Identification of the Determinant Conferring Permissive Substrate Usage in the Telomere Resolvase, ResT*5•

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Linear genome stability requires specialized telomere replication and protection mechanisms. A common solution to this problem in non-eukaryotes is the formation of hairpin telomeres by telomere resolvases (also known as proteolomers). These enzymes perform a two-step transesterification reaction on replication intermediates to generate hairpin telomeres using an active site similar to that of tyrosine recombinases and type IB topoisomerases. Unlike phage telomere resolvases, the telomere resolvase from the Lyme disease pathogen Borrelia burgdorferi (ResT) is a permissive enzyme that resolves several types of telomere in vitro. However, the ResT region and residues mediating permissive substrate usage have not been identified. The relapsing fever Borrelia hermsii ResT exhibits a more restricted substrate usage pattern than B. burgdorferi ResT and cannot efficiently resolve a Type 2 telomere. In this study, we determined that all relapsing fever ResTs process Type 2 telomeres inefficiently. Using a library of chimeric and mutant B. hermsii/B. burgdorferi ResTs, we mapped the determinants in B. burgdorferi ResT conferring the ability to resolve multiple Type 2 telomeres. Type 2 telomere resolution was dependent on a single proline in the ResT catalytic region that was conserved in all Lyme disease but not relapsing fever ResTs and that is part of a 2-amino acid insertion absent from phage telomere resolvase sequences. The identification of a permissive substrate usage determinant explains the ability of B. burgdorferi ResT to process the 19 unique telomeres found in its segmented genome and will aid further studies on the structure and function of this essential enzyme.

Replication and protection of telomeric DNA are required to ensure the genomic stability of all organisms with linear replicons. Until quite recently, it was assumed that linearity is a property confined to the replicons of eukaryotes and certain primarily eukaryotic viruses. However, a growing body of evidence indicates that linear DNA is also found in a broad range of bacteriophages (1–6) and in bacteria themselves (7–10), including the Borrelia species that cause Lyme disease and relapsing fever (11, 12). A common solution to the end replication and protection problem in non-eukaryotes is the covalent sealing of DNA ends in the form of hairpins (2, 4–6, 10, 11, 13–16). Hairpin DNA is not recognized as a double-strand break, and continuous synthesis of DNA around the hairpin loop abolishes the end replication problem. However, mother and daughter replicons are covalently linked at the junction of their telomeres following DNA replication; separation of the two replicons and formation of new hairpin telomeres requires a DNA breakage and reunion process referred to as telomere resolution (17, 18).

Resolution of the linear chromosome and plasmids in Borrelia species and of the linear plasmid prophages from Escherichia coli, Yersinia enterocolitica, and Klebsiella oxytoca is performed by telomere resolvases (also referred to as protelomerases) (5, 19–21). A growing number of candidate telomere resolvases have been identified in the genomes of eukaryotic viruses, phages, and bacteria (22, 23). Telomere resolvases are DNA cleavage and rejoining enzymes related to tyrosine recombinases and type IB topoisomerases (19, 21, 22, 24, 25). Telomere resolvase catalyzes a two-step transesterification reaction in which staggered cuts are introduced 6 bp apart on either side of the axis of symmetry in the replicated telomere substrate (5, 19, 21, 24). Cleavage is accompanied by the formation of a 3′-phosphotyrosyl protein-DNA linkage. Subsequent nucleophilic attack on opposing strands by the free 5′-OH groups in the nicked substrate creates covalently closed hairpin telomeres. A recent crystal structure of the Klebsiella phage telomere resolvase (TelK) in complex with its substrate identified the residues involved in catalysis (25); all but one of these residues are conserved in all telomere resolvases (22), implying that the basic catalytic mechanism underlying telomere resolution is conserved. However, telomere resolvase sequences vary substantially outside of the central catalytic region (25, 26), and the enzymes characterized to date demonstrate important differences in substrate usage that likely reflect functionally distinct mechanisms of substrate interaction.

