The Pif1 family in prokaryotes: what are our helicases doing in your bacteria?

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ABSTRACT Pif1 family helicases, which are found in nearly all eukaryotes, have important roles in both nuclear and mitochondrial genome maintenance. Recently, the increasing availability of genome sequences has revealed that Pif1 helicases are also widely found in diverse prokaryotes, but it is currently unknown what physiological function(s) prokaryotic Pif1 helicases might perform. This Perspective aims to briefly introduce the reader to the well-studied eukaryotic Pif1 family helicases and speculate on what roles such enzymes may play in bacteria. On the basis of our hypotheses, we predict that Pif1 family helicases are important for resolving common issues that arise during DNA replication, recombination, and repair rather than functioning in a eukaryotic-specific manner.

INTRODUCTION TO THE EUKARYOTIC PIF1 FAMILY HELICASES

The prototypical member of the Pif1 family of helicases is the Saccharomyces cerevisiae Pif1 (ScPif1) protein, which was initially isolated in a genetic screen for mutations affecting mitochondrial DNA (mtDNA) recombination (reviewed in Bochman et al., 2010). It was later discovered that ScPif1 is a superfamily Ib helicase with a variety of cellular roles. Aside from affecting mtDNA maintenance, ScPif1 also uses its helicase activity to perform several nuclear functions, including telomerase inhibition (Zhou et al., 2000; Boule et al., 2005), Okazaki fragment processing (Pike et al., 2010), and resolving G-quadruplex (G4) structures (Ribeyre et al., 2009; Paeschke et al., 2011).

Additionally, S. cerevisiae encodes a second Pif1 family helicase known as S. cerevisiae Rrm3 (ScRrm3). Like ScPif1, ScRrm3 has roles in maintaining the stability of both the mitochondrial and nuclear genomes (Bochman et al., 2010). ScRrm3 is a component of the replisome (Azvolinsky et al., 2006), where it is thought to act as an accessory helicase, facilitating timely DNA replication by disrupting stable, nonnucleosomal protein–DNA complexes that would otherwise inhibit replication fork progression (Ivessa et al., 2003; Azvolinsky et al., 2009). Two-dimensional gel electrophoresis of replication intermediates demonstrates that the absence of ScRrm3 results in paused and broken DNA replication forks (Ivessa et al., 2000, 2003). Mutations in RRM3 also result in local increases in recombination at regions containing sites of ScRrm3 action, such as the rDNA (Keil and McWilliams, 1993) and a tRNA-rich stretch of chromosome VII (Ivessa et al., 2003).

Whereas multiple fungi also encode two Pif1 family helicases, other organisms contain variable numbers of homologues, from one in fission yeast and humans to 23 in the fungal arthropod pathogen Meteorhizium anisopliae ARSEF 23. Most multicellular organisms, however, encode only a single Pif1 helicase. In many instances, little is known about these enzymes other than that they share essentially equal sequence similarity to ScPif1 and ScRrm3 and, where examined, seem to be found in both nuclei and mitochondria (Bochman et al., 2010).

PROKARYOTIC PIF1 HELICASES

Searches of the National Center for Biotechnology Information (NCBI) Protein database reveal that many prokaryotic genomes also encode Pif1-like helicases. Due to sequence similarity with RecD (ScPif1 and ScRrm3 are 18.9 and 16.4% identical, respectively, to Escherichia coli RecD), however, many of the prokaryotic Pif1s are misannotated as RecDs (or ambiguously annotated using a variety of other terms). The situation in bacteria is further complicated by the existence of multiple RecD (Montague et al., 2009) and/or Pif1 homologues in some species (see Figure 1 and Table 1 for examples). Pif1s and RecDs are all superfamily Ib helicases that share seven conserved motifs (I, Ia, II, III, IV, V, and VI) common to SFI enzymes, as well as three additional motifs (A, B, and C) found only in Pif1 and RecD family helicases (see Figure 2). Only Pif1-like helicases,
Why do prokaryotes need Pif1 family helicases?

