ABSTRACT

_Bacillus subtilis_ gene _yshC_ encodes a family X DNA polymerase (PolX<sub>BS</sub>), whose biochemical features suggest that it plays a role during DNA repair processes. Here, we show that, in addition to the polymerization activity, PolX<sub>BS</sub> possesses an intrinsic 3′-5′ exonuclease activity specialized in resecting unannealed 3′-termini in a gapped DNA substrate. Biochemical analysis of a PolX<sub>BS</sub> deletion mutant lacking the C-terminal polymerase histidinol phosphatase (PHP) domain, present in most of the bacterial/archaeal PolXs, as well as of this separately expressed protein region, allow us to state that the 3′-5′ exonuclease activity of PolX<sub>BS</sub> resides in its PHP domain. Furthermore, site-directed mutagenesis of PolX<sub>BS</sub> His339 and His341 residues, evolutionary conserved in the PHP superfamily members, demonstrated that the predicted metal binding site is directly involved in catalysis of the exonucleolytic reaction. The implications of the unannealed 3′-termini resection by the 3′-5′ exonuclease activity of PolX<sub>BS</sub> in the DNA repair context are discussed.

INTRODUCTION

Maintenance of genome stability largely relies on the replicative DNA polymerases that accomplish faithful DNA synthesis by virtue of their high nucleotide insertion discrimination, as well as the ability, displayed by most of them, to proofread their own misinsertion errors (1–4). However, the continuous damage of the genomes by genotoxic agents (5) has rendered necessary the emergence of genome surveillance mechanisms to prevent the deleterious effects that the permanence of such damages could cause in the replication and transcription processes.

Base excision repair (BER) is the major pathway involved in the removal of damaged bases, conserved from bacteria to eukaryotes (6–9). BER enzymes remove the damaged base-containing nucleotide, producing as a final step a single nucleotide gap that is filled by a DNA polymerase and sealed by a DNA ligase. Of the various types of DNA damage, the DNA double-strand breaks (DSB) are the most dangerous. These lesions frequently induce various sorts of chromosomal aberrations that can provoke carcinogenesis and cellular death (5). Two pathways deal with this kind of lesions: the error-free homologous recombination, by which an intact double-stranded copy is used as a template to properly restore the broken ends by DNA synthesis; and the error-prone non-homologous end joining (NHEJ), by which the DNA ends are held together and processed to render single-stranded DNA (ssDNA) portions that are annealed with a limited base pair homology, the resulting gap being further filled by a DNA polymerase (10). As in the case of BER, NHEJ is also present in both eukaryotes and bacteria (11–13).

Members of the family X of DNA polymerases (hereafter PolX) have been involved in the DNA synthesis step that occurs during BER (14–17) and NHEJ (18–22). PolXs have been identified in bacteria, archaea, protozoa and viruses. They share several distinctive features, as their relatively small size, they are monomorphic and catalyse, in a relatively inaccurate manner, the insertion of few nucleotides, and no proofreading activity has been identified in any of them (23). In spite of the low sequence conservation among these enzymes, they show a common structural folding that enables them to play a primary role in filling the gapped DNA intermediates arisen during BER and NHEJ. PolXs structure consists of a common Polβ-like core (24) whose N-terminal 8-kDa domain recognizes the downstream 5′-phosphate group of a gap, allowing the correct positioning of the enzyme on the gapped or nicked structure (25–27). In some members, this domain also contains a 5′-deoxyribose 5′-phosphate (dRP) lyase activity, involved in the release of the 5′-dRP moiety...
during short patch BER (14,17), as in mammalian Polβ and Polδ. (15,16), and yeast Pol4 (28) and Trf4 (29). The 3’ primer terminus of the upstream strand is simultaneously bound and further elongated by the C-terminal polymerization domain of the Polβ-like core. This domain exhibits the general semi-opened hand folding comprised of the fingers, palm and thumb subdomains, present in most of the DNA-dependent DNA polymerases (24).

In addition to the above mentioned minimal core, mammalian Polβ, Polδ, terminal deoxyribonucleotide transferase (TdT) and yeast Pol4 have a BRCA1 C-terminal domain, involved in protein–protein interactions with other factors required during NHEJ and V(D)J recombination (18,20–23,30).

Family X members have been also identified in Bacteria and Archaea (19), Deinococcus radiodurans PolX being involved in the repair of the DSB.

The bacterium Bacillus subtilis gene yshC codes for a 570 amino acids family X member (31), B.B., J.M.L., L.V., M.S. and M.V., submitted for publication) (hereafter PolXBs). Its 317 N-terminal amino acids contain all the critical residues involved in DNA and nucleotide binding, as well as those responsible for catalysis of DNA polymerization. PolXBs shares the basic enzymatic features of the DNA polymerase family X members: reliance on the divalent metal ions Mg2+ or Mn2+, strict dependence on the presence of a template strand to direct DNA synthesis; a distributive polymerization pattern when acting on primer/template DNA structures; preference in the use of gapped DNA structures harbouring a downstream 5’-P group to perform efficient DNA synthesis, with a primary role in filling gaps of one to few nucleotides; it inserts preferentially the complementary dNMP, with a primary role in filling gaps of one to few nucleotides; it inserts preferentially the complementary dNMP, with a primary role in filling gaps of one to few nucleotides (19).

As in many bacterial/archaeal members, the PolXBs C-terminal part (residues 333–570) contains a putative PHP domain (24,32) whose biological role remains to be elucidated in family X DNA polymerases (32).

In this article, we show that PolXBs is endowed with an intrinsic 3’–5’ exonuclease activity that depends on highly conserved residues located at its PHP domain, PolXBs being the first reported PolX able to process unannealed 3’-termini. In addition, the catalytic involvement of highly conserved residues of the PHP family is demonstrated.