The Borrelia burgdorferi telomere resolvase, ResT, appears to be particularly divergent. It is substantially smaller than phage telomere resolvases, and unlike its phage counterparts (5, 20, 21), it cannot efficiently resolve negatively supercoiled DNA (19, 27), presumably reflecting differences in the substrates
resolved by phage and Borrelia telomere resolvases in vivo. On the other hand, B. burgdorferi ResT can fuse hairpin telomeres in a reversal of the resolution reaction (28), a function that is not shared with the phage telomere resolvase TelK (25). It can also synapse replicated telomeres and catalyze the formation of Holliday junctions (29). The ability of ResT to promote hairpin fusion has been proposed as the mechanism underlying the ongoing genetic rearrangements that are a prominent feature of the B. burgdorferi genome (18, 28). Finally, B. burgdorferi ResT can tolerate a surprising amount of variation in its substrate (30, 31), a feature that is not shared by phage telomere resolvases (21). Although B. burgdorferi ResT appears to be more permissive with a greater scope of activities than other telomere resolvases, the sequences mediating most of its unique properties have not yet been identified.

The B. burgdorferi genome contains a total of 19 distinct hairpin sequences, all of which must be resolved by ResT (31). These sequences can be classified into three groups based on the presence and positioning of the box 1 motif, which is a critical determinant of activity in phage and Borrelia telomere resolvases (see Fig. 1A) (21, 24, 30). A box 1-like motif is also found in many of the hairpin telomeres sequenced to date (6, 14, 32–35), although its function in telomere resolution is unknown. The box 1 consensus sequence (TAT(a/t)AT) closely resembles the −10/Pribnow box and TATA box consensus sequences of prokaryotic and eukaryotic promoters (TATAAT and TATAA(a/t)A(a/t), respectively), which undergo transient deformations that predispose them to melting (36) and are intrinsically bent and anisotropically flexible (37). Therefore, box 1 may facilitate nucleation of hairpin folding and/or may confer an intrinsic bend or flexibility to substrates that is important for the resolution reaction.

B. burgdorferi ResT can resolve telomeres in which box 1 is located at positions 1 and 4 nucleotides away from the axis of symmetry (Type 1 and 2 telomeres, respectively), as well as AT-rich telomeres without a box 1 sequence (Type 3 telomeres) (see Fig. 1A) (30, 31). B. burgdorferi ResT cleaves telomeres at a fixed position relative to the axis of symmetry, independent of the location of box 1 (30). Positioning of the enzyme for cleavage in all telomere types is most likely driven by sequence-specific interactions between ResT domains (catalytic and/or 3 (C-terminal) and a fixed element upstream of box 1 that is positioned 14 nucleotides from the axis of symmetry in all Borrelia telomeres (box 3 and adjacent nucleotides) (see Figs. 1A and 2) (26, 30, 31). In contrast, box 1 and axis-flanking nucleotides are not involved in high affinity and/or sequence-specific interactions with ResT and require the ResT N-terminal domain for full protection in DNase footprinting assays (26, 27). The most likely candidate for interactions with box 1 and axis-flanking nucleotides is a Borrelia-specific hairpin-binding region in the N terminus, which is thought to promote a pre-hairpinning step involving strand opening at the axis (38).

ResT from the relapsing fever Borrelia species Borrelia henssii exhibits a more restricted substrate usage pattern in vitro when compared with ResT from the Lyme disease pathogen B. burgdorferi (39). Specifically, B. henssii ResT is unable to efficiently resolve a Type 2 telomere. Therefore, B. burgdorferi ResT appears to be a more permissive enzyme than its relapsing fever counterpart. In this study, we investigated the basis for permissive substrate usage by B. burgdorferi ResT. Using a library of chimeric B. henssii/B. burgdorferi ResTs, we mapped the sequence determinants in B. burgdorferi ResT that confer the ability to resolve multiple Type 2 telomeres. Surprisingly, this approach indicated that Type 2 telomere resolution was crucially regulated by a single proline residue located in a small Borrelia-specific insertion in the central catalytic region of ResT. The proline at this position was conserved in the ResTs from all Lyme disease Borrelia species but in none of the ResTs from relapsing fever Borrelia species, which were unable to efficiently resolve Type 2 telomeres in vitro. This study has identified a specific residue in ResT responsible for permissive substrate usage patterns.