We hypothesize that Pif1 family helicases are important for resolving common issues that arise during DNA metabolism rather than performing eukaryotic-specific functions. Thus, based on what we know about eukaryotic Pif1 helicases, prokaryotic Pif1s may serve multiple functions, including maintaining prokaryotic telomeres (in bacteria with long linear chromosomes), resolving DNA and DNA–RNA secondary structures, and complementing the lack of other helicases in vivo. Each of these possibilities is discussed separately.

Prokaryotic telomere maintenance. Whereas Escherichia coli and Bacillus have circular chromosomes, many bacteria have linear chromosomes, plasmids, and/or phages that replicate without a circular intermediate (reviewed in Stewart et al., 2004, and Casjens and Huang, 2008). Well-known examples of such organisms include Borrelia burgdorferi (the causative agent of Lyme disease), many Streptomyces species (sources of various antibiotics), and the φ29 Bacillus subtilis bacteriophage. To solve the end replication problem, at least two different types of prokaryotic telomeres have evolved: 1) those with ends protected by a protein that is covalently linked to the terminal nucleotide (e.g., Streptomyces spp. and φ29), and 2) those with covalently closed terminal hairpins (e.g., Borrelia spp.). In eukaryotes, various Pif1 family helicases are known or hypothesized to associate with telomeres. For instance, ScPif1 is a catalytic inhibitor of telomerase, ScRrm3 is important for replication through telomeric DNA, and mammalian Pif1 helicases interact with telomerase in vivo (Bochman et al., 2010). Although the structures and sequences of prokaryotic telomeres are unrelated to the well-known eukaryotic telomerase-generated telomeres, Pif1 family helicases may nevertheless be involved in prokaryotic telomere maintenance.

The ends of linear chromosomes and plasmids are inherently recombining (Stewart et al., 2004; Casjens and Huang, 2008). In some instances, such as generating antigen variability in Borrelia spp., this recombination is beneficial, and bacterial Pif1 helicases that resolve recombination intermediates may promote such processes. Indeed, ScPif1 promotes recombination in the linear mtDNA of S. cerevisiae (Fouy and Dyck, 1985). Alternatively, prokaryotic Pif1 helicases may possess a nucleoprotein disruption activity similar to that hypothesized for ScRrm3 (Ivesa et al., 2003; Azvolinsky et al., 2009) that is used to displace DNA end-binding proteins during the replication of linear chromosomes and plasmids. Such an activity could be important for the complete replication of DNA ends.

More broadly, it is widely accepted that the genomic DNA in eukaryotic organelles, such as mitochondria, has a prokaryotic origin. It was long overlooked and often disputed, however, that some eukaryotic mtDNA molecules, including S. cerevisiae mtDNA, are linear (Nosek et al., 1998), perhaps reflecting an origin from a prokaryote species with a linear genome. Because Pif1 family helicases are important for mtDNA maintenance (Bochman et al., 2010), it is tempting to speculate that the first eukaryotic Pif1 helicase may have had a mitochondrial (and hence prokaryotic) origin, with nuclear isoforms evolving later. Indeed, there are several examples of mitochondrial proteins with putative prokaryotic origins (e.g., mitochondrial ribosomal proteins; Henze and Martin, 2001) that are encoded in the nuclear genome.