**MATERIALS AND METHODS**

**Nucleotides and proteins**

Unlabelled nucleotides were purchased from Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA [γ-32P]dATP (3000 Ci/mmole) and [α-32P]dATP (3000 Ci/mmole) were obtained from Amersham International Plc, Amersham, Bucks, UK. Restriction endonucleases were from New England Biolabs, Ipswich, MA, England. T4 polynucleotide kinase and T4 DNA ligase were purchased from New England Biolabs. Wild-type PolXBs was expressed and purified from Escherichia coli BL21(DE) strain harbouring plasmid pET28-PolXBs (B.B., J.M.L., L.V., M.S. and M.V., submitted for publication). The purified PolXBs was further loaded onto a 5-m glycerol gradient (15–30%) containing 50 mM Tris–HCl, pH 7.5, 20 mM ammonium sulphate, 180 mM NaCl, 1 mM EDTA and 7 mM β-mercaptoethanol, and centrifuged at 62 000 r.p.m. (Beckman SW65 rotor) for 26 h at 4°C. After centrifugation, 20 fractions were collected from the bottom of the tube for further analysis.

Oligonucleotides, DNA templates and substrates

Oligonucleotides PolXsense (5’-CCCACTGTAACAT ATGCAAAAAAGATATTAC), PHPsense (5’-CC CGATGAAACATATGACCTACGCCGAC), PolXcore (5’-CCCGGATCTTATGCCTGTAGG) and PolXantisense (5’-CCCGCGATCTTATCGTGCACG TTC) were used for polymerase chain reaction amplification of B. subtilis gene yshC to make the PolXBs ΔPHP deletion mutant and to clone separately the PolXBs PHP domain. Oligonucleotides SPI (5’-GATCAGCTGAGTAC), SP1p (5’-GATCACAGTGAGTAG) and SP1 + 3 (5’-GATCAGCTGAGTACCGGG) differing in the 3-terminal bases were used as primer strands. Oligonucleotide SP1c + 18 (5’-CTCGGCCTGCCTTCTATGGTACTCACTGTGAC) that has a 5’-terminal extension of 18 nt in addition to the sequence complementary to SP1, was used as template strand. The 5’-phosphorylated oligonucleotide Dws(P) (5’-ACTGCGCCGTCGTT), complementary to the last 13 5’-nucleotides of SP1c + 18, was used as downstream oligonucleotide to construct gapped structures of 5 nt. All the oligonucleotides described above were obtained from Isogen Life Technologies, Inc., Veldzigt 2a, The Netherlands, Oligonucleotides SPI, SP1p and SP1 + 3 were 5’-labelled with [γ-32P]ATP and T4 polynucleotide kinase. Additionally, oligonucleotide SPI1c + 18 (5’-ACTGCGCCGTCGTT) was also 3’-labelled with [α-32P]dATP and terminal deoxynucleotidyl transferase (TdT). All labelled oligonucleotides were further purified by electrophoresis on 8 M urea–20% polyacrylamide gels. To analyse the DNA-dependent polymerization activity of the protein on different DNA gapped structures, the labelled primers and the downstream Dws(P) oligonucleotide were hybridized to the template SP1c + 18 in the presence of 0.2 M NaCl and 60 mM Tris–HCl, pH 7.5.

Cloning, expression and purification of the PolXBs ΔPHP deletion mutant and the PolXBs PHP domain

Two truncated forms of PolXBs were also expressed and purified: one lacking the amino acid residues 315–570 (mutant ΔPHP) and other lacking amino acid residues 1–315 (PHP domain). For this, the corresponding DNA fragments from genomic B. subtilis DNA were PCR amplified with the following primers: PolXsense and PolXcore (to construct mutant ΔPHP), and PHP sense and PolXantisense (for the PHP domain, PHPdom). Both, PolXsense and PHP sense contain the NdeI restriction site, whereas PolXcore and PolX antisense include a BamHI site. After digesting the amplified DNA with the corresponding restriction enzymes, it was cloned into the pET28a (+) bacterial expression vector from Novagen, which carries an N-terminal His-Tag/thrombin configuration to express recombinant proteins as fusions with an N-terminal hexahistidyl for purification.
on Ni\(^{2+}\)-affinity resins, allowing further releasing of such a His-Tag by digesting with thrombin. The resulting recombinant plasmids pET28-ΔPHP and pET28- PHPdom were further transformed into *E. coli* BL21 (DE3) strain. Expression and purification steps of the PolX\(_{Bs}\) 3′-ΔPHP deletion mutant and of the PHP domain were carried out essentially as described (B.B., J.M.L., L.V., M.S. and M.V., submitted for publication).

### Site-directed mutagenesis of PolX\(_{Bs}\)

PolX\(_{Bs}\) double mutant H339A/H341A was obtained by using the QuickChange site-directed mutagenesis kit provided by Amersham Pharmacia. Plasmid pET28-PolX\(_{Bs}\) containing the PolX\(_{Bs}\) gene was used as template for the mutagenesis reaction. Expression and purification of the mutant proteins were performed as described for the wild-type PolX\(_{Bs}\) (B.B., J.M.L., L.V., M.S. and M.V., submitted for publication).

### 3′–5′ exonuclease assays on 5′-labelled DNA substrates

The incubation mixture contained, in 12.5 μl, 50 mM Tris–HCl, pH 7.5, either 1 mM MnCl\(_2\) or 8 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), 4% glycerol, 0.1 mg/ml BSA and either different fractions (2 μl) from a glycerol gradient containing PolX\(_{Bs}\) or the indicated concentration of the wild-type or mutant PolX\(_{Bs}\). As substrate, either 1.5 nM of 5′-labelled SP1 (ssDNA) or 5-nt gapped structures obtained by hybridization of the indicated SP1, SP1p or Spl + 3 and Dws(P) to the template oligonucleotide SP1c + 18, were used. Samples were incubated at 30°C for the indicated times and quenched by adding 10 mM EDTA. Reactions were analysed by 8 M urea–20% PAGE and autoradiography.