**EXPERIMENTAL PROCEDURES**

**Sequencing and Cloning of Borrelia anserina, Borrelia parkeri, Borrelia recurrentis, and Borrelia turicatae ResT Coding Sequences—**All relapsing fever and avian Borrelia strains were generously provided by Tom Schwan. ResT coding sequences were cloned from B. anserina strain BA.2 (GCB802), B. parkeri strain RML (GCB803), B. recurrentis strain number 132, P6 (GCB804), and B. turicatae strain 91E135 (GCB801). GenBank accession numbers for ResT coding sequences are as follows: B. anserina (FJ882620), B. parkeri (FJ882621), B. recurrentis (FJ882622), and B. turicatae (FJ882623). ResT coding sequences were amplified from the appropriate genomic DNAs using primers containing NdeI and BamH1 sites (described in supplemental Table S2). PCR conditions for the 50-μl reactions were as follows: 1× Phusion High Fidelity (HF) buffer (New England Biolabs, Pickering, Ontario, Canada), 3% DMSO3, 0.2 mM dNTPs, 0.02 units/μl Phusion DNA polymerase (New England Biolabs), 0.5 pmol/μl F primer, 0.5 pmol/μl R primer, 3 ng/μl genomic DNA template. PCR cycling conditions were as follows: 98 °C 45 s followed by 30 cycles of 98 °C, 10 s; 57 °C, 30 s; and 72 °C, 45 s and then 72 °C for 10 min. Appropriately sized PCR products were gel-purified using a Qiagen gel purification kit (Qiagen, Mississauga, Ontario, Canada) and then cloned using the ZeroBlunt®TOPO®PCR cloning kit (Invitrogen, Burlington, Ontario, Canada), all according to the manufacturer’s instructions. Inserts were cut out of TOPO clones using NdeI/BamH1 (New England Biolabs) and cloned into NdeI/BamH1-digested pET15b.

**Construction of ResT Chimeras—**Constructs encoding chimeric proteins were built using site-directed mutagenesis or overlap extension PCR. Site-directed mutagenesis was used to introduce amino acid substitutions or to introduce unique restriction sites that were subsequently used for swapping sequences between B. burgdorferi and relapsing fever ResTs. The methods, templates, and primers used to build each construct are described in supplemental Table S2, and primer sequences are provided in supplemental Table S3. All constructs were sequenced before expression in E. coli.

**Site-directed Mutagenesis—**PCR conditions for the 50-μl reactions were as follows: 1× Phusion High Fidelity (HF) buffer (New England Biolabs), 3% DMSO, 0.1 mM dNTPs, 0.02
units/μl Phusion DNA polymerase (New England Biolabs), 0.4 pmol/μl F primer, 0.4 pmol/μl R primer, 1.5 ng/μl plasmid DNA template. The first three PCR cycles were performed separately for each primer (e.g. one tube for F primer, one tube for R primer). After the third cycle the contents of the F and R primer tubes were mixed, and PCR cycling was continued to completion. PCR cycling conditions were as follows: 98 °C for 45 s followed by 25–30 cycles of 98 °C, 20 s; 65–68 °C, 15 s; 70–72 °C, 3 min and 30 s and then 72 °C for 7 min. PCR products were purified using the QIAQuick PCR purification kit, according to the manufacturer’s instructions (Qiagen), and then template DNA was digested with DpnI (New England Biolabs). Purified, digested DNA was used directly to transform chemically competent DH5α.

