

Characterization of Lhr-Core DNA helicase and manganesedependent DNA nuclease components of a bacterial gene cluster encoding nucleic acid repair enzymes

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Lhr is a large superfamily 2 helicase present in mycobacteria and a moderate range of other bacterial taxa. A shorter version of Lhr, here referred to as Lhr-Core, is distributed widely in bacteria, where it is often encoded in a gene cluster along with predicted binuclear metallo-phosphoesterase (MPE), ATP-dependent DNA ligase, and metallo-*β*-lactamase exonuclease enzymes. Here we characterized the Lhr-Core and MPE proteins from Pseudomonas putida. We report that P. putida Lhr-Core is an ssDNA-dependent ATPase/dATPase (K_m, 0.37 mM ATP; k_{cat} , 3.3 s⁻¹), an ATP-dependent 3'-to-5' single-stranded DNA translocase, and an ATP-dependent 3'-to-5' helicase. Lhr-Core unwinds 3'-tailed duplexes in which the loading/tracking strand is DNA and the displaced strand is either DNA or RNA. We found that P. putida MPE is a manganese-dependent phosphodiesterase that releases p-nitrophenol from bis-p-nitrophenyl phosphate (k_{cat} , 212 s⁻¹) and *p*-nitrophenyl-5'-thymidylate $(k_{cat}, 34 \text{ s}^{-1})$ but displays no detectable phosphomonoesterase activity against p-nitrophenyl phosphate. MPE is also a manganese-dependent DNA endonuclease that sequentially converts a closed-circle plasmid DNA to nicked circle and linear forms prior to degrading the linear DNA to produce progressively smaller fragments. The biochemical activities of MPE and a structure predicted in Phyre2 point to MPE as a new bacterial homolog of Mre11. Genetic linkage of a helicase and DNA nuclease with a ligase and a putative exonuclease (a predicted homolog of the SNM1/Apollo family of nucleases) suggests that these enzymes comprise or participate in a bacterial DNA repair pathway.

Mycobacterium smegmatis Lhr is the founding member of a new clade of superfamily 2 DNA helicases by virtue of its signature domain structure and distinctive DNA interface (1, 2). Lhr is a 1507-amino acid $(aa)^2$ monomeric nucleic acid-dependent ATPase/dATPase that uses ATP hydrolysis to drive unidirec-

tional 3'-to-5' translocation along single-strand DNA (ssDNA) and to unwind duplexes *en route*. Lhr is up-regulated in mycobacteria in response to DNA damage (3, 4), and deletion of the *lhr* gene sensitizes *M. smegmatis* to killing by mitomycin C.³ Full-length Lhr homologs are found in bacteria from eight phyla, being especially prevalent in Actinobacteria and Proteobacteria (1).

The ATPase, DNA translocase, and helicase activities of mycobacterial Lhr are encompassed within the N-terminal 856-aa segment of the 1507-aa polypeptide (1). The crystal structure of Lhr-(1–856) in complex with AMPPNP·Mg²⁺ and a "loading/tracking" 3′ DNA single strand revealed that the enzyme consists of two N-terminal RecA-like domains that bind the AMPPNP·Mg²⁺, a central winged helix domain, and a C-terminal domain of novel tertiary structure (2). All four domains make contributions to Lhr's unique DNA interface.

Homologs of Lhr-(1-856), all of similar size ($\sim 800-900$ aa), are present in the proteomes of scores of diverse bacterial and archaeal taxa. We will henceforth refer to this shorter Lhr-like protein as Lhr-Core. The striking feature of the bacterial Lhr-Core clade is its genetic organization. Most commonly, Lhr-Core is encoded in a co-oriented four-gene cluster comprising a putative exonuclease (Exo) of the metallo- β -lactamase enzyme family, an ATP-dependent DNA ligase, Lhr-Core, and a member of the binuclear metallo-phosphoesterase (MPE) enzyme family. We refer to this operon-like arrangement of the four genes as a "class I" cluster (Fig. 1). Variations on this theme include cases where one or more additional co-oriented ORFs, encoding proteins unrelated to nucleic acid enzymology, are inserted between the DNA ligase and Lhr-Core genes (class Ia cluster) (Fig. 1). For example, in the class Ia cluster of Pseudomonas putida, the intervening ORF encodes a member of the succinyl glutamate desuccinylase/aspartoacylase enzyme family. In another variation, the nuclease and ligase proteins are fused and encoded by a single continuous ORF upstream of Lhr-Core (class Ib cluster) (Fig. 1). A distinctive arrangement of the four genes is found in other bacteria in which the Exo-ligase cassette has been inverted en bloc so that they are transcribed divergently from the Lhr-Core and MPE genes; these are designated class II and IIa clusters (Fig. 1). Finally, other bacteria and archaea have a two-gene Lhr-Core MPE cassette (dubbed a class III cluster) that is not linked to Exo or ligase genes (Fig. 1).

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² The abbreviations used are: aa, amino acids; ssDNA, single-strand DNA; Exo, exonuclease; MPE, metallo-phosphoesterase; SA, streptavidin; NHEJ, non-homologous end-joining; MBL, metallo-β-lactamase; pNPP, *p*-nitrophenyl phosphate; AMPPNP, adenosine 5'-(β, γ-imino)triphosphate.

³ A. Ejaz, A. Fay, M. Glickman, and S. Shuman, unpublished data.

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Figure 1. Genetic clustering of bacterial Lhr-Core and three putative nucleic acid repair enzymes. The bacterial ORF encoding Lhr-Core (green arrows) is typically located within a gene cluster that includes ORFs encoding a binuclear MPE (magenta arrows), an ATP-dependent DNA ligase (gold arrows), and an exonuclease of the metallo- β -lactamase enzyme family (cyan arrows). The order and orientations of the clustered genes may vary among taxa and are classified accordingly in the figure. A bacterial species exemplifying each class is indicated on the *left*. Where additional, functionally unrelated ORFs are denoted by gray arrows. The cases in which only Lhr-Core and MPE comprise a two-gene cluster are designated as class III in the schematic.

The wide prevalence of a heretofore uncharacterized gene cluster specifying putative helicase, nuclease, and ligase enzymes suggests to us the existence of a new bacterial nucleic acid repair pathway centered around Lhr-Core. To understand whether and how these enzymes might contribute to nucleic acid repair, we undertook here to purify and characterize biochemically the Lhr-Core and MPE proteins specified by the class Ia cluster of *P. putida*. We report that the *P. putida* Lhr-Core is a *bona fide* ATP-driven 3'-to-5' translocase and DNA helicase and that the MPE is a manganese-dependent phosphodiesterase/DNA endonuclease with structural and functional similarity to Mre11.

Results

P. putida Lhr-Core

The 816-aa *P. putida* KT2440 Lhr-Core polypeptide is homologous to the 856-aa ATPase/helicase domain of the 1507-amino acid *M. smegmatis* Lhr protein (Fig. 2). A primary structure alignment of *P. putida* Lhr-Core and *M. smegmatis* Lhr highlights 325 positions of amino acid side chain identity/similarity (Fig. 2). The conserved positions include eight side chains that make atomic contacts to AMPPNP·Mg²⁺ in the *M. smegmatis* Lhr crystal structure (shaded *gold* in Fig. 2) and seven side chains that contact the ssDNA loading strand (shaded *green* in Fig. 2) (2). In particular, the four amino acids found to be essential in *M. smegmatis* Lhr for the coupling of ATP hydrolysis to duplex unwinding (Thr-145, Arg-279, Ile-528, and Trp-597; denoted by *triangles* in Fig. 2) are conserved in *P. putida* Lhr-Core. A gap in the sequence alignment at residue 280 of *P. putida* Lhr-Core corresponds to a disordered surface loop in the second RecA domain of the *M. smegmatis* Lhr structure (in *red font* in Fig. 2) that is not conserved in the *P. putida* protein.