### Exonuclease activity on 3′-labelled ssDNA

The 3′-labelling was performed by incubating SP1 oligonucleotide in the presence of \([\alpha\, ^{32}P]dATP\) and TdT. The exonuclease reaction on this substrate was carried out in 12.5 μl, in the presence of 50 mM Tris–HCl, pH 7.5, 1 mM MnCl\(_2\), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 1.5 nM of 3′-labelled SP1 and 125 nM of either wild-type or mutant PolX\(_{Bs}\). Samples were incubated at 30°C for the indicated time and quenched by adding 10 mM EDTA. Reactions were analysed by 8 M urea–20% PAGE and autoradiography. To analyse the dAMP released by the 3′–5′ exonuclease activity, 1 μl of each sample was withdrawn immediately after incubation and analysed by thin layer chromatography (Polygram Cel 300 PEI/UV254) and further autoradiography. The chromatogram was developed with 0.15 M lithium formate (pH 3.0), conditions in which the 5′-dAMP migrates, whereas the DNA substrate remains at the origin, as described (33).

### In situ gel analysis of exonuclease activity

The assay was carried out essentially as described (34). Bacteriophage φ29 DNA was digested with HindIII and the ends were filled-in with \([\alpha\, ^{32}P]dATP\) using the exonuclease-deficient φ29 DNA polymerase [D12A/D66A; (35)]. The unincorporated \([\alpha\, ^{32}P]dATP\) was removed by filtration through Sephadex G-50 spin columns (in the presence of 0.1% SDS). This DNA was denaturalized by boiling and immediately added to the 12% polyacrylamide gel solution. The samples were electrophoresed in these gels followed by *in situ* renaturation of the proteins. Prior to renaturation, the gel was washed twice with 50 mM Tris–HCl, pH 7.5, for 15 min at 4°C. Renaturation was allowed to occur during 14 h at 4°C in buffer A containing 50 mM Tris–HCl, pH 7.5, 1 mM MnCl\(_2\), 40 mM KCl, 16% glycerol, 0.01 mM EDTA, 1 mM DTT and 400 μg/ml BSA. *In situ* exonucleolysis occurred during renaturation; therefore, after drying the gel, the exonuclease activity could be observed as a halo on the exposed black autoradiography film.

### In situ gel analysis of DNA polymerase activity of mutant ΔPHP

The assay was carried out essentially as described (34). The samples containing 500 ng of the ΔPHP mutant were electrophoresed in 12% SDS–PAGE gels containing 1.5 mg/ml activated calf thymus DNA (Pharmacia Biotech Inc., Piscataway, NJ, USA) as template/primer, followed by *in situ* renaturation of proteins and incubation of the gel in a DNA polymerase assay mixture. Prior to renaturation, the gel was washed twice with 50 mM Tris–HCl, pH 7.5, for 15 min at 4°C. Renaturation was allowed to occur during 3 h at 4°C in buffer A. In *in situ* polymerization, the assay was assayed with buffer B (buffer A plus 2 mM each dNTP and 1.2 nM \([\alpha\, ^{32}P]dATP\)) for 12 h at 30°C. After washing from the gel unincorporated \([\alpha\, ^{32}P]dATP\), and *in situ* precipitation of the DNA with a buffer containing 5% trichloroacetic acid and 1% sodium pyrophosphate, the gel was dried and the activity bands (radioactively labelled) were detected by autoradiography.

### DNA polymerization assays on gapped DNA molecules

The incubation mixture contained, in 12.5 μl, 50 mM Tris–HCl, pH 7.5, 1 mM MnCl\(_2\), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 1.5 nM of the hybrid indicated in each case, and the specified concentration of PolX\(_{Bs}\) and dNTPs. When indicated, T4 DNA ligase (0.5 U) was added to the reaction mixture to seal the repaired DNA gap. After incubation for the indicated times at 30°C, reactions were stopped by adding 10 mM EDTA and analysed by 8 M urea–20% PAGE and autoradiography.

### In vitro misincorporation assay

The incubation mixture contained, in 12.5 μl, 50 mM Tris–HCl, pH 7.5, 1 mM MnCl\(_2\), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, the indicated concentrations of dATP (complementary to template positions 1, 2 and 4) as the only nucleotide, 250 nM of either wild-type or mutant H339A/H341A DNA polymerases, and 1.5 nM of the indicated 5′-labelled gapped DNA. After incubation for 10 min at 30°C, the reactions were stopped by adding EDTA up to 10 mM. Samples were analysed by 8 M urea–20% PAGE and autoradiography. Misinsertion of dAMP at non-complementary positions is observed as the appearance of extension products that are longer than the correct 17-mer extension product.
RESULTS

PolX<sub>Bs</sub> contains a 3′–5′ exonuclease activity

Recently, Blasius et al. (36) have shown the presence of an endogenous Mn<sup>2+</sup>-dependent structure-modulated exonuclease activity in the PolP-like core of the DNA polymerase X from the bacterium <i>D. radiodurans</i>. To determine whether PolX<sub>Bs</sub> is endowed with an exonuclease activity, the purified PolX<sub>Bs</sub> was sedimentated through a glycerol gradient, and the collected fractions were incubated with a 5′-labelled ssDNA in the presence of 1 mM MnCl<sub>2</sub> (see Materials and methods section). A nuclease activity peak yielding degradation products shorter than the unit length (15-mer), co-sedimented with the mass peak of the purified PolX<sub>Bs</sub> (Figure 1A). Fractions 11–14 from the glycerol gradient were pooled for further in vitro analysis of PolX<sub>Bs</sub>. As observed in Figure 1B, PolX<sub>Bs</sub> did not render any degradation product in the presence of Mg<sup>2+</sup> ions, indicating that the nucleolytic activity present in PolX<sub>Bs</sub> is Mn<sup>2+</sup>-dependent.