FIGURE 1: Phylogenetic tree revealing the relationships between selected Pif1 and RecD helicases. Pif1 (solid boxes) and RecD (dashed boxes) protein sequences were aligned using CustalX (v. 2.0.12) by iterating each step of the alignment. The same program was used to both draw and bootstrap a neighbor-joining tree. TreeView (v. 1.6.6) was used to visualize the phylogenetic tree and root it with the outgroup (human β-actin, unpublished data). The scale bar indicates the number of substitutions per site. Red boxes indicate prokaryotes, blue boxes denote eukaryotes, the green box indicates a bacteriophage, and the purple boxes designate viruses. Ps, Psychrobacter sp. PRWF-1; Mp, Muclaginibacter paludis DSM 18603; Bb, Borrelia burgdorferi N40; Ec, E. coli; Sa, Staphylococcus aureus; Da, Desulfbacterium autotrophicum HRM2; Ba, Bacteroides sp. 2.1_16; rV5, E. coli phage rV5; IIV3, Invertebrate Iridescent Virus-3; Hs, Homo sapiens; Bl, Bifidobacterium longum subsp. longum F8; Sc, S. cerevisiae; V99B1, Emiliania huxleyi virus 99B1; and Sp, Schizosaccharomyces pombe. Protein sequences are available upon request. However, contain the Pif1 family signature sequence (DKLxXVRKxRCKxKPFGGGQ), a degenerate sequence located between motifs II and III, the function of which is currently unknown (Bochman et al., 2010). Aside from CLUSTAL alignments of protein sequences to determine phylogenies, one way to differentiate between Pif1 and RecD helicases is with the signature sequence. Using the sequence above as a Basic Local Alignment Search Tool (BLAST) query of the NCBI database returns only Pif1 homologues, although the more distantly related a Pif1 family helicase is from the canonical ScPif1, the more degenerate the signature sequence becomes (compare BaPif1 and BbPif1 in Figure 2). Careful analysis using CLUSTAL alignments, however, reveals that prokaryotic Pif1 enzymes are generally more closely related to eukaryotic Pif1 helicases than they are to any of the various RecD subgroups (Figure 1).
Thus G4 sequences are likely to have roles in diverse cellular functions, such as telomere maintenance (Paeschke et al., 2008) and transcriptional regulation (Rawal et al., 2006; Huppert et al., 2008). Moreover, a recent analysis of 18 prokaryotic genomes revealed that the association of G4 motifs with transcriptional regulatory regions is not limited to eukaryotes, as evidenced by conserved G4 motifs in the promoters of gene orthologues in distantly related bacteria (Rawal et al., 2006). Although helicases such as E. coli RecQ are also known to unwind G4 structures (Wu and Maizels, 2001), not all bacterial genomes encode RecQ homologues (Table 1). It is therefore plausible that prokaryotes that lack RecQ helicases may instead encode Pif1 helicases to resolve G4 structures.

Previously, it was shown that ScPif1 preferentially unwinds DNA–RNA hybrids relative to DNA–DNA substrates (Boule and Zakian, 2007), much like the E. coli UvrD helicase (Matson, 1989). It is hypothesized that ScPif1 inhibits telomerase activity by unwinding the DNA–RNA hybrid formed between telomeric DNA and the telomerase RNA template (Boule et al., 2005). Although DNA/RNA secondary structure resolution. Eukaryotic Pif1 helicases have recently been implicated in the processing of G4 structures (Ribeyre et al., 2009; Sanders, 2010; Paeschke et al., 2011). A G4 structure is a four-stranded RNA or DNA secondary structure that is held together by Hoogsteen G-G base pairing. Human Pif1 and ScPif1 both bind and efficiently unwind G4 structures in vitro (Ribeyre et al., 2009; Sanders, 2010). It is known that guanine-rich sequences that have the potential to form G4 structures are also found at transcriptional regulatory regions and are enriched in telomeric DNA and rDNA, and G4 structures are also found at transcriptional regulatory regions and preferred meiotic double-stranded break sites (Neidle and et al., 2006). It is therefore intriguing that Pif1 family helicases are found throughout the Bifidobacteriales (a large order of Actinobacteria) and the phylum Bacteroidetes (which includes human gut commensals), the genomes of which are particularly GC-rich.