Lhr-Core is a DNA-dependent ATPase

To evaluate the enzymatic and physical properties of P. putida Lhr-Core, we produced the protein in Escherichia coli as a His₁₀Smt3 fusion and purified it from a soluble extract by serial nickel affinity, tag removal, and gel filtration steps. Lhr-Core eluted as a monomer during gel filtration. SDS-PAGE affirmed the purity of the Lhr-Core preparation (Fig. 3A). Reaction of Lhr-Core with 1 mM [α -³²P]ATP in the presence of 5 mM magnesium and 5 μ M 24-mer ssDNA oligonucleotide cofactor resulted in the hydrolysis of $[\alpha^{-32}P]ATP$ to $[\alpha^{-32}P]ADP$, as monitored by polyethyleneimine-cellulose TLC. The extent of ATP hydrolysis increased in proportion to the amount of input Lhr-Core, and 60% of the available ATP was hydrolyzed at 1 μ M enzyme (Fig. 3A). The ATPase specific activity, calculated from the slope of the titration curve in the linear range of enzyme dependence, was 2.56 nmol ATP hydrolyzed per 1 pmol Lhr-Core in 15 min, which translates into an estimated turnover number of 2.8 s⁻¹. ATPase activity depended on addition of a divalent cation. Divalent cation cofactor specificity was assessed at 0.5, 1, 2.5, 5, and 10 mM magnesium, manganese, calcium, cobalt, copper, nickel, and zinc (Fig. 3B). Magnesium and calcium were the superior cofactors, supporting vigorous



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RecA fold RecA fold		Winged Helix	LhrC (novel structure)	1	V	
Lhr (core) – <i>Pseudomonas putida</i> 816-aa						
Ppu Msm	LANAWFAKRGWKPF# LTREWFTTAFAAPTH	AF <mark>Q</mark> RRVWAA PA <mark>Q</mark> ADAWSA		AG <mark>KT</mark> YAVWLAAL-RAFKPRPQGR SG <mark>KT</mark> LAAFLWAIDRLADPAREPS	65 74	
Ppu Msm	HLAPIQVLWVTPMRA QGTQVLYVSPLKA	ALAADTARA ALAVDVERN	LQAPLDELQLPW LRTPLTGITRVAER	SVGVRSGDTNSAERA HGLPAPSITVGVRSGDTPPNQRR	115 132	
Ppu Msm	RQARRLPSVLIT <mark>T</mark> PI AMIANPPDVLIT <mark>T</mark> PI Å	E <mark>SLTLLL</mark> TR E <mark>S</mark> LFLM <mark>L</mark> TS	AKAREDFATLQLVV A-ARETLTSVRTVI	V <mark>DE</mark> WHELLGNKRGVQLQLALARL V <mark>DE</mark> VHAVAATKRGAHLALSLERL	175 191	
Ppu Msm	RHWHPGLPTWGLS DQLL-DTPAQRIGLS	SATLGNLQH SATVRPPEE	ARDVLLPQ-GGLLV VARFLSGQAPTTIV	QGRQDKALQVDTLLPKAIERFPW CPPAAKTFDLSVQVPVPDMANLD	232 250	
Ppu Msm	AGHMGLKMLDQVCHI NNSIWPDVEERIVDI	EIDASASSI LVEAHNSSI	VFTNT <mark>R</mark> AQAEIWYQ VFANS <mark>R</mark> RLAERLTS ∆	ALLEARPDWAG RLNEIHAERSGIELPAGPNPEVG	280 310	
Ppu Msm	GGAPAHLMGSGQANO	LIAL GAPPLLARA	HHASLARDTRDWVE	RSLKQGSLKAVICTSSLDLGV <mark>D</mark> F DDLKSGRLRAVVATSSLELGI <mark>D</mark> M	321 370	
Ppu Msm	LPVERVLQIGSAKG GAVDLVIQVEAPPSV	IARLMQRAG VASGLQRVG	RSGHAPGRRSRVTL RAGHQVGEISQGVL	VPTHSLELVEAAAARQALLAGHI FPKHRTDLIGCAVTVQRMQTGDI	381 430	
Ppu Msm	EARFSPRLCMDVLV ETLRVPANPLDVLA	QHLVSMALG QHTVAVAAL	SGFRPEQLLAEVRS EPVDADAWFDAVRR	TWAFRQLRDSQWQWALDFVCHGG SAPFATLPRSAFEATLDLLS	441 487	
Ppu Msm	SSLTAYPDYQRVER(GKYPSTEFAELI	 QADGVY RPRLVYDRD	RVTSERLARRHR TGTLTARPGAQ <mark>R</mark> LA	MGIGTI <mark>V</mark> SDANLQLKYWSKGGGG VTSGGAIPDRGMFTVYLASETEK	496 544	
Ppu Msm	KT-LGSVEEAFIARI PSRVGELDEEMVYES	LRPGDTLVF SRPGDVISI	GATSWRITEITHDR	AYVRRSTARKAAVAR <mark>W</mark> NGGRMPL VLVIPAPGQPARLPF <mark>W</mark> RGDSVGR	555 604	
Ppu Msm	SSELADAL PAELGAAVGAFTGEI	VEQLDAAAH LASLDRKAF	FEGPEM DKRCQKMGFAGYAT	RAVRPLLALQAQ-WSALPTTSTL DNLHQLLREQREATGVVPSDTTF	602 664	
Ppu Msm	LAETFKSRQG-WHLI VVERFRDELGDWRVI	FLYPFAGRM ILHSPYGLR	IANLGLANLIAWRVS VHGPLALAVGRRLR	RAQPLSVSIAVNDYGFELLSPAQ ERYGIDEKPTASDDGIIVRLPDS	661 724	
Ppu Msm	VDWASHLPQ-ALGTA GDTPPGADLFVFDAI	ADLLEDVLA DEIEPIVTA	SLNAGEMALRRFRE EVGGSALFASRFRE	IAQIAGLVFGGYPAAQKSTRQIQ CAARALLLPRRHPGKRSPLWHQR	720 784	
Ppu Msm	ASSGLFYEVFRKHDA QRAAQLLDIARKYPI	AGNLLLGQA DFPIVL-EA	RDEVLSEELEIERL VRECLQDVYDVPAL	HRQLLKMSELQLDLQALRRPG IELMHKIAQRRLRIVEVETATPS	778 843	
Ppu Msm	 PLAFALLVEGMRETI PFAASLLFGYVGA	LSTEKLADR 856	IARMVAELEQAANA	G* 816		

Lhr (full-length) - Mycobacterium smegmatis 1507-aa

Figure 2. Homology of Lhr-Core and Lhr. The *top panel* depicts, in cartoon form, the domain organization of the 1507-aa *M. smegmatis* Lhr protein, as revealed by the crystal structure of its autonomous helicase domain (aa 1–856). Lhr-Core proteins, exemplified by the 816-aa *P. putida* enzyme, lack the large, structurally uncharacterized C-terminal domain present in full-length Lhr. The *bottom panel* shows an alignment of the primary structures of *P. putida* Lhr-Core (*Ppu*) and *M. smegmatis* Lhr (*Msm*). Positions of side chain identity/similarity are denoted by **O**. Gaps in the alignment are indicated by –. A disordered segment of the Msm Lhr tertiary structure that has no counterpart in Ppu Lhr-Core is shown in *red font*. Conserved Lhr amino acids that contact ATP-Mg²⁺ are highlighted in *gled shading*; conserved side chains that contact the ssDNA loading strand are shaded in *green*. Four conserved Lhr amino acids essential for coupling of ATP hydrolysis to duplex unwinding are denoted by *triangles*.

