one or two additional ribonucleotides in the D10R2 and D9R3 substrates (Fig. 2, F and G).

AtuLigD2 specific activity was 7-fold higher in sealing the D11R1 substrate versus the all-DNA nick (Fig. 2, B and E). AtuLigD2 was less sensitive to the penultimate ribonucleotide of D10R2 than were the other NHEJ ligases analyzed (Fig. 2E).

To verify the specificity of the ribonucleotide effect on NHEJ sealing, AtuLigD1 was extremely feeble at sealing the D12 substrate (~0.09 fmol of ends sealed per fmol of input ligase in a 20-min reaction), but was stimulated 140-fold by the 3'-monoribonucleotide of the D11R1 nick (Fig. 2, A and D). The additional flankng ribonucleotides of the D10R2 and D9R3 substrates were frankly inhibitory to sealing by AtuLigD1 (Fig. 2D).

AtuLigC3 was also very poor at sealing the D12 nick, and its activity was 97-fold higher on the D11R1 substrate (Fig. 2, C and F). AtuLigC2 displayed a similarly strong preference for sealing the 3'-monoribonucleotide substrate (Fig. 2G). LigC2 and LigC3 sealing activities were both suppressed by the introduction of one or two additional ribonucleotides in the D10R2 and D9R3 substrates (Fig. 2, F and G).
A 2′-OCH₃ Ribonucleotide at the Nick Does Not Stimulate Sealing by the NHEJ Ligases—None of the five bacterial NHEJ ligases were able to seal a substrate containing a single 2′-OCH₃ ribonucleotide at the nick with efficiency comparable with that of the D11R1 substrate (Fig. 4, A–E). Rather, the OCH₃ substitution reduced activity to the basal level seen with the all-DNA substrate D12 (data not shown). The OCH₃ ribonucleoside is expected to adopt a RNA-like 3′-endo-sugar pucker (28). The fact that the NHEJ ligases discriminated between a ribose and a 2′-OCH₃ ribose is subject to several interpretations. One view is that terminal 3′-endo-sugar conformation does not suffice per se to promote sealing by bacterial LigC and LigD, e.g. because the ribose 2′-OH directly stimulates catalysis of sealing, by contacting a constituent of the LigD and LigC active sites (an amino acid of the ligase, the divalent cation cofactor, or a bridging water). Alternatively, it is conceivable that the advantage of a ribonucleotide-like nucleoside conformation is negated by steric hindrance effects of the bulky methyl group at the nick. A control experiment showing that the 2′-OCH₃ ribonucleotide at the nick suppressed sealing by Chlorella virus DNA ligase (Fig. 4F) indicated that even a ligase that is indifferent to a 2′-OH versus a 2′-OCH₃ at the nick is inhibited by the extra bulk of a 2′-OCH₃.

Ribonucleotide Effects on the Kinetics of Ligation—The ribonucleotide effect was probed further by assaying the rates of strand sealing under conditions in which the concentration of ligase (250–300 nM) was in 5–6-fold excess over that of the nicked duplex substrate (50 nM) (Fig. 5). The initial rates of sealing of the D11R1 nick by AtuLigC2 and AtuLigC3 were 275- and 580-fold greater than the respective rates of sealing of the D11R1 substrate (Fig. 4, B–D). The Penultimate ribonucleotide at the nick suppressed sealing by Chlorella virus DNA ligase (Fig. 4F) indicated that even a ligase that is indifferent to a 2′-OH versus a 2′-OCH₃ at the nick is inhibited by the extra bulk of a 2′-OCH₃.

It is worth emphasizing that the suppressive penultimate ribonucleotide effect on sealing by the LigC enzymes is a direct readout of their substrate specificity because LigC enzymes have no associated 3′-processing function. However, with their 3′-ribonuclease activities, LigD enzymes might conceivably provide an explanation for the reversal of the salutary effects of the bulky methyl group at the nick. A control experiment showing that the 2′-OCH₃ ribonucleotide at the nick suppressed sealing by Chlorella virus DNA ligase (Fig. 4F) indicated that even a ligase that is indifferent to a 2′-OH versus a 2′-OCH₃ at the nick is inhibited by the extra bulk of a 2′-OCH₃.