To further characterize the type of the nucleolytic activity of PolX<sub>Bs</sub>, the enzyme was incubated with a [α-<sup>32</sup>P]dAMP 3′-labelled ssDNA substrate in the presence of 1 mM MnCl<sub>2</sub> (see Materials and methods section; Figure 1C). Under these conditions, it could be observed the appearance of 1-nt product, the absence of intermediate degradation products and a progressive decrease of the amount of radioactive substrate with time. Altogether, the above results indicate that PolX<sub>Bs</sub> nucleolytic activity acts unidirectionally at the 3′-ends. The φ29 DNA polymerase was used as control of such unidirectional degradation, as it possesses a well-characterized 3′–5′ exonuclease activity [(33,37); Figure 1C]. In addition, the radioactive 1-nt product released by PolX<sub>Bs</sub> was characterized as dAMP by thin layer chromatography (see Materials and methods section and Figure 1D). Thus, nucleolytic activity displayed by PolX<sub>Bs</sub> meets the criteria established to categorize a nucleolytic activity as a 3′–5′ exonuclease.

The 3′–5′ exonuclease activity of PolX<sub>Bs</sub> resides in its PHP domain

As mentioned above, many of the putative bacterial and archaeal PolX possess a C-terminal PHP domain...
predicted to be involved in metal-dependent catalysis of phosphoester bond hydrolysis (32). To ascertain the involvement of the PHP domain in the 3′−5′ exonuclease activity exhibited by PolX_Bs, both, a deletion mutant lacking the C-terminal amino acid residues 316−570 (mutant ΔPHP), and the 29 kDa C-terminal PHP domain (PHPdom, residues 315−570) of PolX_Bs were expressed, purified (see Materials and methods section) and further underwent to in situ nuclease activity assays. Renaturation and analysis of the activity were performed in the presence of 1 mM MnCl2, as described in Materials and methods section. As shown in Figure 2A, the wild-type PolX_Bs gave rise to an expected activity band coincident with its electrophoretic mobility (64 kDa), ruling out any contamination by nuclease, particularly the endogenous E. coli ε subunit [25 kDa; (38)], described to bind the PHP scaffold of the α subunit of the E. coli replicase (39), allowing us to assign unambiguously this activity to PolX_Bs. In contrast, the in situ nuclease assay in Figure 2A shows that the absence of the PHP domain in the purified mutant ΔPHP precluded the appearance of a detectable nucleolytic activity. Additionally, the polymerization band observed in an in situ polymerization assay (see Materials and methods section), coincident with the expected 35-kDa electrophoretic mobility of the purified PolX_Bs truncated product (Figure 2B), rules out a general misfolding of this mutant under the renaturation conditions used, allowing us to conclude the absence of a 3′−5′ exonuclease activity in the Polβ-like core (ΔPHP) of PolX_Bs. MALDI-TOF analysis of the proteins migrating faster than the mutant ΔPHP PolX_Bs indicated that they correspond to C-terminal truncated forms of

Figure 2. The 3′−5′ exonuclease activity of PolX_Bs resides in the C-terminal PHP domain. (A) In situ analysis of 3′−5′ exonuclease activity of wild-type and ΔPHP mutant PolX_Bs. Left panel, Coomassie blue staining after SDS–PAGE separation of control non-induced (-Ind.) and IPTG-induced extracts of E. coli BL21(DE3) cells transformed with the recombinant plasmids pET28-PolX_Bs or pET28-ΔPHP (+IPTG). The electrophoretic analysis of the highly purified fractions obtained after Ni-NTA chromatography (Ni2+) and further digested with thrombin (+ Thr) is also shown. Arrows show the expected position for the wild-type (64 kDa) and deletion mutant (35 kDa) PolX_Bs. Right panel shows the autoradiography of the in situ gel analysis of the 3′−5′ exonuclease activity, carried out as described under ‘Materials and methods’ section, after the SDS–PAGE separation shown in the left panel. The 3′−5′ exonuclease activity band corresponding to the electrophoretic migration of the wild-type PolX_Bs is indicated. (B) In situ polymerization activity of purified PolX_Bs ΔPHP mutant. The assay was performed as described in Materials and methods section. Left panel, Coomassie blue staining after SDS–PAGE separation of the purified fraction obtained after Ni-NTA chromatography (Ni2+) and further digested with thrombin (+ Thr). Arrows show the position for the PolX_Bs ΔPHP mutant. Right panel, autoradiography of the in situ polymerization analysis carried out as described under Materials and methods section, after the SDS–PAGE separation (using an activated DNA-containing gel) shown in the left panel. The DNA polymerase activity bands corresponding to the electrophoretic migration of the PolX_Bs ΔPHP mutant are indicated. (C) In situ exonuclease activity of purified PolX_Bs PHP domain. The assay was performed essentially as described in (A), by transforming E. coli BL21(DE3) cells with the recombinant plasmids pET28-PHPdom.
the mutant polymerase (data not shown). Conversely, when purified PHPdom was subjected to 3′–5′ exonuclease in situ analysis, an activity band at the expected position of 29 kDa was detected (Figure 2C). The coincidence of the activity bands with the different electrophoretic mobility of the purified PHPdom before (Ni2+ lane) and after (Thr lane) removal of the fused N-terminal His-Tag serves as an additional control to unequivocally assign the 3′–5′ exonuclease activity of PolX Bs to its PHP domain.

Site-directed mutagenesis in the PHP domain of PolX Bs: selective inactivation of the 3′–5′ exonuclease activity

Aravind and Koonin (32) predicted the presence of a catalytic site in the PHP domains that consists of four motifs containing conserved histidine residues. Figure 3A shows an alignment of the C-terminal PHP domain of bacterial/archaeal family X DNA polymerases with the E. coli YcdX protein, an isolated 27-kDa molecular weight archaeal family X DNA polymerases with the E. coli DH4 strain. An alignment of the C-terminal PHP domain of bacterial/archaeal family X DNA polymerases (32), and that shows a 26% of sequence identity with the PolX Bs PHP sequence from Leu338 to Glu531. YcdX has an unusual topology of a β/β-ββ-barrel whose C-terminal side has a deep cleft that contains three metal binding sites ligated to the imidazole group of residues His7, His9 and His15 from motif I; His40 from motif II; His101 from motif III, His131 and His194, the latter belonging to motif IV; as well as to the carboxylate group of Glu73, Glu156 and Asp192, the latter belonging also to Motif IV [marked with asterisks in Figure 3A; (40)].