ATP hydrolysis across the full range of concentrations tested. Manganese was effective at 0.5 to 2.5 mM and slightly less so at 5 and 10 mM. Cobalt and nickel functioned best at 0.5 and 1 mM and became progressively less active at 2.5, 5, and 10 mm. Zinc supported activity narrowly at 0.5 to 1 mm but was ineffective at a concentration of 2.5 mm or higher. By contrast, copper was





Figure 3. Characterization of the Lhr-Core ATPase. *A*, *left panel*, an aliquot (5 μ g) of the Superdex-200 fraction of *P*. *putida* Lhr-Core was analyzed by SDS-PAGE. The Coomassie Blue-stained gel is shown. The positions and sizes (kilodatons) of marker polypeptides are indicated on the *left. Right panel*, ATPase reaction mixtures (10 μ l) containing 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 5 mm MgCl₂, 1 mm DTT, 1 mm [$(m^{-32}P)$ ATP (10 nmol ATP), 5 μ m 24-mer ssDNA oligonucleotide (5'-GGGTCGCAATTGATTCCGATAGTG), and Lhr-Core protein as specified (0.125, 0.25, 0.5, 1, 2, 4, 6, 8, or 10 pmol, corresponding to 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, or 1 μ m Lhr-Core) were incubated at 37 °C for 15 min. The extent of [^{32}P]ADP formation is plotted as a function of input enzyme. Each datum is the average of three independent enzyme titration experiments \pm S.E. *B*, divalent cation specificity. Reaction mixtures (10 μ l) containing 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 1 mm DTT, 1 mm [$(m^{-32}P]$ ATP (10 nmol ATP), 5 μ m 24-mer ssDNA, 0.5 μ m (5 pmol) Lhr-Core, and increasing concentrations. Each datum is the average of three separate metal titration experiments \pm S.E. *C*, NTP specificity. Reaction mixtures (10 μ l) containing 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 5 mm MgCl₂, 1 mm DTT, 5 μ m 24-mer ssDNA, 0.5 μ m (5 pmol) Lhr-Core, and increasing concentrations. Each datum is the average of three separate metal titration experiments \pm S.E. *C*, NTP specificity. Reaction mixtures (10 μ l) containing 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 5 mm MgCl₂, 1 mm DTT, 5 μ m 24-mer ssDNA, 0.5 μ m (5 pmol) Lhr-Core, and interpolating 4 measured in control reaction mixtures (10 μ l) containing 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 5 mm MgCl₂, 1 mm DTT, 5 μ m 24-mer ssDNA, 0.5 μ m (5 ro 30 min. The reaction mixtures (10 μ l) containing 20 mm Tris-HCl (pH 7.5), 30 mm NaCl, 5 mm MgCl₂, 1 mm DTT, 5 μ m 24-mer ssDNA, 1 μ m Lhr-Core, and interpolating 1 mm of the indicated NTP/dNTP but no

ineffective at 0.5 and 1 mm but supported activity at 5 and 10 mm. All subsequent assays of Lhr-Core activity employed magnesium as the metal cofactor.

hydrolyzed ATP and dATP; the other rNTPs and dNTPs were hydrolyzed poorly or not at all (Fig. 3*C*).

NTP substrate specificity

Nucleotide specificity was examined by colorimetric assay of the release of P_i from unlabeled ribonucleotides ATP, GTP, CTP, or UTP and the deoxynucleotides dATP, dGTP, dCTP, and dTTP, each at 1 mm concentration. Lhr-Core specifically

Steady-state kinetics of ATP hydrolysis

We determined steady-state kinetic parameters by measuring the velocity of ATP hydrolysis as a function of ATP concentration in the presence of 5 mM magnesium and 5 μ M 24-mer ssDNA cofactor (Fig. 3*D*). From a nonlinear regression curve fit of the data to the Michaelis–Menten equation, we calculated





Figure 4. Dependence of ATP hydrolysis by Lhr-Core on ssDNA cofactor. Reaction mixtures (10 μ l) containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM [α ⁻³²P]ATP, 0.4 μ M Lhr-Core, and increasing amounts of 24-mer, 18-mer, 12-mer, or 6-mer ssDNA oligodeoxynucleotides as specified (0.06, 0.13, 0.25, 0.5, 1, 2, 3, 4, 5, or 10 pmol, corresponding to 0.006, 0.013, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, or 1 μ M ssDNA) were incubated at 37 °C for 15 min. The extent of ATP hydrolysis is plotted as a function of input oligonucleotide. Each datum is the average of three separate DNA titration experiments \pm S.E. A nonlinear regression curve fit of the data to a one-site specific binding model (in Prism) is shown. The nucleobase sequences of the ssDNA oligonuc cleotides and the calculated K_d values are shown below the graph.

that Lhr-Core has a K_m of 0.37 \pm 0.038 mM ATP and a $k_{\rm cat}$ of 3.33 \pm 0.12 s⁻¹.

Single-strand DNA length requirement for ATP hydrolysis

We tested the ability of ssDNAs of varying length to activate ATP hydrolysis by Lhr-Core. Titration of the oligonucleotides revealed a hyperbolic dependence of ATP hydrolysis on the amount of 24-mer, 18-mer, or 12-mer strands with similar extents of hydrolysis at saturating DNA concentrations (Fig. 4). Nonlinear regression fitting of the data to a one-site binding model in Prism yielded apparent K_d values as follows: 44 nM 24-mer, 65 nM 18-mer, and 113 nM 12-mer. Shortening the DNA cofactor to a 6-mer caused a marked shift to the right in the titration curve, with an apparent K_d value of 725 nM 6-mer.

Lhr-Core is a 3'-to-5' DNA translocase

NTP hydrolysis by nucleic acid–dependent NTP phosphohydrolases is often coupled to mechanical work, either duplex unwinding or displacement of protein–nucleic acid complexes, as a consequence of translocation of the phosphohydrolase enzyme along the nucleic acid. To address whether Lhr-Core has translocase activity, we employed a streptavidin (SA) displacement assay (1, 2, 6) as follows. 5' ³²P-labeled 34-mer DNA oligonucleotides containing a single biotin moiety at the fourth inter-nucleotide from the 5' end or the second inter-nucleotide from the 3' end were preincubated with excess SA to form a stable SA–DNA complex that was easily resolved from the free

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biotinylated 34-mer DNA during native PAGE (Fig. 5). The translocation assay scores the motor-dependent displacement of SA from DNA in the presence of ATP and excess free biotin, which instantly binds to free SA and precludes SA rebinding to the labeled DNA. The rationale of the assay is that directional tracking of the motor along the DNA single strand will displace SA from one DNA end but not the other. As depicted in Fig. 5A, the enzyme acts as a "cow catcher" on a locomotive engine. When moving 3' to 5', it can displace SA as it collides with the 5' biotin-SA. In contrast, a 3' biotin-SA complex is not expected to be displaced by a 3'-to-5' translocase because the motor moves away from the SA and simply falls off the free 5' end. The converse outcomes apply to a 5'-to-3' translocase, whereby it displaces a 3' biotin-SA complex but not a 5' biotin-SA adduct. The instructive finding was that Lhr-Core displaced the SA from a 5' biotin-SA complex on the 34-mer ssDNA to yield the free ³²P-labeled 34-mer strand but was unable to displace SA from the 3' biotin-SA complex tested in parallel. Stripping of the 5' biotin–SA complex by Lhr-Core to liberate free DNA depended on ATP and magnesium (Fig. 5A). Enzyme titration experiments with 0.1 μ M 5' biotinylated 34-mer substrate in the presence of 1 mM ATP and 5 mM magnesium revealed that the extent of SA displacement was proportional to the amount of input enzyme and that 90% of the biotin-SA complex was dissociated at saturating enzyme. In the linear range of enzyme dependence, Lhr-Core displaced 0.99 ± 0.05 pmol of 5' biotin-SA per 1 pmol of Lhr-Core (Fig. 5B). The kinetic profile of SA displacement by 0.5 μ M Lhr-Core during a 10-min reaction with 0.1 μ M of 5' biotinylated substrate is shown in Fig. 5C. The data fit well by nonlinear regression to a two-phase association with apparent rates constants of $3.35 \pm 0.87 \text{ min}^{-1}$ (percent fast 53 ± 11) and $0.49 \pm 0.17 \text{ min}^{-1}$ (Fig. 5*C*).