3, B–D). The penultimate ribonucleotide also inhibited the formation of the DNA-adenylate intermediate by AtuLigD2 and AtuLigC3 (Fig. 3, B and E). Third, a monoribonucleotide two bases away from the nick terminus was typically benign, i.e. the D9RD2 nicks were sealed with the same efficacy as the D12 nicks by AtuLigD1, AtuLigD2, AtuLigC3, and the PaeLigD LIG domain, although somewhat less effectively by AtuLigC2. The directly suppressive effects of the penultimate ribonucleotide provide an explanation for the reversal of the salutary effects of the 3′-terminal ribonucleotide on NHEJ ligases when two or more ribonucleotides are present on the 3′-OH side of the nick.

It was to exclude this possibility that we tested the isolated LIG domain of PaeLigD. The PaeLigD LIG domain has no 3′-processing activity, yet its sealing activity is inhibited by the penultimate monoribonucleotide (Fig. 3). Control experiments showed that Chlorella virus DNA ligase was not affected adversely by a penultimate ribonucleotide, i.e. the viral enzyme was equally adept at sealing the D11R1 and D10R1D nicks (data not shown). These findings point to a unique shared substrate specificity among Pseudomonas and Agrobacterium NHEJ ligases.
**Bacterial NHEJ Ligases**

**A**

![Graph of AtuLigC2 and AtuLigC3](Image)

**B**

![Graph of AtuLigD1 and AtuLigD2](Image)

**FIGURE 5. Ribonucleotide effects on the rate of ligation.** Reaction mixtures (120 μl) containing either 50 μm Tris·HCl (pH 7.5) (for AtuLigD1, AtuLigC2, PaeLigD, Chlorella virus ligase, and E. coli LigA) or Tris acetate (pH 6.0) (for AtuLigD2 and AtuLigC3); 5 mM DTT; 5 mM MnCl₂; either 0.25 mM ATP (for LigD, LigC, and Chlorella virus ligase enzymes) or 20 μM NAD⁺ (for E. coli LigA); 50 mM 32P-labeled nicked duplex substrate D12, D11R1, D10R2, or D9R3; and 300 nM AtuLigD1 or 250 nM AtuLigD2, AtuLigC3, PaeLigD, E. coli LigA, or Chlorella virus ligase (ChVLig). Aliquots (20 μl, containing 1 pmol of nicked substrate) were withdrawn at the times specified and quenched immediately with EDTA/formamide. A, the extents of strand sealing by AtuLigC2, AtuLigC3, and E. coli LigA (EcoLigA) are plotted as a function of time. B, the initial rates of sealing of each nicked substrate by the indicated ligases were determined from the kinetic profiles (i.e., the experiments in A and others not shown).

Additional ribonucleotides compared with the other NHEJ ligases (Fig. 5B). LigA and Chlorella virus ligase were unaffected by up to three ribonucleotides flanking the nick 3′-OH terminus (Fig. 5B).

**A Model DSB Repair Reaction of PaeLigD Depends on the POL Domain**—The stimulatory effect of a ribonucleotide on sealing of a nicked duplex suggests a rationale for the physical coupling of POL and LIG functions within the single multifunctional LigD protein, whereby POL would add a ribonucleotide to the 3′-OH terminus at a DSB and thereby enhance break sealing by the LIG component. This is in addition to the role imputed to the POL domain in promoting the physical interaction between LigD and Ku during bacterial NHEJ (4, 5, 18, 36). To explore whether POL influences LigD-mediated DSB joining in vitro in the absence of Ku, we recreated full-length PaeLigD, the isolated LIG domain, and the bifunctional PE-LIG and LIG-POL domains (14) with two model substrates comprising 35-bp duplex DNAs with either a single 3′-overhang deoxyribonucleotide (Fig. 6A) or a single 5′-overhang deoxyribonucleotide (Fig. 6B) at the DSB ends. The repair reaction mixtures contained 50 nM 32P-labeled DNA and 50 μM ATP. The salient finding was that whereas full-length PaeLigD and the LIG-POL fragment could join the DSB substrates to form a dimer-sized 32P-labeled product, neither the isolated LIG domain nor the PE-LIG fragment was able to do so (Fig. 6). This disparity is specific to the DSB repair reaction insofar as all four of the recombinant LigD derivatives display comparable activity in sealing at a nick (Ref. 14 and data not shown). Further analysis of the dimer-sized reaction product formed by PaeLigD with the 3′-monoribonucleotide overhang DSB substrate revealed that it was alkaline-sensitive, i.e., overnight treatment with 0.26 M NaOH at 37°C resulted in near quantitative decay of the dimer-sized species and the appearance of a radiolabeled monomer-sized oligonucleotide (Fig. 6C). These findings indicate that POL incorporates a ribonucleotide at the DSB repair junction prior to sealing by the LIG module.