Overlap Extension PCR—A description of the three-step PCR reaction and primer design is provided in supplemental Fig. S3. In the first step (25 PCR cycles), primers A and B were used to amplify the first approximately one-half of the chimera, and primers C and D were used to amplify the second half (e.g. B. burgdorferi ResT), in separate 50-μl reactions. Primers A and D contained 5’-Ndel and -BamHI sites, respectively. Primers B and C were partially complementary to one another and contained sequences that annealed to both B. burgdorferi and B. hermsii ResT coding sequences (60 °C of sequence complementarity for each). In the second PCR step (six PCR cycles), 2.5 μl of each of the two PCR products from Step 1 were mixed together directly (5-μl final reaction volume) and extended by DNA polymerase already present in the PCR mixture to generate a small amount of chimeric product containing all 1,347 nucleotides of the ResT coding sequence. In Step 3 (25 PCR cycles), 2.5 μl of full-length chimeric Step 2 product were amplified in a 50-μl PCR reaction, using primers A and D. PCR reaction conditions were as follows: 1 × Phusion High Fidelity (HF) buffer (New England Biolabs), 3% DMSO, 0.1 mM dNTPs, 0.02 units/μl Phusion DNA polymerase (New England Biolabs), 0.1 pmol/μl F primer, 0.1 pmol/μl R primer, 1.5 ng/μl plasmid DNA template. PCR cycling conditions were as follows: 98 °C 3 min followed by 25 cycles of 98 °C, 15 s; 63 °C, 15 s; 72 °C, 30 s and then 72 °C for 7 min. Following PCR, products were gel-purified from agarose gels using the QIAQuick gel extraction kit (Qiagen). Cleaned products were cloned into pJET1/blunt using the GeneJET PCR cloning kit, according to the manufacturer’s instructions (MBI Fermentas, Burlington, Ontario, Canada). Ndel/BamHI-flanked ResT coding sequences were cloned into Ndel/BamHI-digested pET15b.

ResT Expression and Purification—ResT expression and purification were performed as described previously (38) with the following exceptions. Bacteria were collected by centrifugation at 6,000 × g for 15 min at 4 °C. Pellets were resuspended in an EDTA-free buffer (25 mM Hepes-NaOH, pH 7.6). Resuspended cells were subjected to three freeze-thaw cycles followed by lysozyme treatment followed by ultracentrifugation for 45 min at 100,000 × g for 4 °C. Columns for His tag affinity purification were prepared using 1 ml of nickel-nitrilotriacetic acid slurry (Qiagen). All purification buffers contained 0.5 M NaCl. Fractions were diluted to 50 ng/μl in elution buffer, and dithiothreitol was added to a final concentration of 1 mM.

Requirements for Permissive Substrate Usage by ResT

ResT Reaction Conditions—Previously described telomere substrates (30, 31) were prepared from Qiagen midipreps (Qiagen), linearized with PstI (New England Biolabs), and cleaned by phenol extraction/ethanol precipitation. Twenty-μl telomere resolution reactions contained 25 mM Tris-HCl (pH 8.5), 100 mM NaCl, 1 μM EDTA, 100 μg/ml bovine serum albumin, 5 mM spermidine, 10 ng/μl PstI-linearized substrate DNA, and 10 ng/μl ResT. Time course reactions (140 μl) were incubated at 30 °C with 20-μl aliquots removed at 0, 1, 2, 4, 8, and 16 min. Reactions were performed in duplicate or triplicate and stopped by the addition of SDS to a final concentration of 0.2%. Samples were resolved on 20-cm 1% agarose gels in 1× Tris-acetate-EDTA (TAE) buffer at 100 V for 2 h. The gels were stained with ethidium bromide, and fluorescence of DNA bands was quantified using the Alphalnnotech software. The percentage of telomere resolution was determined by dividing the fluorescence of the reaction products by the total fluorescence (products plus substrate). Initial velocity values were calculated from the graphical plots of reaction kinetics. Statistical analyses of data were performed using Microsoft Excel and a two-tailed Student’s t test with unequal variance.