Lhr-Core is a 3'-to-5' helicase

In light of the translocase activity demonstrated above, we tested Lhr-Core for helicase activity with a series of 3'-tailed duplex substrates consisting of a 24-bp duplex with a 15-mer 3' single-strand tail to serve as a loading strand (Fig. 6). The 39-mer loading/tracking strand was a synthetic DNA or RNA oligonucleotide of identical nucleobase sequence (excepting U for T in RNA). The 5' ³²P-labeled 24-mer displaced strand was a DNA or RNA oligonucleotide of identical nucleobase sequence complementary to that of the 39-mer strand. The helicase assay format entailed preincubation of Lhr-Core and labeled nucleic acid substrate, followed by initiation of unwinding by addition of ATP, with simultaneous addition of a "trap" of excess unlabeled 24-mer displaced strand. The trap strand minimizes reannealing of any radiolabeled 24-mer that was unwound by Lhr-Core, and it competes with the loading strand for binding to any free Lhr-Core or Lhr-Core that dissociated from the labeled 3'-tailed duplex without unwinding it. Consequently, the assay predominantly gauges a single round of strand displacement by Lhr-Core bound to the labeled 3'-tailed duplex prior to the onset of ATP hydrolysis. The salient findings from the assay were as follows. Lhr-Core unwound 3'-tailed RNA-DNA and DNA-DNA substrates to yield a radiolabeled free single-strand RNA and DNA, respectively,



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Figure 5. Lhr-Core DNA translocase activity. *A*, streptavidin displacement assays were performed as described under "Experimental procedures." The nucleobase sequences of the 5' or 3' biotinylated 34-mer ssDNAs are shown at *bottom; B* denotes the position of the biotin spacer. The complete translocase reaction mixtures contained 20 mM Tris-HCI (pH 7.5), 30 mM NaCl, 5 mM MgCl₂ (where denoted by + over the lane), 1 mM DTT, 1 mM ATP (where indicated by +), 0.1 μ M ³²P-labeled 5' biotinylated DNA attached to SA (on the *left side*) or 3' biotinylated DNA attached to SA (on the *left side*), and 2 μ M Lhr-Core (where indicated by +). Control mixtures lacking Lhr-Core and SA were included in the *leftmost lanes* of each series. Other components were omitted where indicated by - above the lanes. Reactions lacking magnesium were supplemented with 10 mM EDTA where indicated by +. The reaction products were analyzed by native PAGE, and the ³²P-labeled DNA was visualized by autoradiography of the dried gel. The species corresponding to the SA–DNA complex and free DNA are indicated on the *left. B*, translocase reaction mixtures (10 μ I) containing 20 mM Tris-HCI (pH 7.5), 30 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.1 μ M ³²P-labeled 5' biotinylated SA–DNA complex, and Lhr-Core as specified (0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5, or 10 pmol, corresponding to 0.008, 0.016, 0.031, 0.063, 0.125, 0.25, 0.5, or 1 μ M Lhr-Core) were incubated at 37 °C for 15 min. The products were analyzed by native PAGE. The extent of translocation ((free DNA)/(SA-DNA + free DNA)) is plotted as a function of input enzyme. Each datum represents the average of three separate enzyme titrations ± S.E. *C*, reaction mixtures (100 μ I) containing 20 mM Tris-HCI (pH 7.5), 30 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.1 μ M ³²P-labeled 5' biotinylated SA-DNA complex, and 0.5 μ M Lhr-Core) and SA or 15, 30 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.1 μ M ³²P-labeled 5' biotinylated SA-DNA complex, and 0.5 μ M Lhr-Core) were incubated at 37 °

that migrated faster than the input tailed duplex during native PAGE and comigrated with free 24-mer generated by thermal denaturation of the substrates (Fig. 6, *lanes* Δ). Lhr-Core failed to unwind an RNA–RNA duplex, and Lhr-Core did not unwind a DNA–RNA hybrid with a 39-mer RNA loading strand (Fig. 6). These results establish that Lhr-Core is a unidirectional motor, powered by ATP hydrolysis, that tracks 3' to 5' along a DNA loading strand and unwinds duplexes *en route*.

P. putida MPE

P. putida gene PP_1102 encodes a 216-aa protein (Fig. 7, *top panel*) that is a member of the binuclear MPE enzyme superfamily. MPE enzymes characteristically utilize a binuclear tran-

sition metal ion center to catalyze phosphomonoester or phosphodiester hydrolysis (7). Structurally and biochemically wellstudied binuclear MPE enzymes play diverse physiological roles, *e.g.* as agents of signal transduction (phosphoprotein phosphatase calcineurin), RNA processing (RNA debranching enzyme Dbr1), DNA repair (DNA nuclease Mre11), and RNA repair (bacterial Pnkp phosphoesterase) (8–12). Submission of the *P. putida* MPE amino acid sequence to the Phyre2 structure prediction server (13) generated a "top hit" structure model (99.7% confidence) of MPE based on homology to the crystal structure of *Methanocaldococcus jannaschii* Mre11 bound to manganese (PDB code 3AZU). The modeled active site of *P. putida* MPE is shown in Fig. 7, *bottom panel*, with two





● GGGUCGCAAUUGAUUCCGAUAGUG	RNA
3'UUUUUUUUUUUUUUUCCCAGCGUUAACUAAGGCUAUCAC	RNA
● GGGUCGCAAUUGAUUCCGAUAGUG	RNA
$_{3'}$ TTTTTTTTTTTTTTTTTCCCAGCGTTAACTAAGGCTATCAC	DNA
● GGGTCGCAATTGATTCCGATAGTG	
3'UUUUUUUUUUUUUUUUUCCCAGCGUUAACUAAGGCUAUCAC	RNA
3'TTTTTTTTTTTTTTTTTCCCAGCGTTAACTAGGCTATCAC	DNA DNA

Figure 6. Lhr-Core helicase activity. Reaction mixtures (10 μ l) contained 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 5 mM MgCl₂ 1 mM DTT, 1 mM ATP, 0.1 μ M of the indicated 3'-tailed duplex substrate (depicted at the *bottom*, with the 5' ³²P label denoted by ●), and 0.5 μ M Lhr-Core (indicated by +). Lhr-Core was omitted from the reaction mixtures in lanes marked –. Reaction mixtures lacking Lhr-Core that were heat-denatured prior to PAGE are included in lanes marked Δ . The reaction products were analyzed by native PAGE and visualized by autoradiography.

Pseudomonas putida MPE

MNYQPIEHRGQALWLLADKAIYWPARRALLVADVHIGKAASYRALHQPVPRGTT 54 EATLARLDRLLAEHDCEQLIILGDFLHARTARAPATLAKVEDWRKRHKNLKVVL 108 IRGNHDRNAGDPPASLDIQVVDEPWVLEPFALQHEPQPHGTHPVLAGHVHPVFV 162 LRGKARQRLRLPCFVIDEQVSLLPAFGEFTGGWEITPASASRVYLAGAERVWPL 216



Figure 7. *P. putida* **MPE** and a homology model of its active site. *Top panel*, the amino acid sequence of *P. putida* MPE polypeptide, with the predicted metal and phosphate ligands highlighted in *green shading. Bottom panel*, stereo view of the model of the *P. putida* MPE active site generated in Phyre2 based on homology to the crystal structure of *M. jannaschii* Mre11 in complex with manganese (PDB code 3AUZ). The *P. putida* MPE active site side chains are depicted as *stick models*. The two manganese ions are shown as *magenta spheres*.

Bacterial nucleic acid repair enzyme gene cluster

manganese ions (superimposed from the *M. jannaschii* Mre11 structure) coordinated by conserved MPE side chains Asp-33, His-35, Asp-78, Asn-112, His-113, His-142, His-156, and His-158. MPE His-113 is predicted to interact with the scissile phosphate of the substrate. These eight active site residues are highlighted in *green shading* in the amino acid sequence in Fig. 7.