**DISCUSSION**

Our demonstration that bacterial LigC and LigD are stimulated by a 3′-monoribonucleotide illuminates a new functional signature of bacterial NHEJ ligases that distinguishes them from all other polynucleotide ligases characterized to date. Previous studies of cellular and viral ATP-dependent DNA ligases underscored several shared themes of substrate recognition, including (i) a requirement for B-form helical conformation on the 5′-PO₄ side of the nick, as revealed by the inability of most DNA ligases to seal a 5′-PO₄ RNA strand, and (ii) lack of discrimination between nicks containing an all-DNA versus all-RNA strand at the nick 3′-OH terminus (23, 29–31). An entirely different set of specificity rules applies to T4 RNA ligase 2 (Rnl2), the prototype of a ligase family dedicated to sealing nicks in duplex RNAs (32–34). Rnl2 is indifferent to whether the 5′-PO₄ side of the nick is RNA or DNA, but Rnl2 is incapable of sealing an all-DNA 3′-OH strand. Rnl2 requires at least two ribonucleotides at the 3′-OH terminus, with all other positions of the substrate being amenable to substitution by DNA (24). The bacterial NHEJ ligases are special in that they require a
Bacterial NHEJ Ligases

The appreciation from crystal structures that DNA ligases force the 3′-OH strand into an RNA-like state neatly accounts for the biochemical findings that human LIG1, Chlorella virus ligase, and other ATP-dependent DNA ligases do not discriminate between DNA and RNA in the single 3′-OH ribonucleotide for optimal sealing activity, but are then inhibited by a second ribonucleotide at the penultimate position of the 3′-OH strand.

The magnitude of the stimulation of sealing by a 3′-monoribonucleotide, whether expressed as -fold increase in specific activity or initial rate for the D11R1 versus D12 substrate, is quite striking. The LigC paralogs in particular are activated by >2 orders of magnitude. Sealing was assayed in the presence of 0.25 mM ATP and 5 mM manganese, which are the empirically determined optima for all of the LigC and LigD enzymes studied. (Note that manganese is the requisite divalent cation cofactor for the POL and PE activities of the bacterial LigD enzymes.) The 3′-ribonucleotide effect on sealing by bacterial NHEJ ligases is not an aberration of using manganese as the metal cofactor. We observed similar differences in the rates of sealing of the D11R1 versus D12 substrate in the presence of 5 mM magnesium (data not shown).

Although a detailed understanding of the structural basis for the 3′-ribonucleotide effect on sealing by NHEJ ligases awaits their crystallization in complex with nucleic acid substrates, we can draw some inferences from the crystal structures of human LIG1 (27), Chlorella virus ligase (30), and E. coli LigA (35) bound to nicked duplexes with 5′-PO₄ or 5′-AppN ends. All DNA ligases have a core catalytic domain composed of two structural modules: (i) a nucleotidyltransferase domain that binds the adenylate moieties of ATP (or NAD⁺) and AppDNA and (ii) an OB domain that forms part of a C-shaped protein clamp around the DNA duplex. The requirement for B-form secondary structure on the 5′-PO₄ side of the nick is enforced (at least in part) by the OB domain, which binds across the DNA minor groove and measures its width on the 5′-PO₄ side. An important theme first revealed by the human LIG1-DNA co-crystal and verified by subsequent ligase-DNA structures is that the binding of ligase to the duplex induces a local distortion immediately flanking the nick, whereby the terminal base pair(s) on the 3′-OH side of the nick adopt an RNA-like A helical conformation. The distortion of the 3′-OH strand into an A-like conformation is assisted by insertion of amino acids of the nucleotidyltransferase and OB domain into the minor groove flanking the nick.

The appreciation from crystal structures that DNA ligases force the 3′-OH strand into an RNA-like state neatly accounts for the biochemical findings that human LIG1, Chlorella virus ligase, and other ATP-dependent DNA ligases do not discriminate between DNA and RNA in the...
Bacterial NHEJ Ligases

3′-OH strand. We can speculate that bacterial NHEJ ligases are unable to force the DNA 3′-OH terminus into an A-like conformation and are therefore dependent on a 3′-monoribonucleotide to facilitate productive nick sensing and/or catalysis. We attempted to test this model by assaying the binding of NHEJ ligases to the D11R1 versus D12 substrate via an electrophoretic mobility shift assay that had proved useful previously for studying nick recognition by viral DNA and RNA ligases (23, 24). The gel shift assay must be performed in the absence of a divalent cation, lest the stoichiometric amounts of ligase proceed to seal all of the nicked duplex ligand. Our finding that the NHEJ ligases failed to form a stable ligase-nucleic acid complex under these conditions with any of the ligands tested thwarted this line of study. (We found that E. coli LigA also does not form a stable complex with nicked DNA under these conditions.)