Previous alignments of the PHP domains, along with that shown in Figure 3A has permitted the identification of PolX Bs residues His339 and His341 (motif I), His371 (motif II), Asp410, His437 (motif III), His465, Asp496, and Asp526 and His528 (motif IV), highly conserved in bacterial/archaeal family X DNA polymerases (32), as the homologs to 9 out of the 10 YcdX residues mentioned above [coloured in blue (histidines) and red (glutamates and aspartate) in Figure 3A]. Thus, it can be inferred that these residues would form also a catalytic site involved in the coordination of metal ions.

In addition, the protein structure homology modelling server Swiss-Model (41–43) has provided a structural model for the PolX Bs PHP domain (Figure 3B), obtained by using as the best template the crystallographic structure of E. coli YcdX protein [E-value 1.89e-5; PDB 1B Ip0, (40)]. The model suggests that PolX Bs PHP domain would show a β/β/ββ-barrel folding pattern with seven β-strands surrounded by six α-helices. The absence of sequence homology between YcdX and the last 34 residues of PolX Bs PHP precluded the modelling of the folding of this C-terminal segment of the PHP domain. However, secondary structure predictions suggest that it is folded also as an α-helix (44–46), leading us to propose a topology of a β/ββ-barrel for the PHP domain of PolX Bs.

To ascertain the functional importance of the putative PHP active site residues described above in supporting the catalysis of the 3′–5′ exonuclease activity of PolX Bs, a mutant containing the double change to Ala of motif I residues His339 and His341 (H339A/H341A) was obtained by site-directed mutagenesis, overproduced and purified as described in Materials and methods section. This double change drastically abolished the 3′–5′ exonuclease activity present in the wild-type PolX Bs, paralleling the behaviour displayed by mutant ΔPHP (Figure 4). The presence of polymerization activity in mutant H339A/H341A (see subsequently) excluded a general misfolding as responsible for the absence of exonuclease activity.

These results indicate that residues His339 and His341 of the PolX Bs PHP domain are critical for the exonuclease activity and could be part of a catalytic core, proposed here to be a conserved 3′–5′ exonuclease active site in bacterial/archaeal family X DNA polymerases.

The 3′–5′ exonuclease of PolX Bs edits unpaired 3′-termini

As most of the reported family X DNA polymerases, previous studies have shown the gapped DNAs as the optimal substrates for PolX Bs (B.B., J.M.L., L.V., M.S. and M.V., submitted for publication). To determine the DNA substrate specificity displayed by the 3′–5′ exonuclease activity of PolX Bs, time-course experiments on 5-nt gapped DNA structures bearing either a matched (C-G) or mismatched (G-G) 3′-terminus (see scheme in Figure 5) were carried out. As shown in Figure 5, although the enzyme could excise both termini, the 3′–5′ exonuclease of PolX Bs showed a clear preference for the mispaired substrate. These results indicate that a mismatched terminus favours the initiation of degradation by the 3′–5′ exonuclease activity of PolX Bs. According to the preference for gapped DNA substrates shown by PolXs, exonuclease activity of PolX Bs on overhanged primer/template dsDNA substrates was much lower than that displayed on the gapped structures (data not shown).

Interestingly, the 3′–5′ exonuclease activity of PolX Bs was much more active with a substrate harbouring a 3′-flap, as most of the mispaired nucleotides were specifically removed at the shortest reaction time, the degradation rate slowing down drastically once reached the paired dsDNA region (Figure 5). In fact, degradation of the ssDNA portion of this flapped structure was much more efficient than that of the ssDNA molecule (compare Figures 5 and 6). These results allow us to suggest that the 3′–5′ exonuclease activity of PolX Bs is well suited to remove unannealed DNA tails arisen in gapped DNA structures.

Figure 6 shows the ability of both, the wild-type and the exonuclease deficient H339A/H341A mutant PolX Bs to fill-in the 5-nt gap when the primer strand harbours a matched 3′-terminus (C-G). As expected, in the presence of the four dNTPs the pair C-G was extended by both the wild-type and, in a lower extent, the mutant polymerases to complete gap filling (20-mer product in Figure 6A). In this assay, a partial strand-displacement took place as a 21-mer band was also detected. On the gapped substrate with a mismatched 3′-terminus (G-G, see scheme in Figure 6B), and in the presence of dATP (the first complementary nucleotide that should be added if a direct elongation of the mispaired 3′-terminus could take place), PolX Bs was exclusively able to remove the G-G
Figure 3. (A) Alignment of the C-terminal PHP domain of bacterial family X DNA polymerases. Numbers between slashes indicate the amino acid position relative to the N-terminus of each DNA polymerase. PHP domains are aligned among them and with respect to E. coli YcdX protein. Because of the large number of sequences, only selected representatives from the Eubacteria and Archea genus are aligned. Names of organisms are abbreviated as follows: Bsub, Bacillus subtilis (GenBank accession number NP_390737); Lmon, Lysteria monocytogenes (GenBank accession number YP_013839); Ssap, Staphylococcus saprophyticus (GenBank accession number YP_301742); Saur, Staphylococcus aureus (GenBank accession number YP_001246578); Dred, Desulfotomaculum reducens (GenBank accession number YP_001112987); Aaeo, Aquifex aeolicus (GenBank accession number NP_213981); Tthe, Thermus thermophilus (GenBank accession number YP_144416); Tden, Thiobacillus denitrificans (GenBank accession number AAZ97399); Mmaz, Methanosarcina mazei (GenBank accession number NP_633918); Faci, Ferroplasma acidarmanus (GenBank accession number ZP_01709777); Mthe, Methanothermobacter thermautotrophicus (GenBank accession number NP_275693); Drad, Deinococcus radiodurans.
mispair (14-mer band), without rendering any polymerization product. The absence of products shorter than 14-mer can be explained considering that dATP could serve to repolymerize after a next degradation event. Therefore, direct elongation of the primer terminus by PolX Bs was hindered by the presence of the mismatch. In this sense, when dCTP, the first correct nucleotide that should be inserted after removal of the mispair, was supplied in addition to dATP, the 14-mer band was elongated to give a 17-mer product by the sequential insertion of dCMP and the two following complementary dAMP molecules (Figure 6B). In the presence of the four dNTPs, and after mismatch removal, the wild-type PolX Bs resumed gap filling (20-mer product). The impairment showed by mutant H339A/H341A to elongate the mispair G-G (Figure 6B), demonstrates that the 3′–5′ exonuclease activity located in the PHP domain of PolX Bs is the one responsible for editing the mismatch. Furthermore, the previous action of the 3′–5′ exonuclease activity of PolX Bs makes the polymerase well suited to repair gapped molecules even with a 3′-flap structure (Figure 6C). In all cases, once the gap was filled, DNA polymerase would dissociate to allow further sealing.