MPE is a manganese-dependent p-nitrophenyl phosphodiesterase

To gain insight into the catalytic activities of *P. putida* MPE, we produced the protein in *E. coli* as a His₁₀Smt3 fusion and purified it from a soluble extract by serial nickel affinity, tag removal, and gel filtration steps. MPE eluted as a monomer during gel filtration. The protein was tested for its ability to hydrolyze the generic non-nucleotide phosphoester substrates *p*-nitrophenyl phosphate (a phosphomonoester) and bis-*p*-nitrophenyl phosphate (a phosphodiester). Hydrolysis of these compounds liberates a chromogenic product, *p*-nitrophenol, that is easily quantified by its absorbance at 410 nm. MPE readily converted 10 mM bis-p-nitrophenyl phosphate into p-nitrophenol in the presence of a divalent cation. When various metals were tested at 2 mM concentration, the divalent cation requirement for bis-p-nitrophenyl phosphate hydrolysis was satisfied best by manganese (Fig. 8A). Cobalt and cadmium were 69% and 50% as effective as manganese; nickel was 6% as effective. Other divalent cations added at the same concentration (calcium, magnesium, copper, and zinc) were ineffective. No *p*-nitrophenol release was detected when 1 μ g of MPE was reacted with 10 mM p-nitrophenyl phosphate and any of the divalent metals tested. We conclude that MPE is a manganesedependent phosphodiesterase.

The extent of hydrolysis of 10 mM bis-p-nitrophenyl phosphate during a 30-min reaction with MPE in the presence of 2 mm manganese was proportional to the amount of input enzyme (Fig. 8B). The amount of product formed by 1 μ g of MPE in 30 min represented hydrolysis of 87% of the input bis*p*-nitrophenyl phosphate. From the slope of the titration curve in the linear range of enzyme dependence, we calculated that 46.2 ± 1.6 nmol of bis-*p*-nitrophenyl phosphate were hydrolyzed per 1 pmol of MPE during a 30-min reaction. The kinetic profiles of *p*-nitrophenol release by 3.125, 6.25, and 25 pmol MPE during a 30-min reaction with 10 mM bis-*p*-nitrophenyl phosphate revealed that the rates and extents of product formation were proportional to the input enzyme (Fig. 8C). Formation of p-nitrophenol by MPE displayed a hyperbolic dependence on the concentration of bis-p-nitrophenyl phosphate (Fig. 8D). From a nonlinear regression fit of the data to the Michaelis–Menten equation, we calculated a K_m of 4.85 mM and a k_{cat} of 212 s⁻¹.

To further query the substrate specificity of MPE, we assayed the enzyme for activity with *p*-nitrophenyl-5'-thymidylate (pNP-TMP), a phosphodiesterase-nuclease substrate (Fig. 9). MPE catalyzed the hydrolysis of 10 mM pNP-TMP to yield *p*-nitrophenol. pNP-TMP hydrolysis was even more strictly dependent on manganese compared with bis-*p*-nitrophenyl phosphate hydrolysis under the same experimental conditions. Other divalent metals added at the same concentration were largely ineffective as cofactors for pNP-TMP hydrolysis, with





Figure 8. MPE is a manganese-dependent *p***-nitrophenyl phosphodiesterase.** *A*, reaction mixtures (50 μ l) containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, 10 mM pNPP or bis-pNPP, 1 μ g MPE (41.2 pmol, corresponding to 0.82 μ M MPE), and either no divalent cation (lane –) or 2 mM calcium, cadmium, cobalt, copper, magnesium, manganese, nickel, or zinc (as chloride salts) were incubated at 37 °C for 30 min. The extents of *p*-nitrophenol formation are plotted in bar graph format. Each datum represents the average of three separate experiments \pm S.E. *B*, reaction mixtures (50 μ l) containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, 2 mM MnCl₂, 10 mM bis-pNPP, and MPE as specified (0.78, 1.56, 3.13, 6.25, 12.5, 25, or 50 pmol, corresponding to 0.016, 0.031, 0.063, 0.25, 0.25, 0.5, or 1 μ M MPE) were incubated 37 °C for 30 min. The extent of *p*-nitrophenol formation is plotted as a function of input enzyme. Each datum represents the average of three independent enzyme titration experiments \pm S.E. *C*, reaction mixtures (500 μ l) containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, 2 mM MnCl₂, 10 mM bis-pNPP, and either 3.125 pmol (*filled circles*), 6.25 pmol (*open squares*), or 25 pmol (*open circles*) MPE protein were incubated at 37 °C. Aliquots (50 μ l) were withdrawn at specified times and quenched with EDTA and Na₂CO₃. The extent of *p*-nitrophenol formation is plotted as a function of freaction of reaction mixtures (50 μ l) containing 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 2 mM MnCl₂, 0.125 μ M (6.25 pmol) (*DPE*, containing 20 mM Tris-HCl (pH 7.5), 30 mM NaCl, 2 mM MnCl₂, 0.125 μ M (6.25 pmOl) MPE, and 0.625, 1.25, 2.5, 5, or 10 mM bis-pNPP. Were incubated at 37 °C for 30 min. The extend of bis-pNPP concentration. Each datum represents the average of three independent time course experiments \pm S.E. *D*, steady-state kinetic parameters for hydrolysis of bis-pNPP. Were incubated at 37 °C for 30 min. The extend of bis-pNPP concentration is plotted as a function. Each datum is the avera

cadmium, cobalt, and nickel supporting 4–10% the level of activity seen with manganese (Fig. 8A). We determined steady-state kinetic parameters for Mn²⁺-dependent hydrolysis of pNP-TMP as a function of pNP-TMP concentration. From a nonlinear regression fit of the data to the Michaelis–Menten equation, we calculated a K_m of 11.3 mM and a k_{cat} of 34 s⁻¹. These results indicate that MPE has vigorous phosphodiesterase activity and does not hydrolyze phosphomonoesters.

MPE is a Mn²⁺-dependent DNase

In light of the predicted homology of *P. putida* MPE to Mre11 and its ability of hydrolyze *p*-nitrophenyl-5'-thymidylate to *p*-nitrophenol and TMP (Fig. 9), we tested MPE for deoxynuclease activity by reacting the protein with pUC19 plasmid DNA in the presence of 2 mM manganese. Aliquots of the reaction mixture were withdrawn at 1, 2, 5, 10, 20, 30, and 45 min and analyzed by native agarose gel electrophoresis in the presence of ethidium bromide to stain the DNA (Fig. 10*A*). The input pUC19 DNA substrate comprised a mixture of closed circle (the major species), nicked circle, and dimer circle forms (Fig. 10*A*, *time 0*). Reaction with MPE converted the closedcircle DNA into nicked-circle DNA within 1 to 2 min, and the nicked-circle DNA was in turn converted to 2.7-kb linear DNA between 2 and 10 min (Fig. 10*A*). Between 5 and 45 min, the linear DNAs were progressively shortened (Fig. 10*A*).

The order of events in the time course of the MPE reaction with pUC19 was echoed when we analyzed the products of a 30-min reaction at varying levels of input MPE (Fig. 10*B*). At limiting MPE (0.5 μ g), the closed-circle DNA was converted to nicked-circle DNA. Increasing MPE to 1 and 2 μ g prompted the appearance of linear DNA, and a further increase to 4 μ g MPE resulted in digestion to DNA fragments of less than 1 kb (Fig. 10*B*). No digestion of the pUC19 DNA substrate was observed when 2 mM magnesium was added as the divalent cation (Fig. 10*B*). We conclude that *P. putida* MPE is a manganese-dependent DNA endonuclease.