Alternatively, we gauged the initial rates of sealing using D12 substrate concentrations in excess of the 50 nM level present in the standard assay. The reasoning was that if raising the DNA concentration elicited a proportional increase in rate, then one could infer that binding is a limiting factor. However, if increasing the DNA concentration did not result in progressive rate increases, we could surmise that the initial binding was not rate-limiting and that some other event (chemistry or a pre-chemical conformational step) accounts for why the all-DNA nick is a feeble substrate for the NHEJ ligases. We measured the rates of sealing of the D12 nick at 50, 100, 250, and 500 nM during a 20-min reaction. In the case of AtuLigD2, AtuLigC2, and AtuLigC3, there was <50% variation in the initial rates over the 10-fold range of substrate concentrations, implying that substrate binding was not rate-determining. The rates of sealing by AtuLigD1 were within a factor of 2 over the same 10-fold range of DNA concentrations. Only in the case of PaeLigD was there a steady increase in rate with DNA concentration, from which we calculated a \( K_m \) of 260 nM (data not shown). We noted, however, that PaeLigD displayed a similar DNA concentration dependence for its rate of sealing of the D11R1 substrate (\( K_m = 250 \text{ nM} \)) (data not shown), signifying that substrate binding was probably not the key factor in the positive ribonucleotide effect on PaeLigD.

The unusual substrate preference of the bacterial NHEJ ligase clade makes sense in light of the ensemble of 3′-end-healing activities associated with LigD. The POL component preferentially adds a single non-templated ribonucleotide to a blunt-end DSB in vitro, and it preferentially fills in gaps at 5′-overhang ends with a short tract of ribonucleotides. The PE domain can trim the ribonucleotide tract to the point at which only a 3′-monoribonucleotide remains at the ligatable end. In effect, the POL and PE domains can conspire to provide the very 3′-end configuration that is favored for sealing by LigD and LigC. Because other ATP-dependent ligases and the bacterial NAD\(^+\)-dependent DNA ligase do not share the 3′-ribonucleotide preference of LigD and LigC, we surmise that the NHEJ ligase domains evolved their substrate specificity in tandem with the domain fusions that produced the polyfunctional LigD proteins, which are apparently unique to prokaryotic NHEJ systems.

In support of these ideas, we found that the ability of *Pseudomonas* LigD to join model DSB substrates in vitro was dependent on both POL and LIG domains. The LIG domain alone was not competent to do so. Potential contributions of the POL domain to DSB joining include promoting synopsis of the DSB ends (36, 37) and adding a ligation-enhancing ribonucleotide at the repair junction. Our finding here that the junction formed during *in vitro* repair of a 35-bp duplex with a single 3′-mononucleotide overhang is alkaline-labile is consistent with a pathway of ribonucleotide addition prior to sealing.