**Figure 4.** Mutations introduced in residues His339 and His341 of the PHP domain eliminate the 3′–5′ exonuclease activity of PolX Bs. The assay was carried out in the conditions described in Materials and methods section, using the indicated concentration of either the wild-type or mutant derivatives ΔPHP and H339A/H341A of PolX Bs and 1.5 nM of a 32P 5′-labelled ssDNA oligonucleotide (15-mer), in the presence of 1 mM MnCl2. After incubation for 30 min at 30°C, degradation of the labelled DNA was analysed by 8 M urea–20% PAGE and autoradiography. The position of the undegraded primer strand (15-mer) is indicated.

**Figure 5.** The 3′–5′ exonuclease activity on 5-nt gapped molecules bearing either a matched or a mismatched primer 3′-terminus. The different molecules used in the analysis are depicted on top of the figure. Asterisks indicate the 32P 5′-labelled end of the primer strand. The assay was carried out as described in Materials and methods section, using 125 nM of PolX Bs and 1.5 nM of each of the DNA molecules in the presence of 1 mM MnCl2. After incubation for the indicated times at 30°C, samples were analysed by 8 M urea–20% PAGE and autoradiography. The position of the undegraded primer strand (15-mer) is indicated.

(GenBank accession number NP_294190); Tvol, Thermoplasma volcanium (GenBank accession number NP_111375). The highly conserved histidine, glutamic and aspartic residues proposed to act as metal ligands at the PHP domain are indicated in blue (histidines) and red (glutamates and aspartates) letters, over a black background. Other highly conserved residues are shown with white letters. Asterisks indicate the 10 metal ligands observed in the crystallographic structure of E. coli YcdX protein. Corresponding residues for PolX Bs are also indicated. Motifs I–IV, present in the Histidinol phosphatase family correspond to the boxed areas. Alignment was done with the Mutalin tool (http://bioinfo.genopole-toulouse.prdf.fr/multalin/multalin.html) and further adjusted by hand. (B) Proposed model for the PolX Bs PHP domain. Left panel shows a ribbon representation of the structural model for PolX Bs PHP domain. Model was provided by the homology modelling server Swiss-Model, using as template the crystallographic structure of E. coli YcdX protein [PDB 1B1p, (40)]. Residues proposed to form the catalytic core are represented in red (aspartate and glutamates) and blue (histidines) sticks. The molecular surface of the modelled PolX Bs PHP domain is shown in the right panel.
of the final nick by the T4 DNA ligase to render the expected 33-mer product, a situation that would mimic the final step of DNA repair pathways. Taken together, the results presented allow the assignment of an editing function to PolX$_{Bs}$ 3'→5' exonuclease, suggesting a mechanism where an unannealed 3'-terminus is removed before elongation.

Exonucleases said to have proofreading activity should satisfy the following criteria: they have to act unidirectionally at the 3'-end releasing dNMPs, they have to preferentially excise mispaired primer terminus and they have to functionally interact with the polymerase to increase fidelity of DNA synthesis (47,48). To ascertain whether the 3'→5' exonuclease activity of PolX$_{Bs}$ can prevent the fixation of polymerization errors made by PolX$_{Bs}$, the exonuclease deficient mutant and the wild-type protein were compared in their capacity to incorporate dAMP onto the non-complementary position 18 of the gapped structure harbouring the matched 3'-terminus. As shown in Figure 7, with both proteins dAMP incorporation occurred mainly at complementary positions 16 and 17. The faint band at position 19 indicated that dAMP was poorly misincorporated (A–A) on position 18. The similar misincorporation levels obtained with both proteins suggest that the 3'→5' exonuclease activity of PolX$_{Bs}$ does not contribute to the fidelity of the polymerization process.

Thus, in spite of meeting the main biochemical criteria satisfied by proofreading 3'→5' exonucleases, the 3'→5' exonuclease activity of PolX$_{Bs}$ does not prevent the fixation of polymerization errors made by PolX$_{Bs}$, its role most probably being restricted to the resection of unannealed 3'-termini.

**DISCUSSION**

**PolX$_{Bs}$: a family X DNA polymerase endowed with an editing activity**

Members of the Pol X family are folded as a structural unit composed by two main domains: an N-terminal 8-kDa domain, with strong affinity for a downstream 5'-P terminus (23,26,49–51), and a 31-kDa polymerization domain that anchors the 3'-OH group of the upstream primer terminus at the polymerization site (24), this fact allowing them to perform DNA synthesis on gapped DNAs (23), intermediate products during the DNA repair processes NHEJ and BER. As mentioned in the
Introduction section, PolX<sub>Bs</sub> shares the basic enzymatic features of the DNA polymerase family X members. However, and as shown here, contrarily to eukaryotic PolXs, PolX<sub>Bs</sub> is endowed with an intrinsic Mn<sup>2+</sup>-dependent 3′–5′′ exonuclease activity. It is physically associated to the DNA polymerase activity, acting in concert with it; it acts unidirectionally from the 3′-end releasing dNMPs, and it preferentially degrades mispaired/flapped 3′-termini, favouring further re-synthesis by the DNA polymerase activity and final DNA ligation, this being the first report of a PolX member endowed with an editing activity of unannealed 3′-termini. These facts lead us to propose that PolX<sub>Bs</sub> should be playing a DNA repair role in vivo.