To check that the observed phosphodiesterase and nuclease activities are inherent to *P. putida* MPE, we produced and purified a mutated version of MPE in which the putative active site residue Asp-78 (Fig. 7) was changed to alanine. SDS-PAGE showed comparable purity of the WT and D78A proteins (Fig. 11*A*). The D78A mutation reduced manganese-dependent bis-





Figure 9. Hydrolysis of *p*-nitrophenyl-5'-thymidylate. *A*, divalent cation specificity. Reaction mixtures (50 μ l) containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, 10 mM pNP-TMP, 1 μ g MPE (41.2 pmol, corresponding to 0.82 μ M MPE), and either no divalent cation (lane –) or 2 mM calcium, cadmium, cobalt, copper, magnesium, manganese, nickel, or zinc (as chloride salts) were incubated at 37 °C for 30 min. The extent of *p*-nitrophenol formation is plotted in bar graph format. Each datum represents the average of three separate experiments ± S.E. *B*, kinetic parameters. Reaction mixtures (50 μ l) containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, 2 mM MnCl₂, 0.5 μ M (25 pmol) MPE, and 0.625, 1.25, 2.5, 5, or 10 mM pNP-TMP were incubated at 37 °C for 1 min. *p*-nitrophenol formation (nanomoles per minute) is plotted as a function of pNP-TMP concentration. Each datum is the average of three separate experiments ± S.E. A nonlinear regression curve fit of the data to the Michaelis–Menten equation (in Prism) is shown. The K_m and k_{cat} values are indicated.



Figure 10. MPE is a manganese-dependent DNase. *A*, reaction mixtures containing (per 20 μl) 20 mM Tris-HCI (pH 8.0), 30 mM NaCl, 2 mM MnCl₂, 800 ng of circular pUC19 DNA, and 4 μg MPE (165 pmol, 8.24 μM MPE) were incubated at 37 °C. Aliquots (20 μl) were withdrawn at the specified times and quenched immediately by adding 10 μl of a solution containing 50% glycerol, 50 mM EDTA, and 0.3% SDS. The reaction products were analyzed by electrophoresis through a 0.7% native agarose gel and visualized by staining with ethidium bromide. The closed circle, nicked circle, and linear of pUC19 DNA are indicated on the *left*. The positions and sizes (kilobase pairs) of linear DNA markers are shown on the *right. B*, reaction mixtures (20 μl) containing 20 mM Tris-HCI (pH 8.0), 30 mM NaCl, 800 ng of circular pUC19 DNA, 2 mM MnCl₂ or MgCl₂ (where indicated by +), and MPE as indicated (0.5, 1, 2, or 4 μg, corresponding to 1.03, 2.06, 4.12, or 8.24 μM MPE) were incubated at 37 °C for 30 min. The reaction products were analyzed as specified in A.

p-nitrophenylphosphatase activity to 6% of the WT (Fig. 11*B*) and similarly attenuated the DNA endonuclease activity (Fig. 11*C*).

Discussion

Interest in Lhr helicase is driven by its distinctive domain organization and mode of ssDNA binding and the strong induction of its expression after DNA damage in mycobacteria (1–4). Having solved the crystal structure of the *M. smegmatis* Lhr-(1–856) (2) and noting the widespread distribution of Lhr-Core homologs in diverse bacteria as part of a gene cluster of putative nucleic acid repair enzymes (Fig. 1), we were eager to biochemically characterize Lhr-Core and its cluster neighbor MPE from an exemplary bacterium; in this case, *P. putida*.

We show here that *P. putida* Lhr-Core is an ssDNA-dependent ATPase/dATPase, an ATP-dependent 3'-to-5' ssDNA translocase, and an ATP-dependent 3'-to-5' helicase. Lhr-Core unwinds 3'-tailed duplexes in which the loading/tracking strand is DNA and the displaced strand is either DNA or RNA. These properties of *P. putida* Lhr-Core echo those of *M. smegmatis* Lhr, with a few differences: *P. putida* Lhr-Core ATPase is most active with magnesium as the metal cofactor, whereas *M. smegmatis* Lhr-Core ATPase is most active with calcium (1), and *M. smegmatis* Lhr prefers to unwind a 3'-tailed duplex with a DNA loading strand in which the displaced strand is RNA (1).

We report that *P. putida* MPE is a manganese-dependent phosphodiesterase that releases *p*-nitrophenol from bis-*p*-nitrophenyl phosphate and *p*-nitrophenyl-5'-thymidylate but





Figure 11. Effect of mutating MPE active site residue Asp-78. *A*, MPE purity. Aliquots (5 μ g) of the Superdex preparations of WT MPE and the D78A mutant were analyzed by SDS-PAGE. The Coomassie Blue–stained gel is shown. The position and sizes (kilodaltons) of marker polypeptides are indicated on the *left. B*, phosphodiesterase reaction mixtures (50 μ l) containing 100 mM Tris-HCl (pH 8.0), 30 mM NaCl, 10 mM bis-pNPP, 2 mM MnCl₂, and 1 μ g (41.2 pmol, 0.82 μ M) WT or D78A MPE were incubated at 37 °C for 30 min. The extent of formation of *p*-nitrophenol is plotted. Each datum represents the average of three separate experiments \pm S.E. *C*, DNase reaction mixtures (20 μ l) containing 20 mM Tris-HCl (pH 8.0), 30 mM NaCl, 800 ng of circular pUC19 DNA, 2 mM MnCl₂, and either no enzyme (lane –) or 4 μ g WT or D78A MPE (165 pmol, 8.24 μ M MPE) were incubated at 37 °C for 30 min. The reaction products were analyzed by telectrophoresis through a 0.7% native agarose gel and visualized by staining with ethidium bromide. The closed circle, nicked circle, and linear forms of pUC19 DNA are indicated on the *right*. The positions and sizes (kilobase pairs) of linear DNA markers are shown on the *left*.

displays no detectable phosphomonoesterase activity against *p*-nitrophenyl phosphate. The key finding regarding a putative nucleic acid repair function is that MPE is a manganese-dependent DNA endonuclease that sequentially converts a closedcircle plasmid DNA to nicked-circle and linear forms prior to degrading the linear DNA to produce progressively smaller fragments. The biochemical activities of MPE and structure prediction in Phyre2 point to MPE as a new bacterial homolog of Mre11. Mre11 is the manganese-dependent nuclease subunit of a DNA end-processing complex (which also includes an SMC-like ATPase subunit, Rad50) that functions in cellular pathways of double-strand break repair, e.g. homologous recombination and nonhomologous end-joining (NHEJ) (14). The purified recombinant *P. putida* MPE enzyme is active as a DNA nuclease in the absence of other *P. putida* proteins. Although Lhr-Core is an ATPase, it is structurally and functionally distant from Rad50-like SMC proteins, particularly because it lacks their characteristic coiled-coil structures. Preliminary experiments using the recombinant P. putida Lhr-Core and MPE proteins showed that they did not form a heteromeric complex when mixed in vitro and that addition of Lhr-Core and ATP did not stimulate the DNase activity of MPE.

Clustering on the bacterial chromosome of genes encoding enzymes with related biochemical activities is suggestive of their participation in a common physiological pathway. In this case, we find that *bona fide* helicase (Lhr-Core) and DNA endonuclease (MPE) enzymes are genetically linked to a putative DNA ligase and a putative exonuclease. The 552-aa *P. putida* DNA ligase belongs unambiguously to the ATP-dependent DNA ligase clade; per BLAST search with its amino acid sequence and analysis in Phyre2 (13), the ligase consists of an N-terminal DNA-binding domain, a central adenylyltransferase domain, and a C-terminal OB domain, which are homologous to the equivalent domains of human DNA ligase I (15).