REFERENCES

1. Gong, C., Bongiorno, P., Martins, A., Stephanou, N. C., Zhu, H., Shuman, S., and Glickman, M. S. (2005) *Nat. Struct. Mol. Biol.* 12, 304–312
2. Aravind, L., and Koonin, E. V. (2001) *Genome Res.* 11, 1365–1374
3. Doherty, A. J., Jackson, S. P., and Weller, G. R. (2001) *FEBS Lett.* 500, 186–188
4. Weller, G. R., Kysela, B., Roy, R., Tonkin, L. M., Scanlan, E., Della, M., Devine, S. K., Day, J. P., Wilkinson, A., di Fagagna, F., Devine, K. M., Bowater, R. P., Jeggo, P. A., Jackson, S. P., and Doherty, A. J. (2002) *Science* 297, 1686–1689
5. Gong, C., Martins, A., Bongiorno, P., Glickman, M., and Shuman, S. (2004) *J. Biol. Chem.* 279, 20594–20606
6. Della, M., Palmbos, P. L., Tseng, H. M., Tonkin, L. M., Daley, J. M., Topper, L. M., Pitcher, R. S., Tomkinson, A. E., Wilson, T. E., and Doherty, A. J. (2004) *Science* 306, 683–685
7. Korycka-Machala, M., Brzostek, A., Rozalska, S., Rumijowska-Galewicz, A., Dziedzic, R., Bowater, R., and Dziadek, J. (2006) *FEBS Microbiol. Lett.* 258, 83–91
8. Akey, D., Martins, A., Aniukwu, J., Glickman, M. S., Shuman, S., and Berger, J. M. (2006) *J. Biol. Chem.* 281, 13412–13423
9. Zhu, H., Nandakumar, J., Aniukwu, J., Wang, L. K., Glickman, M. S., Lima, C. D., and Shuman, S. (2006) *Proc. Natl. Acad. Sci. U. S. A.* 103, 1711–1716
10. Wang, S. T., Setlow, B., Conlon, E. M., Lyon, J. L., Imamura, D., Sata, T., Setlow, P., Losick, R., and Eichenberger, P. (2006) *J. Biol. Chem.* 32, 16–37
11. Moeller, R., Stackerbrandt, E., Reitz, G., Berger, T., Rettgert, P., Doherty, A. J., Hornreck, G., and Nicholson, W. L. (2007) *J. Bacteriol.* 189, 3306–3311
12. Stephanou, N. C., Gao, F., Bongiorno, P., Erht, S., Schnappinger, D., Shuman, S., and Glickman, M. S. (2007) *J. Bacteriol.* 189, 5237–5246
13. Pitcher, R. S., Green, A. J., Brzostek, A., Korycka-Machala, M., Dziadek, J., and Doherty, A. J. (2007) *DNA Repair* 6, 1271–1276
14. Zhu, H., and Shuman, S. (2005) *J. Biol. Chem.* 280, 418–427
15. Zhu, H., and Shuman, S. (2005) *J. Biol. Chem.* 280, 25973–25981
16. Zhu, H., and Shuman, S. (2007) *Nucleic Acids Res.* 35, 3631–3645
17. Pitcher, R. S., Brissett, N. C., Picher, A. J., Andrade, P., Juarez, R., Thompson, D., Fox, G. C., Blanco, L., and Doherty, A. J. (2007) *J. Biol. Chem.* 36, 391–405
18. Pitcher, R. S., Tonkin, L. M., Green, A. J., and Doherty, A. J. (2005) *J. Biol. Chem.* 281, 531–544
19. Yakovleva, L., and Shuman, S. (2006) *J. Biol. Chem.* 281, 25026–25040
20. Zhu, H., and Shuman, S. (2005) *J. Biol. Chem.* 280, 33707–33715
21. Zhu, H., and Shuman, S. (2006) *J. Biol. Chem.* 281, 13873–13881
22. Zhu, H., and Shuman, S. (2005) *J. Biol. Chem.* 280, 12137–12144
23. Srisvand, V., and Shuman, S. (1998) *Nucleic Acids Res.* 26, 525–531
24. Nandakumar, J., and Shuman, S. (2004) *Mol. Cell* 16, 211–221
25. Lehman, I. R. (1974) *Science* 186, 790–797
26. Odell, M., Srisvand, V., Shuman, S., and Nikolov, D. (2000) *Mol. Cell* 11, 1183–1193
27. Nair, P. A., Nandakumar, J., Smith, P., Odell, M., Lima, C. D., and Shuman, S. (2007) *Nat. Struct. Mol. Biol.* 14, 770–778
28. Lubini, P., Zürcher, W., and Egli, M. (1994) *Chem. Biol.* 1, 39–45
29. Sekiguchi, J., and Shuman, S. (1997) *Biochemistry* 36, 9073–9079
30. Pascal, J. M., O’Brien, P. J., Tomkinson, A. E., and Ellenberger, T. (2004)
31. Ballard, D. R., and Bowater, R. P. (2006) Biochem. J. 398, 135–144
32. Ho, C. K., and Shuman, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 12709–12714
33. Nandakumar, J., Ho, C. K., Lima, C. D., and Shuman, S. (2004) J. Biol. Chem. 279, 31337–31347
34. Nandakumar, J., Shuman, S., and Lima, C. D. (2006) Cell 127, 71–84
35. Nandakumar, J., Nair, P. A., and Shuman, S. (2007) Mol. Cell 26, 257–271
36. Aniukwu, J., Glickman, M. S., and Shuman, S. (2008) Genes Dev. 22, 512–527
37. Brissett, N. C., Pitcher, R. S., Juarez, R., Picher, A. J., Green, A. J., Dafforn, T. R., Fox, G. C., Blanco, L., and Doherty, A. J. (2007) Science 318, 456–459