Due to sequence divergence from their better-characterized homologues, it was difficult to definitively determine whether Pif1 and RecD, as well as Rep and UvrD, family helicases in some organisms. Thus, some of the proteins listed in these categories may represent intermediate forms. Therefore, it was difficult to definitively determine the difference between Pif1 and RecD, as well as Pif1 helicases to resolve G4 structures. Further, as might be expected, bacteria with GC-rich genomes are predicted to contain more G4-forming sequences than are organisms with lower GC content (Rawal et al., 2006; Capra et al., 2006). It is therefore intriguing that Pif1 family helicases are known to unwind G4 structures (Wu and Maizels, 2001), not all bacterial genomes encode RecQ homologues (Table 1). It is therefore plausible that prokaryotes that lack RecQ helicases may instead encode Pif1 helicases to resolve G4 structures. Furthermore, as might be expected, bacteria with GC-rich genomes are predicted to contain more G4-forming sequences than are organisms with lower GC content (Rawal et al., 2006; Capra et al., 2006). It is therefore intriguing that Pif1 family helicases are found throughout the Bifidobacteriales (a large order of Actinobacteria) and the phylum Bacteroidetes (which includes human gut commensals), the genomes of which are particularly GC-rich. Previously, it was shown that ScPif1 preferentially unwinds DNA–RNA hybrids relative to DNA–DNA substrates (Boule and Zakian, 2007), much like the E. coli UvrD helicase (Matson, 1989). It is hypothesized that ScPif1 inhibits telomerase activity by unwinding the DNA–RNA hybrid formed between telomeric DNA and the telomerase RNA template (Boule et al., 2005). Although

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| Organism | Phylum | Pif1 | RecD | DinG | RecQ | Rep | UvrD | PcrA |
|----------|--------|------|------|------|------|-----|------|------|
| *Mucilaginibacter paludis* DSM 18603 | Bacteroidetes | 4 | 0 | 0 | 3 | 1 | 1 | 0 |
| *Psychrobacter sp. PRwf-1* | γ-proteobacteria | 1 | 0 | 0 | 0 | 2 | 2 | 1 |
| *Rhodobacterium vanniellii* ATCC 17100 | α-proteobacteria | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Sulfurovum* sp. NBC37–1 | ε-proteobacteria | 1 | 0 | 0 | 0 | 1 | 2 | 0 |
| *Desulfbacterium autotrophicum* HRM2 | δ-proteobacteria | 1 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Gardnerella vaginalis* AMD | Actinobacteria | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Deferribacter desulfuricans* SSM1 | Deferribacteres | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Anaerobaculum hydrogeniformans* ATCC BAA-1850 | Synergistetes | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| *Thermodesulfovibrio yellowstonii* DSM 11347 | Nitrospirae | 1 | 0 | 0 | 0 | 0 | 0 | 2 |
| *Chlororheterpton thalassium* ATCC 35110 | Chlorobi | 1 | 0 | 0 | 0 | 1 | 0 | 1 |
| *Borrelia burgdorferi* N40 | Spirochaetes | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Escherichia coli* | γ-proteobacteria | 0 | 1 | 1 | 1 | 1 | 1 | 0 |
| *Staphylococcus aureus* | Firmicutes | 0 | 0 | 0 | 0 | 1 | 1 | 1 |

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TABLE 1: Repertoire of helicases in evolutionarily diverse prokaryotes as revealed by BLAST searches and ClustalX alignments.
the prokaryotic telomeres just discussed are not replicated by a conventional telomerase-based mechanism, perhaps Pif1 helicases function in bacteria to resolve DNA–RNA hybrids (i.e., R-loops) that form during transcription. Because R-loop accumulation leads to genomic instability in organisms from E. coli to mammals, resolution of R-loops is important for maintaining genome integrity (Li and Manley, 2006).