The fourth gene in the Lhr-Core cluster encodes a putative nuclease of the metallo- β -lactamase (MBL) family. MBL nucleases that play key roles in RNA and DNA transactions include the endoribonucleases tRNAase Z (3' processing of tRNA) and CPSF-73 (3' end formation of mRNA), the DNA 5' exonucleases SNM1A and SNM1B/Apollo (repair of interstrand DNA cross-links), and the DNA endonuclease Artemis (DNA break repair via NHEJ) (16, 17). The catalytic unit of MBL nucleases consists of an MBL domain and a β -CASP domain. The phosphodiesterase active site is formed by two catalytic zinc ions coordinated by an ensemble of histidine and aspartate side chains in the MBL domain (16). Phyre2 analysis of the putative 338-aa P. putida Exo protein returned a structural model (100% confidence) based on homology to the crystal structure of human SNM1B/Apollo (18). The fold of the Phyre2 model of *P. putida* Exo (Fig. 12A) consists of MBL and β -CASP domains. The Phyre2-generated primary structure alignment of P. putida Exo and human Apollo highlights 79 positions of side chain identity/similarity spanning the segment of P. putida Exo from amino acids 8 to 304 (Fig. 12C). A stereo view of the structural model of the active site of *P. putida* Exo is shown in Fig. 12B, superimposed on the active site ligands of the Apollo crystal structure (PDB code 5AHO), these being two zinc ions, a zinc-bound water, and tartrate anion posited to mimic the scissile phosphodiester (18). The histidine and aspartate side chains that coordinate the zinc ions and tartrate in Apollo are conserved in P. putida Exo (Fig. 12C). Although this in silico exercise affirms that P. putida Exo is likely to be a nuclease, our aim to biochemically characterize P. putida Exo has been stymied by the insolubility of the protein when produced in E. coli via the same protocols used to make Lhr-Core and MPE.

Nonetheless, it is tempting at this juncture to speculate that Exo is a candidate ortholog of SNM1A, SNM1B/Apollo, or Artemis and thereby might play a role, along with the helicase Lhr-Core, endonuclease MPE, and DNA ligase, in a dedicated





Figure 12. Structural model of *P. putida* **Exo.** *A*, Phyre2-modeled tertiary structure of Exo based the on the crystal structure of the human 5' exonuclease Apollo (18) and consisting of metallo- β -lactamase and β -CASP domains. The N and C termini are indicated. *B*, stereo view of the active site of the Phyre2-modeled Exo superimposed on the active site ligands of the Apollo crystal structure (PDB code 5AHO), these being two zinc ions (*green spheres*), a zinc-bound water (*red sphere*), and a tartrate anion (*stick model* with *gray carbons*) posited to mimic the scissile phosphodiester (18). The Exo histidine and aspartate side chains that coordinate the zinc ions and tartrate are shown as *stick models* with *beige carbons*. Atomic contacts are denoted by *dashed lines*. *C*, Phyre2-based alignment of the amino acid sequences of Ppu Exo and human Apollo. Positions of side chain identity/similarity are indicated by *dots* above the sequence. Gaps in the alignment are denoted by *dashes*. The conserved active site residues depicted in *B* are indicated by *arrowheads* below the Apollo sequence.

DNA repair pathway, potentially involving inter-strand DNA cross-links (an appealing idea in light of the apparent role of mycobacterial Lhr and eukaryal SNM1s in mitomycin C sensitivity)³ or NHEJ.

It is worth noting that the *P. putida* KT2440 proteome (5) has, in addition to the Lhr-Core helicase that is part of the class Ia cluster, a full-length Lhr protein (1434 aa; NCBI NP_743222.1) encoded elsewhere on the bacterial chromosome (locus tag PP_1061). To our knowledge, there has been no report of the effects of genetically ablating these enzymes in *P. putida*. However, a recent study via genome-wide transposon mutagenesis of *P. fluorescens* indicated that the full-length Lhr and each of the four enzymes of the Exo-ligase-Lhr-Core·MPE cluster were inessential for viability and that strains with transposon inserts in these genes were not sensitized to the DNA cross-linking agent cisplatin (20). It is conceivable that *Pseudomonas fluorescens* Lhr and Lhr-Core are genetically redundant in this regard. On the other hand, two bacteria that

have a class III Lhr-Core-MPE cluster, *Caulobacter crescentus* and *Dinoroseobacter shibae* (neither of which has a full-length Lhr), are sensitized to cisplatin when the Lhr-Core or MPE genes are disrupted by transposons (20).

A potential role for the MPE nuclease in NHEJ is attractive given MPE's relatedness to Mre11. Bacterial NHEJ, as understood in *M. smegmatis*, is genetically dependent on the endbinding protein Ku and the multifunctional end processing and sealing enzyme LigD (21). Mycobacterial NHEJ is mutagenic, by virtue of the addition and excision of nucleotides at the DSB ends prior to end sealing by DNA ligase (21). Nucleotide addition at DSB ends is performed by the polymerase module of LigD (21), but the nuclease(s) responsible for end resection during bacterial NHEJ are unknown. Although *M. smegmatis* and *Mycobacterium tuberculosis* lack the Exo·ligase·Lhr-Core·MPE gene cluster, the cluster is present in many *Pseudomonas* species (albeit not in *Pseudomonas aeruginosa*). *P. putida*, like *P. aeruginosa* (22), has both Ku and LigD. We speculate that one or more of the enzymes in the Exo·ligase·Lhr-Core·MPE gene cluster can contribute to NHEJ, either in a pathway parallel to Ku-LigD or in the Ku-LigD pathway as backup activities, *e.g.* for the DNA ligase (21).

Finally, we refer the interested reader to an Lhr-Core– based subsystem in the SEED viewer (19) that tabulates the distribution of Lhr-Core in bacterial and archaeal genomes and its clustering with MPE, DNA ligase, and/or metallo- β -lactamase Exo enzymes. In brief, Lhr-Core is clustered genetically with the other three enzymes in various bacteria belonging to the phyla Proteobacteria, Bacteroidetes, Chlamydiae, Cyanobacteria, Planctomycetes, Verrucomicrobia, and Gemmatimonadetes. The class III cluster arrangement Lhr-Core·MPE is found in several archaeal taxa from the phyla Euryarchaeota and Korarchaeota.

Experimental procedures

Purification of recombinant Lhr-Core and MPE proteins

The ORFs encoding full-length P. putida Lhr-Core (PP 1103; NCBI accession number NP 743264) and MPE (PP_1102; NCBI accession NP_743263.1) were PCR-amplified from P. putida strain KT2440 genomic DNA (5) with primers that introduced a BamHI site immediately flanking the start codon and a HindIII site downstream of the stop codon. The PCR products were digested with BamHI and HindIII and inserted between the BamHI and HindIII sites of pET28b-His10Smt3 to generate expression plasmids encoding the Lhr-Core or MPE polypeptides fused to an N-terminal His₁₀Smt3 tag. MPE mutation D78A was introduced into the expression plasmid by PCR with mutagenic primers. All of the plasmid inserts were sequenced to verify that no unintended coding changes were acquired during amplification and cloning. pET28b-His10Smt3-Lhr-Core and pET28b-His10Smt3-MPE plasmids were transformed into E. coli BL21(DE3) cells. Cultures (2 liters) amplified from single kanamycin-resistant transformants were grown at 37 °C in Terrific Broth containing 60 μ g/ml kanamycin until the A_{600} reached 0.8–1.0. The cultures were chilled on ice for 1 h, adjusted to 2% (v/v) ethanol and 0.3 mM isopropyl- β -D-thiogalactopyranoside, and incubated for 16 h at 17 °C with constant shaking. All subsequent steps were performed at 4 °C. Cells were harvested by centrifugation and resuspended in 25 ml of buffer A (50 mM Tris-HCl (pH 8.0), 500 mм NaCl, 1 mм DTT, 20 mм imidazole, 10% sucrose) containing one protease inhibitor mixture tablet (Roche). Lysozyme was added to a concentration of 1 mg/ml. After incubation for 1 h, the lysate was sonicated to reduce viscosity, and the insoluble material was removed by centrifugation at 38,000 \times *g* for 1 h. The supernatant was mixed for 1 h with 3 ml of nickelnitrilotriacetic acid-agarose resin (Qiagen) that had been equilibrated with buffer A. The resin was recovered by centrifugation and resuspended in 30 ml of buffer A. The resin was again recovered by centrifugation and then resuspended in 20 ml of buffer B (50 mм Tris-HCl (pH 8.0), 500 mм NaCl, 1 mм DTT, 10% glycerol) containing 50 mM imidazole and poured into a column. After washing the column with 10 ml of 3 M KCl solution, the bound material was eluted stepwise with 5-ml aliquots of buffer B containing 100, 200, and 500 mM imidazole.