The PHP domain of PolX<sub>Bs</sub> contains the catalytic active site responsible for the 3′–5′′ exonuclease activity

The presence of a PHP domain linked to the α subunit of bacterial DNA polymerase III, to PolC, to the small subunits of archaeal DNA polymerases, to eukaryotic DNA polymerases α, δ and ε, and to bacterial/archaeal family X polymerases was previously anticipated (32). It was proposed to act as a phosphatase to hydrolyse the pyrophosphate by-product of DNA synthesis, shifting the equilibrium of the reaction towards polymerization. However, the functional role of the PHP domain in DNA polymerases is not yet fully understood, as the nuclease activity showed by several of the above-mentioned DNA polymerases resides in the canonical and well-characterized 3′–5′′ exonuclease domain, acting as proofreaders of the polymerization errors (1,35).

Up-to-date, polymerase α subunit of Thermus thermophilus DNA polymerase III holoenzyme is the only example for which the presence of an endogenous 3′–5′′ exonuclease activity has been attributed to its N-terminal PHP domain, that could function in cis to proofread polymerization errors, in addition to the 3′–5′′ exonuclease activity of the ε subunit, that would act in trans (52). In contrast, crystallographic resolution of the z subunit of E. coli (53) and T. aquaticus (54) DNA polymerase III shows that both, the PHP and the polymerization active sites lie on opposite faces of the enzyme. This fact would imply a gross structural relocation of the PHP domain to proofread the polymerization errors suggesting that, at least in these two DNA polymerases, the PHP domain could be playing a different role.

Results presented here allow us to assign unequivocally the 3′–5′′ exonuclease activity of PolX<sub>Bs</sub> to its PHP domain. Inspection of the alignment of the C-terminal PHP domains of bacterial/archaeal family X DNA polymerases with the E. coli YcdX protein [this work; (32)], together with the specific absence of exonuclease activity in the PolX<sub>Bs</sub> double-mutant H339A/H341A, validate the hypothesis of a nucleolytic activity residing in this domain, as well as of the residues likely acting as metal ligands (32), making our results in PolX<sub>Bs</sub> potentially extrapolative to other bacterial/archaeal DNA polymerases with a PHP domain. In this work, essential PHP active site residues, conserved in many of the bacterial/archaeal DNA polymerase X members in particular, and in most of the PHP domain-containing DNA polymerases, in general, have been shown to play a catalytic role responsible for the 3′–5′′ exonuclease activity.

Modelling of PolX<sub>Bs</sub> PHP has led us to propose a topology of a β<sub>1β</sub>₂<sub>α</sub>₅ barrel for this protein domain, similar to that of the catalytic α subunit of E. coli and T. aquaticus DNA polymerase III (53,54), and for the PHP domains fused to the rest of bacterial/archaeal PolX. As shown in Figure 3B, the predicted catalytic residues would be placed in a solvent-accessible cavity that would allow the 3′-terminus of a mispaired strand to reach the catalytic core. Although placed at the C-terminus of PolX<sub>Bs</sub>, the orientation of the PHP domain respect to the Polβ-like core cannot be anticipated, further crystallographic resolution of PolX<sub>Bs</sub> being required to understand the functional coupling between the polymerization and 3′–5′′ exonuclease activities.

The lack of histidine and aspartic residues proposed to act as metal ligands in the PHP domain of some members of the bacterial/archaeal PolX family, as T. volcanium (Tvpol in Figure 3A), could suggest alternative functions for PHP in these DNA polymerases, as DNA or protein ligands. In this sense, Wieczorek and McHenry (39) have recently reported the importance of the N-terminal PHP domain of the α subunit of E. coli Pol III in binding the ε proofreading subunit upstream from the polymerase domain, as deletion mutants destroyed ε binding. In addition, they made a subset of mutations on the Asp43, a conserved residue present in the PHP domain of all bacterial replicases. Their results showed how mutations affected differently to the binding of the ε subunit and to the polymerase activity, suggesting that the PHP domain may assist the primer terminus transference between both the polymerization and exonuclease active sites. Alignments of the PHP domain of bacterial replicases with that of bacterial/archaeal PolXs (32) shows the conservation of this Asp also in the latter group, and corresponding to D370 in PolX<sub>Bs</sub>. Interestingly, it is also present in the E. coli ycdX protein (D39), although from the crystallographic resolution of the structure of this protein, a catalytic involvement of D40 was not inferred (40). However, the nearly absolute conservation of this residue could suggest that the PHP domain of bacterial/archaeal PolXs could be used also as a platform to bind other cellular factors. In addition, the presence of ancillary 3′–5′′ exonuclease activities placed at the Polβ-like core domain of family X DNA polymerases cannot be excluded, as it has been described to occur for the PolX from the radioresistant bacterium D. radiodurans (36).