Identification and Alignment of Known and Putative Telomere Resolvase Sequences; Protein Structure Prediction and Threading—The methods used to identify and align telomere resolvase sequences are described in the legends for Fig. 2 and supplemental Fig. S2. The method used to thread ResT sequences onto the TelK structure is described in the legend for Fig. 4.

RESULTS

The Inability to Process Type 2 Telomeres Is a Feature of All Relapsing Fever Telomere Resolvases—Our previous work showed that B. burgdorferi (Lyme disease) and B. hermsii (relapsing fever) ResTs exhibit differences in substrate usage in vitro (39). Specifically, B. hermsii ResT resolves a Type 2 telomere very inefficiently when compared with B. burgdorferi, whereas both enzymes can resolve a Type 1 telomere efficiently (Fig. 1, A and B). The basis for this species-specific difference in substrate usage is unknown.

To determine whether ResTs from other relapsing fever Borrelia exhibit similar Type 2 telomere resolution defects, we cloned the ResT coding sequences from three other relapsing fever strains, B. parkeri, B. recurrentis, and B. turicatae, as well as the avian Borrelia species B. anserina. These sequences are shown in Fig. 2, together with the ResT sequences from other Lyme disease (Borrelia afzelii, Borrelia spielmanii, Borrelia garinii, and B. burgdorferi) and relapsing fever (B. hermsii and Borrelia duttonii) Borrelia species (39–42). In all Borrelia ResTs, positions corresponding to the active site residues in tyrosine recombinases, type 1B topoisomerases and the Klebsiella phage telomere resolvase, TelK (also known as a proteolomerase) were conserved (22, 25) (Fig. 1). Supplemental Fig. S1 shows the alignment of B. burgdorferi ResT with TelK, and supplemental Fig. S2 shows the alignment of 29 known and putative telomere resolvase family members, including all of the Borrelia ResTs (supplemental Fig. S2).
Requirements for Permissive Substrate Usage by ResT

FIGURE 1. Species-specific resolution of Type 1 and 2 telomeres. A, a schematic showing the three types of hairpin telomere found on the linear replicons of the B. burgdorferi genome (see Ref. 31). The box 1 sequence in Type 1 and 2 telomeres is situated 1 and 4 nucleotides away from the axis of symmetry, respectively. The Type 3 telomere contains no clear box 1. B, a schematic illustrating the telomere resolution reaction substrate and products is shown along with two ethidium bromide-stained agarose gels showing telomere resolution assays. The gels show resolution kinetics for B. burgdorferi and B. hermsii ResT on Type 1 and 2 telomeres (plasmid substrates pYT1/lp17L and pYT92/chromL, respectively).

The ResTs from B. anserina, B. burgdorferi, B. hermsii, B. parkeri, B. recurrentis, and B. turicatae were expressed as recombinant proteins, purified, and tested for their ability to resolve Type 1 and 2 telomeres. All ResTs could resolve Type 1 telomeres, but all relapsing fever and avian ResTs exhibited profound defects in Type 2 telomere resolution when compared with B. burgdorferi (<11% of the initial velocity values for B. burgdorferi control). The inability to efficiently resolve Type 2 telomeres is a property common to all relapsing fever ResTs.

