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Enzymatic Properties of an Ecto-nucleoside Triphosphate Diphosphohydrolase from *Legionella pneumophila*

SUBSTRATE SPECIFICITY AND REQUIREMENT FOR VIRULENCE[*]

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Legionella pneumophila* is the predominant cause of Legionnaires disease, a severe and potentially fatal form of pneumonia. Recently, we identified an ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) from *L. pneumophila*, termed Lpg1905, which enhances intracellular replication of *L. pneumophila* in eukaryotic cells. Lpg1905 is the first prokaryotic member of the CD39/NTPDase1 family of enzymes, which are characterized by the presence of five apyrase conserved regions and the ability to hydrolyze nucleoside tri- and diphosphates. Here we examined the substrate specificity of Lpg1905 and showed that apart from ATP and ADP, the enzyme catalyzed the hydrolysis of GTP and GDP but had limited activity against CTP, CDP, UTP, and UDP. Based on amino acid residues conserved in the apyrase conserved regions of eukaryotic NTPDases, we generated five site-directed mutants, Lpg1905E159A, R122A, N168A, Q193A, and W384A. Although the mutations E159A, R122A, Q193A, and W384A abrogated activity completely, N168A resulted in decreased activity caused by reduced affinity for nucleotides. When introduced into the *lpg1905* mutant strain of *L. pneumophila*, only N168A partially restored the ability of *L. pneumophila* to replicate in THP-1 macrophages. Following intratracheal inoculation of A/J mice, none of the Lpg1905 mutants was able to restore virulence to an *lpg1905* mutant during lung infection, thereby demonstrating the importance of NTPDase activity to *L. pneumophila* infection. Overall, the kinetic studies undertaken here demonstrated important differences to mammalian NTPDases and different sensitivities to NTPDase inhibitors that may reflect underlying structural variations.

Legionella pneumophilia is the major causative agent of Legionnaires disease, a severe systemic disease characterized by acute pneumonia. During infection of the human lung, *L. pneumophila* is internalized by alveolar macrophages where the bacteria replicate within an intracellular vacuole that evades fusion with the endocytic pathway. Instead the *L. pneumophila* containing vacuole intercepts early secretory vesicles and following maturation exhibits properties of the endoplasmic reticulum (1–3). Establishment of the unique *Legionella* vacuole and the ability to replicate within eukaryotic cells requires the Dot/Icm type IV secretion system, which translocates bacterial effector proteins into the cytoplasm of the host cell (4–7).

The ability of *L. pneumophila* to replicate within mammalian cells appears to be a consequence of its association in the natural environment with free living protozoa. The three sequenced *L. pneumophila* genomes encode an unusually high number of proteins with similarity to eukaryotic proteins (8–10), which may interfere with host cell processes by functional mimicry (11). We recently showed that Lpg1905 from *L. pneumophila* is a secreted member of the CD39/NTPDase1 family of ecto-nucleoside triphosphate diphosphohydrolases (NTPDases; gene family ENTPD).[5] NTPDases are extremely rare in bacteria, and Lpg1905 is the first prokaryotic member of the CD39/NTPDase1 family to be characterized (12).

NTPDases are important ectonucleotidases that are characterized by the presence of five apyrase conserved regions (abbreviated as ACR1 to ACR5) and by the ability to hydrolyze NTPs and nucleoside diphosphates (NDPs) to the monophosphate form. Mammalian NTPDases play a major role in the regulation of blood clotting, inflammatory processes, and

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[5] The abbreviations used are: NTPDase, nucleoside triphosphate diphosphohydrolase; ACR, apyrase conserved region; NDP, nucleoside diphosphate; CI, competitive index; P2, purinergic type 2.