**Complementing the lack of other helicases.** Most genomes encode multiple distinct helicases that have specialized functions in DNA and RNA metabolism. For instance, there are 134 open reading frames in the *S. cerevisiae* genome that encode proteins containing helicase structural motifs (Shiratori et al., 1999). The *E. coli* genome also encodes a variety of predicted helicases, with more than dozen such enzymes verified by direct biochemical assays. A universal collection of helicase types that is required for viability, however, has not been identified in bacteria or eukaryotes, likely due to functional redundancy in vivo. Thus, although *E. coli* encodes one member each of the DinG, RecD, RecQ, Rep, and UvrD helicase families (Table 1), other bacteria contain multiple homologues of these helicases, are devoid of them altogether, and/or possess other types of helicases (e.g., PcrA and Pif1) that may complement the lack of one or more of these enzymes. In *E. coli*, DinG is a 5′-3′ helicase with a putative role in homologous recombination (HR), RecD is the 5′-3′ member of the RecBCD complex (which is also involved in HR), RecQ (3′-5′) is involved in HR and double-stranded break repair, Rep is a 3′-5′ accessory replicative helicase needed for timely DNA replication, and UvrD is a 3′-5′ repair helicase that can remove RecA from single-stranded DNA (see Wu and Maizels, 2001; Montague et al., 2009; Boubakri et al., 2010; and references therein).

Given their sequence similarity to RecD enzymes, one might predict that Pif1 family helicases are found in the subset of bacterial species that lack a RecD homologue. Indeed, this situation is true in species such as *Rhodococcus rubrum* vannielii and *Gardnerella vagina-lis*, which lack RecD. Other bacterial species, however, encode one or more homologues of both Pif1 and RecD (e.g., *Psychrobacter* sp. PRwf-1 and *Desulfobacterium autotrophicum*; Table 1). Additionally, across the diverse prokaryotic phyla (Ciccarelli et al., 2006), many prokaryotes that encode Pif1 family helicases appear to lack both RecQ and DinG helicase homologues. As stated earlier in text, Pif1 helicases may function in place of RecQs to resolve G4 DNA and/or to remove R-loops, as DinG does in *E. coli* (Boubakri et al., 2010).

Additionally, DinG acts in concert with either Rep or UvrD in *E. coli* to remove RNA polymerase from the path of replication forks when replication and transcription complexes collide (Boubakri et al., 2010). This activity is reminiscent of the nucleoprotein disruption activity of ScRrm3 (Ivesa et al., 2003), and the increased replication fork pausing seen at inverted ribosomal operons in *E. coli* dinG rep mutants is similar to the fork pausing observed at rDNA repeats in *rnm3A* *S. cerevisiae* (Ivesa et al., 2000). Therefore, because eukaryotic Pif1 helicases have functions similar to those observed for the *E. coli* DinG, UvrD, and Rep helicases, prokaryotic Pif1 helicases may complement the lack of one or more of these enzymes in bacteria that do not encode DinG, UvrD, and/or Rep homologues.

**Outlook**

The possibilities presented here are by no means comprehensive, and none of these hypotheses has been tested. Additionally, if prokaryotic Pif1-like helicases were acquired by horizontal gene transfer, they may not be expressed or active in vivo. Transcriptome data from *Bdellovibrio bacteriovorus* indicate, however, that BbPif1 mRNA is expressed (Lambert et al., 2010), and biochemical results from a number of purified prokaryotic Pif1 helicases demonstrate that these enzymes are active in vitro (Bochman, Judge, and Zakian, unpublished). In any event, more bioinformatic analyses are needed to determine exactly how widespread Pif1 family helicases are among prokaryotes. Furthermore, molecular-genetic examination of nontraditional model organisms that encode Pif1-like helicases (e.g., *Bifidobacteria* and *Bacteroides* spp.) is required to begin examining these enzymes in vivo. To the best of our knowledge, no in vivo functional research has been performed on a prokaryotic Pif1 family helicase, but we hope that this article will generate interest in experimental tests of the roles of Pif1 helicases in bacterial cells.

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