Mapping of the ResT Region Required for Resolution of a Type 2 Telomere—To map the ResT region(s) involved in utilization of a Type 2 telomere substrate, we used overlap extension PCR (supplemental Fig. S3) to assemble a library of 36 chimeric ResTs in which relapsing fever B. hermsii or B. recurrentis sequences were systematically replaced by B. burgdorferi (Lyme disease) sequences (see supplemental Table S1 for a list chimeric constructs, protein purification yields, and telomere resolution characteristics). B. hermsii and B. recurrentis ResTs were chosen for these experiments because they were as active as B. burgdorferi ResT in Type 1 telomere resolution. Of 36 chimeric proteins, approximately half (19) were stably expressed. Twelve of the stably expressed proteins resolved Type 1 telomeres as efficiently as B. burgdorferi ResT (>70% initial velocity values for B. burgdorferi ResT), indicating that these ResTs were stable and functional enzymes. In general, chimeric proteins generated using B. hermsii and B. burgdorferi ResT sequences were more stable and functional in Type 1 telomere resolution than those built from B. recurrentis and B. burgdorferi sequences. Therefore, B. hermsii/B. burgdorferi chimeric ResTs were used for most subsequent mapping experiments.
Mapping of the ResT region(s) required for resolution of a Type 2 telomere was performed by measuring the initial velocities of B. burgdorferi, B. hermsii, and chimeric ResTs on Type 1 and 2 substrates. Fig. 3 shows a schematic of the ResT hybrid proteins used and the ratio of the initial velocities relative to the B. burgdorferi Type 2/Type 1 velocity ratio (the raw data can be found in supplemental Fig. S4). This approach indicated that N-terminal B. burgdorferi sequences up to residue 321 were not required for Type 2 telomere resolution as chimeras containing B. burgdorferi sequences in the C-terminal portion of the protein displayed wild-type ratios or better through h321. However, further removal of the B. burgdorferi residues from 322 to 340 (h340b) resulted in a dramatic loss in the ability to process a Type 2 telomere. Similar results were obtained for B. recurrentis/B. burgdorferi chimeras when these could be successfully generated (supplemental Table S1).

Finally, the ability of B. hermsii ResT to process a Type 2 telomere was fully restored (Fig. 3B) by substitution of B. hermsii residues 309–340 with the corresponding B. burgdorferi sequences (h309b340h). These findings, therefore, identified sequences 309–340 as the crucial determinant in B. burgdorferi ResT that imparts the ability to process a Type 2 telomere. This region contains the active site residues His-324 and the tyrosine nucleophile Tyr-335 (Figs. 2 and 4).

Identification of Proline 326 in ResT as the Crucial Determinant for Resolution of a Type 2 Telomere—The sequences required for Type 2 telomere processing fell within the central catalytic domain of ResT (residues 164–352). This was the only ResT region that showed any significant homology to the sequences of other telomere resolvase family members (supplemental Fig. S2). To explore possible structural differences in B. burgdorferi and B. hermsii ResTs that could account for the difference in substrate usage, we first threaded the B. burgdorferi catalytic domain sequences (163–352) onto the three-dimensional crystal structure of TelK in complex with a telomere substrate (25) (overall root mean square value: 0.64 Å, see supplemental Fig. S5 and supplemental Video S1). B. hermsii ResT catalytic domain sequences were then threaded onto the B. burgdorferi ResT model (Fig. 4 and supplemental Video S2).
Proline 326 Is Necessary for Efficient Processing of All Type 2 Telomeres—We recently sequenced and cloned a total of 19 unique hairpin telomeres found in the *B. burgdorferi* genome; eight of these are Type 2, whereas four are Type 1 and seven are Type 3 (31). To characterize the substrate preferences of *B. hermsii* ResT in greater detail, we compared the resolution activities of *B. hermsii* and *B. burgdorferi* ResTs using this sizeable library of natural telomere variants (Fig. 6). The initial velocities of *B. hermsii* and *B. burgdorferi* ResTs were similar on Type 1 and 3 telomeres. In contrast, *B. hermsii* ResT was impaired in the ability to resolve all Type 2 telomeres, with levels of inhibition ranging from 29% (lp36R) to 98% (chromL) of *B. burgdorferi* ResT activity levels (Fig. 6C). Thus, *B. hermsii* ResT exhibited specific defects in the resolution of all Type 2 telomeres.