Potential roles of PolX<sub>Bs</sub> in DNA repair pathways

Based on the above results, and considering PolX<sub>Bs</sub> as a repair enzyme, two scenarios could be envisioned. BER is the main pathway responsible for repairing DNA damage produced by oxidation, alklylation, deamination or hydroxylation of DNA bases (6). Several family X DNA polymerases have been involved in the filling-in step of BER, as mammalian polβ (16,55) and polλ (15,49,56), yeast Pol4 (28,57), as well as the ASFV PolX (58). In bacteria, the gap-filling step has been classically attributed to DNA polymerase I (59,60). In BER, DNA damage is recognized...
by mono- and/or bi-functional DNA N-glycosylases (61). Whereas monofunctional glycosylases exclusively leave an AP site, bifunctional glycosylases, in addition to removing the damaged base, incise the phosphoester bond at the 3′-side of the deoxyribose via β-elimination, leaving a single-strand break with 3′-blocking ends. Such ends have to be ‘cleaned’ to allow further polymerization and ligation steps (6,62). The action of an AP endonuclease cleaves the 5′-side of the abasic site, generating an extendable 3′-end. B. subtilis codes for two AP endonucleases, ExoA and Nfo (63–65). Interestingly, the lost of both enzymes neither decreases the resistance to H2O2 and tert-butyl hydroperoxide (tBHP) nor increases the spontaneous mutation frequency (66). This fact could indicate that in addition to ExoA and Nfo, other DNA repair systems are sufficient to compensate for the lack of both enzymes to generate extendable 3′-ends that allow the further polymerization step. The biochemical properties displayed by PolXBs enable it to play an active role in this repair pathway, as it is specifically active on gapped structures. This fact, together with its 3′–5′ exonuclease activity, lead us to speculate that this protein could, under certain circumstances, clean the blocked 3′-ends by means of its 3′–5′ exonuclease activity. As it has been shown here, there is a functional coupling between the exonucleolytic and polymerization activities, as once the 3′-terminus is processed, it reaches the polymerization active site to be elongated, allowing the completion of the gap-filling step. In addition, an indirect argument that would support a potential role of PolXBs in BER consists in the fact that a B. subtilis knockout mutant of the yshD gene that belongs to the same operon as yshC (31), has been suggested to play a role in BER of oxidative damaged bases (67).

Additionally, we have shown that PolXBs is very active in degrading 3′-flap structures. They can arise during the limited base pairing realignment of the DNA ends produced by DSB, this fact being a general hallmark during NHEJ. Very briefly, in eukaryotes the heterodimer Ku binds DNA ends in a sequence independent manner at DSBs allowing the alignment of both ends and protecting them against nucleases (68–70). Once bound to DNA, Ku recruits the DNA-dependent protein kinase catalytic subunit (71) and both carry out the synapsis of the broken ends. Once brought together, and prior to ligation, the ends are processed by nucleases (Artemis, Fen1), phosphatases and kinases generating gap structures that are filled by a DNA polymerase belonging to family X (λ, μ, Pol IV) (72–74). Finally, the complex Ligase IV/XRCC4/XLF will join the ends together. The identification in bacterial genomes of genes homologous to the eukaryotic Ku factor (75,76) led to the discovery of NHEJ complex in bacteria (11,13). Bacterial Ku homologs are genetically associated to putative ATP-dependent DNA ligase genes (LigD) (75–78), that in many bacteria, as in Mycobacterium tuberculosis, are multidomain proteins containing polymerase, ligase and nuclease activities that could account for the end processing, gap filling and ligation steps during NHEJ (79). B. subtilis LigD has been also involved in NHEJ (13). However, it is interesting to point out that, although provided with ligase and polymerization domains, it lacks the nuclease one. The fact that in eukaryotes the gap filling step during NHEJ is carried out by a family X DNA polymerase (72–74), that deletion of the gene coding for the bacterial D. radiodurans DNA polymerase X sensitizes cells against DSB provoked by γ-rays (19), together with the presence of a 3′–5′ exonuclease activity in PolXBs (this article), suggest a potential

![Diagram](Image 379x237 to 413x274)

The 8 kDa domain of PolXBs binds to the downstream 5′–P and the PHP domain to the unannealed upstream 3′ terminus.

![Diagram](Image 399x341 to 465x379)

Flapped 3′ terminus is recessed by the 3′–5′ exonuclease activity of the PHP domain and the resulting matched 3′ end is placed at the polymerization active site.

![Diagram](Image 452x238 to 486x275)

The gap is filled by the polymerization activity.

![Diagram](Image 452x455 to 486x491)

Finally, the resulting nick will be sealed by a DNA ligase.

Figure 8. Scheme of the potential role of PolXBs in the resection of unannealed 3′-termini. After the 3′-protruding ends have been brought together, PolXBs would recognize specifically the 5′-P through its 8-kDa domain (coloured in red), the unannealed 3′-terminus being bound and further resected by the 3′–5′ exonuclease activity located at the PHP domain (coloured in orange). Subsequent to the resection, the resulting matched 3′-terminus would switch to the polymerization domain (coloured in cyan) to be elongated. After filling the gap, the nick would be sealed by a DNA ligase. PolXBs representation has been made by fusing the Polβ structure (PDB 1BPX) and the modelled PHP domain shown in Figure 3B.
role for PolX_{Bs} in processing the unannealed 3'-termini that could arise during DNA ends rejoining, the same PolX_{Bs} molecule performing the subsequent gap-filling step (Figure 8). Thus, it is tempting to speculate that PolX_{Bs} could form part of a NHEJ backup system under circumstances in which processing of 3'-flap structures were required.

The pathway described above would be the simplest one to be followed by PolX_{Bs} during DNA repair. However, the relatively low rate of its exonuclease activity, although comparable to other Mn^{2+}-dependent nucleases involved in DNA repair as yeast Mre11 (80–85), the Clostridium thermocellum polynucleotide kinase/phosphatase (86), the E. coli SbcCD (87) and yfE (88) proteins and the recently reported exonuclease activity of D. radiodurans (36), raises the question regarding the physiological substrate on which PolX_{Bs} acts. As in the repair processes a great variation of intermediates arise, the PHP domain could work even in trans on other DNA molecules. In addition, the high PolX_{Bs}/DNA substrate ratios that are required to accomplish the in vitro activity of PolX_{Bs}, are related to the poor DNA binding stability shown by this enzyme, analysed by EMSA and foot printing (data not shown). This fact opens the possibility that PolX_{Bs} could depend on other cellular factors to be recruited and stabilized to the DNA repair intermediates, as well as to increase its activity. In addition, proper coordination between synthetic and degradative activities of PolX_{Bs} could be modulated by some of these potential factors.

In any case, the determination of the in vivo relevance of the exonuclease activity displayed by PolX_{Bs} relies on the analysis of B. subtilis variants harbouring point mutants at the exonuclease catalytic residues of the PolX_{Bs} PHP domain.

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