The polypeptide compositions of the eluate fractions were monitored by SDS-PAGE. The recombinant proteins eluted in the 200 and 500 mM imidazole fractions, which were pooled, supplemented with the Smt3-specific protease Ulp1 (at a His10Smt3-Lhr-Core:Ulp1 or His10Smt3-MPE:Ulp1 ratio of 1000:1), and then dialyzed overnight against 4 liters of buffer C (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM DTT, 20 mM imidazole, 10% glycerol). The tag-free proteins were separated from His10Smt3 by applying the dialysates to a 2-ml nickelnitrilotriacetic acid-agarose column that had been equilibrated with buffer C; the Lhr-Core and MPE proteins were recovered in the flow-through fractions. The tag-free Lhr-Core and MPE preparations were then subjected to gel filtration through a 120-ml Superdex-200 column equilibrated with buffer D (20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM DTT, 10% glycerol). The gel filtration column had been calibrated with a mixture of size standards: thyroglobulin, γ -globulin, ovalbumin, myoglobin, and vitamin B12. Lhr-Core chromatographed as a single symmetrical peak at an elution volume of 75 ml; interpolation to the standard curve (of the log of the molecular weights of standards versus their elution volume) indicated a native size of 88 kDa, consistent with the calculated size of 90 kDa from the Lhr-Core amino acid sequence. MPE eluted as a single peak at 86 ml, corresponding to a native size of 28 kDa, versus the calculated size of 24 kDa. We surmise that Lhr-Core and MPE are monomers in solution. Peak Superdex fractions were pooled and concentrated by centrifugal ultrafiltration. Protein concentrations were determined with Bio-Rad dye reagent using BSA as the standard. The final yield of Lhr-Core was 7 mg per liter of bacterial culture. The yields of WT MPE and mutant MPE-D78A were 25 mg per liter and 3 mg per liter, respectively.

Lhr-Core ATPase assay

Reaction mixtures (10 μ l) containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM [α -³²P]ATP, 5 μ M 24-mer ssDNA oligonucleotide, and Lhr-Core were incubated for 15 min at 37 °C. Reactions were quenched by adding 2 μ l of 5 M formic acid. An aliquot (2 μ l) of the mixture was spotted on a polyethyleneimine– cellulose TLC plate. Ascending TLC was performed with 0.45 M ammonium sulfate as the mobile phase. The extent of conversion of [α -³²P]ATP to [α -³²P]ADP was quantified by scanning the TLC plate with a Fujix BAS2500 imager.

Streptavidin displacement assay of Lhr-Core translocation on DNA

Synthetic 34-mer oligodeoxynucleotides containing a Biotin-ON inter-nucleotide spacer at the fourth position from the 5' end or the second position from the 3' end were 5' 32 Plabeled by reactions with T4 Pnk and [γ - 32 P]ATP. The labeled DNAs were purified by electrophoresis through a native 18% polyacrylamide gel and eluted from an excised gel slice by overnight incubation at 4 °C in 400 μ l of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl. Translocation reaction mixtures (10 μ l) containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 5 mM MgCl₂, 1 mM ATP, 0.1 μ M (1 pmol) biotinylated ³²P-labeled DNA, and 4 μ M streptavidin (Sigma) were preincubated at room temperature for 10 min to form SA–DNA complexes.





The mixtures were supplemented with 40 μ M free biotin (Fisher), and the translocation reactions were initiated by adding Lhr-Core. After incubation at 37 °C for the specified times, the reactions were quenched by adding 3 μ l of a solution containing 200 mM EDTA, 0.6% SDS, 25% glycerol, and 20 μ M unlabeled single-stranded 59-mer DNA oligonucleotide. The reaction products were analyzed by electrophoresis through a 15-cm native 18% polyacrylamide gel containing 89 mM Tris borate and 2.5 mM EDTA. The free ³²P-labeled 34-mer DNA and the SA–DNA complexes were visualized and quantified by scanning the gel with a Fujix BAS-2500 imaging apparatus.

Lhr-Core helicase assay

5' ³²P-labeled synthetic 24-mer DNA or RNA oligonucleotides were prepared by reaction with T4 polynucleotide kinase (Pnk) and $[\gamma^{-32}P]$ ATP. The kinase reaction mixture was heated to 95 °C to inactivate T4 Pnk. The labeled DNA or RNA strand was then annealed to a 3-fold excess of complementary 39-mer DNA or RNA strand to form 3'-tailed duplex helicase substrates. The tailed duplexes were purified by electrophoresis through a native 12% polyacrylamide gel and eluted from an excised gel slice by overnight incubation at 4 °C in 400 μ l of either 10 mM Tris-HCl (pH 6.8), 1 mM EDTA, 50 mM NaCl for RNA-containing substrates or 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl for the all-DNA substrate. Helicase reaction mixtures (10 µl) containing 20 mM Tris-HCl (pH 7.5), 1 тм DTT, 5 тм MgCl₂, 50 пм (0.5 pmol) ³²P-labeled tailed duplex substrate, and Lhr-Core as specified were preincubated for 10 min at room temperature. The unwinding reaction was initiated by addition of 1 mM ATP and a 10-fold excess of an unlabeled oligonucleotide identical to the labeled strand of the helicase substrate. Addition of an excess of unlabeled strand was necessary to prevent the spontaneous reannealing of the unwound ³²P-labeled strand. The reaction mixtures were incubated for 30 min at 37 °C and then guenched by adding 1.5 μ l of a solution containing 3.5% SDS, 80 mM EDTA, and 10 μ g proteinase K. After protease digestion for 5 min at 37 °C, the mixtures were supplemented with 4 μ l of 50% glycerol, 0.3% bromphenol blue. The reaction products were analyzed by electrophoresis through a 15-cm 10% polyacrylamide gel in 89 mM Tris borate, 2.5 mM EDTA. The products were visualized by autoradiography of the dried gel.

MPE hydrolysis of p-nitrophenyl phosphoesters

p-nitrophenyl phosphate (pNPP), bis-*p*-nitrophenyl phosphate (bis-pNPP) and *p*-nitrophenyl-5'-thymidylate (pNP-TMP) were purchased from Sigma. Reaction mixtures (25 μ l) containing 100 mM Tris-HCl (pH 8.0), 10 mM *p*-nitrophenyl phosphoester, divalent cation, and MPE as specified were incubated for 30 min at 37 °C. The reactions were quenched by adding 50 μ l of 50 mM EDTA and then 0.9 ml of 1 M Na₂CO₃. Release of *p*-nitrophenol was determined by measuring A_{410} and interpolating the value to a *p*-nitrophenol standard curve.

MPE DNA nuclease assay

Reaction mixtures (20 μ l) containing 20 mM Tris-HCl (pH 8.0), 2 mM MnCl₂, 800 ng of pUC19 plasmid DNA, and MPE as specified were incubated at 37 °C. The reactions were quenched

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at the specified times by adding 10 μ l of a solution containing 50% glycerol, 50 mM EDTA, and 0.3% SDS. The mixtures were extracted serially with equal volumes of phenol-CHCl₃ and CHCl₃. The aqueous phase samples were then analyzed by electrophoresis through a horizontal 0.7% agarose gel containing 45 mM Tris borate, 1.2 mM EDTA, and 0.5 μ g/ml ethidium bromide. Ethidium bromide–stained DNA was visualized via UV transillumination.

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