Within the region required for Type 2 telomere resolution (residues 309–340), *B. burgdorferi* and *B. hermsii* sequences differed at a total of six positions (Fig. 2). Of these 6 amino acids, only 2 (positions 326 and 327) were predicted to lie close enough to interact with the DNA substrate, specifically with the first 2 nucleotides flanking the axis of symmetry in the replicated telomere substrate (<4.0 Å). Interestingly, the side chains of residues Pro-326 and His-326 in *B. burgdorferi* and *B. hermsii* ResT, respectively, were predicted to exhibit substantially different orientations with respect to neighboring residues and the DNA substrate (Fig. 4 and supplemental Video S2). These observations suggested that sequence differences at positions 326 and 327 in *B. burgdorferi* and *B. hermsii* ResTs might be involved in the differential abilities of the two enzymes to process Type 2 telomeres. To test this hypothesis, we substituted His-326 and Asp-327 in *B. hermsii* ResT with the corresponding residues from *B. burgdorferi* ResT (proline and asparagine, respectively). As shown in Fig. 5, the H326P substitution (hH326P) fully rescued Type 2 telomere resolution. Additional substitution of Asp-327 in *B. hermsii* ResT (hH326P, D327N) reduced the level of rescue, indicating that introduction of a proline at position 326 was sufficient to restore activity on a Type 2 telomere.

One possible explanation for the Type 2 resolution defect in *B. hermsii* ResT is that the charged, basic histidine residue at position 326 impedes the movement of DNA substrate in the open space near the axis of symmetry (Fig. 4 and supplemental Fig. S5). Thus, replacing the histidine at position 326 with a proline could restore Type 2 resolution simply by removing a source of steric hindrance. To address this possibility, we substituted an alanine at position 326 in *B. hermsii* ResT. The hH326A variant exhibited wild-type levels of Type 1 telomere resolution (Fig. 5A) but showed no detectable activity on a Type 2 telomere (Fig. 5B). Hence, the defect in Type 2 resolution by *B. hermsii* ResT is apparently not the result of steric hindrance by His-326 but appears to result from a specific requirement for proline at this position. This is supported by the fact that *B. parkeri* ResT contains a glutamine at position 326 but is also unable to resolve Type 2 telomeres (Fig. 2 and supplemental Table S1). Therefore, we concluded that Type 2 telomere resolution is dependent on properties specifically conferred by the proline at position 326.

**DISCUSSION**

In the study described here, we used chimeric ResT proteins to map the region of the enzyme that confers the ability of ResT from Lyme disease but not relapsing fever spirochetes to resolve Type 2 telomeres. Using site-directed mutagenesis, we subsequently identified a single, differentially conserved amino acid in Lyme disease ResTs that is required for efficient resolution of Type 2 telomeres. This residue is part of a small, 2-amino acid insertion in the ResT catalytic domain that is not found in most other telomere resolvases from other organisms (supplemental Fig. S2). Our work has uncovered an important deter-
minant of differential substrate usage in *B. burgdorferi* and relapsing fever ResTs and will provide insight into the permis- 
vivo substrate usage patterns of *Borrelia* telomere resolvases.

How Does ResT Pro-326 Promote Telomere Resolution?—At the outset of this study, we expected that the *B. burgdorferi* ResT sequences mediating resolution of Type 2 telomeres...
would be found in the N-terminal domain because this region is required for protection of box 1 and axis-flanking nucleotides in footprinting assays (26). We were therefore surprised to find that Type 2 telomere resolution was dependent on a single proline residue in the catalytic domain. This amino acid was predicted to be located within 4 Å of the first 2 nucleotides flanking the axis of symmetry in the replicated DNA substrate by threading of ResT on the TelK co-crystal structure. Proline residues typically interact with DNA through van der Waals interactions, which occur at a distance of 3.6 Å or less (43). At this distance, Pro-326 was predicted to interact with only the first nucleotide at the axis of symmetry, precluding interactions with box 1 sequences in both Type 1 and Type 2 telomeres. How, then, could this residue promote Type 2 telomere resolution?

One possible explanation is that because of its proximity to the axis of symmetry, Pro-326 plays a generalized role in substrate usage, in a manner that is not dependent on direct contact with box 1. This hypothesis is supported by several observations. First, the Pro-326 substitution in B. hermsii ResT not only promoted Type 2 telomere resolution but also significantly augmented Type 1 telomere resolution when compared with both the wild-type B. hermsii and the wild-type B. burgdorferi ResTs (Fig. 5A). Second, replacing the Pro-326 residue in B. burgdorferi ResT with the histidine found in relapsing fever ResTs resulted in a >5-fold reduction in Type 1 activity (supplemental Table S1: hP326H). Third, B. hermsii H326P ResT was actually more efficient than B. burgdorferi ResT in resolving certain Type 2 telomeres (Fig. 7: Ip28-2L). Finally, we have also found that B. hermsii H326P ResT is more efficient than either B. burgdorferi or B. hermsii ResT in resolving negatively supercoiled substrates and in catalyzing telomere fusion, independent of telomere sequence and box 1 spacing (data not shown). These observations argue for a generalized role for Pro-326 in the resolution and fusion of diverse telomere substrates.

Proline and histidine generally interact with DNA using one of three interaction modes: 1) base-independent interactions in which the plane of the amino acid ring faces the DNA, maximizing contact surface area (43); 2) ring-stacking interactions with deformed DNA that destabilize base pairing (e.g. TATA box-binding proteins) (44); and 3) proline can also intercalate between adjacent base steps, resulting in kinking (e.g. integration host factor, HU, and Hbb proteins) (45–47). Our in silico modeling of the B. burgdorferi ResT catalytic domain suggested that as per interaction mode 1, the plane of the Pro-326 ring flanks the substrate in a base-independent type of interaction that limits the movement of axis-flanking nucleotides with respect to the active site and may, therefore, influence DNA bending (Fig. 4 and supplemental Video S2). However, the possibility that Pro-326 engages in ring-stacking interactions (interaction mode 2) that destabilize base pairing at the axis (25, 38) is equally appealing from a functional point of view. The possibility of proline intercalation (interaction mode 3) cannot be ruled out but would require a substantial conformational change to allow insertion between adjacent base pairs. Finally, it is possible that proline 326 exerts its effect upon the reaction without contacting the DNA substrate, by instead imposing a conformational change upon the enzyme relative to the histidine-containing ResT variants. Evaluation of these hypotheses will require a co-crystal structure of the ResT-DNA complex, which will also provide insight into the role of Borrelia-specific N- and C-terminal ResT sequences in substrate interaction.

**FIGURE 7. Effect of proline at position 326 in B. hermsii ResT on resolution of several naturally occurring Type 2 telomeres.** The average initial velocities of B. burgdorferi (b), B. hermsii (h), and B. hermsii H326P (hH326P) ResTs on Type 2 telomeres chromL, Ip28-3R, and Ip28-2L are shown. Initial velocities are expressed as a percentage of the B. burgdorferi initial velocity on these substrates. Error bars represent the standard error for two time courses.
telomere resolvasomes from many other organisms. The importance of the Pro-326 residue for permissive substrate usage raises several intriguing questions. First, if this amino acid confers relaxed substrate specificity, why is it not present at the equivalent position in relapsing fever ResTs, and is it associated with a selective advantage or disadvantage with respect to genome stability and rearrangement in B. burgdorferi? Second, are the telomeres of relapsing fever Borrelia species similar to those of B. burgdorferi, and if so, are additional cellular factors required for resolution of Type 2 telomeres in vivo (39)? The capacity of B. burgdorferi ResT for permissive, multifunctional substrate usage is a fascinating phenomenon that distinguishes this enzyme from other known members of the telomere resolvase family.

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