TABLE 1
Bacterial strains and plasmids used in this study

Bacterial strain/plasmid	Relevant characteristics	Source
L. pneumophila		
130b	O1; clinical isolate	Ref. 81
130b (pMIP)	130b carrying pMIP	Ref. 12
lpg1905::*km*	*lpg1905* insertion mutant of 130b (Km^R)	Ref. 12
lpg1905::*km* (pMIP)	*lpg1905*::*km* carrying pMIP	Ref. 12
lpg1905::*km* (pMIP:*lpg1905*)	*lpg1905*::*km* carrying pMIP:*lpg1905*	Ref. 12
lpg1905::*km* (pMIP:*lpg1905*E159A)	*lpg1905*::*km* carrying pMIP:*lpg1905*E159A	Ref. 12
lpg1905::*km* (pMIP:*lpg1905*R122A)	*lpg1905*::*km* carrying pMIP:*lpg1905*R122A	This study
lpg1905::*km* (pMIP:*lpg1905*N168A)	*lpg1905*::*km* carrying pMIP:*lpg1905*N168A	This study
lpg1905::*km* (pMIP:*lpg1905*Q193A)	*lpg1905*::*km* carrying pMIP:*lpg1905*Q193A	This study
lpg1905::*km* (pMIP:*lpg1905*W384A)	*lpg1905*::*km* carrying pMIP:*lpg1905*W384A	This study
E. coli		
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F′*proAB*$^+$ *lacI*^{q}*lacZ*ΔM15 Tn10) (Tet^R)	Stratagene
BL21 (DE3)	F^- *ompT hsdS*$_B$(r_B^-*mB*$^-$)*gal dcm* (DE3)	Novagen
Plasmids		
pGEM-T Easy	3015 bp, Amp^R, high copy cloning	Promega
pGEM:*lpg1905*	Full-length *lpg1905* cloned into BamHI/HindIII	This study
pGEM:*lpg1905*R122A	Arg^{122} mutated to Ala in pGEM:*lpg1905*	This study
pGEM:*lpg1905*N168A	Asn^{168} mutated to Ala in pGEM:*lpg1905*	This study
pGEM:*lpg1905*Q193A	Gln^{193} mutated to Ala in pGEM:*lpg1905*	This study
pGEM:*lpg1905*W384A	Trp^{384} mutated to Ala in pGEM:*lpg1905*	This study
pRSETb	2897bp, Amp^R, *E. coli* expression vector with a bacteriophage T7 promoter and N-terminal polyhistidine (His_6) tag	Invitrogen
pRSET:*lpg1905*	Truncated enzymatically active form of *lpg1905* cloned into EcoRI/HindIII (missing the first 34 amino acids corresponding to a putative signal peptide sequence)	Ref. 12
pRSET:*lpg1905*E159A	Glu^{159} mutated to Ala in pRSET:*lpg1905*	Ref. 12
pRSET:*lpg1905*R122A	Arg^{122} mutated to Ala in pRSET:*lpg1905*	This study
pRSET:*lpg1905*N168A	Asn^{168} mutated to Ala in pRSET:*lpg1905*	This study
pRSET:*lpg1905*Q193A	Gln^{193} mutated to Ala in pRSET:*lpg1905*	This study
pRSET:*lpg1905*W384A	Trp^{384} mutated to Ala in pRSET:*lpg1905*	This study
pMIP	9523 bp, pMMB207 with the promoter region of *mip* cloned into SacI/XbaI (Cm^R)	Ref. 37
pMIP:*lpg1905*	pMIP with full-length *lpg1905* cloned into BamHI and HindIII	Ref. 12
pMIP:*lpg1905*E159A	pMIP with full-length *lpg1905*E159A cloned into BamHI and HindIII	Ref. 12
pMIP:*lpg1905*R122A	pMIP with full-length *lpg1905*R122A cloned into BamHI and HindIII	This study
pMIP:*lpg1905*N168A	pMIP with full-length *lpg1905*N168A cloned into BamHI and HindIII	This study
pMIP:*lpg1905*Q193A	pMIP with full-length *lpg1905*Q193A cloned into BamHI and HindIII	This study
pMIP:*lpg1905*W384A	pMIP with full-length *lpg1905*W384A cloned into BamHI and HindIII	This study

immune responses (13–16). At least eight NTPDases exist in humans, and of these CD39/NTPDase1, CD39L1/NTPDase2, CD39L3/NTPDase3, and NTPDase8 are located on the cell surface. Other members of this family may be associated with intracellular organelles and membranes or may be secreted (17, 18).

A number of parasites also possess functional NTPDases, and these have been implicated in host-pathogen interactions and virulence. *Toxoplasma gondii* is an important pathogen of immunocompromised people, including pregnant women, that expresses two isoforms of NTPDase, one of which is particularly associated with virulent strains (19–22). *Schistosoma mansoni*, the cause of a chronic debilitating disease known as intestinal schistosomiasis (15, 23), also exhibits classic ecto-NTPDase activity. Other parasites that express virulence-associated NTPDase activity include *Trypanosoma* spp. *Trichomonas vaginalis*, *Leishmania* spp., and *Entamoeba histolytica* (24–29).

In mammalian systems, CD39 on both T regulatory cells (30) and endothelial cells (31) suppresses immune responses and thrombotic reactions, respectively. It is possible that microbiological expression of NTPDase-like ecto-enzymes may also decrease protective host responses. However, investigations into the role of parasite NTPDases in virulence have been hampered by the difficulty of genetic manipulation in these pathogens and/or the requirement of NTPDases for intrinsic purine scavenging and therefore parasitic viability. Despite the lack of mechanistic roles for these enzymes in infection, NTPDases have been proposed as possible targets for the development of novel anti-infective agents (32).

The relative ease of genetic manipulation in *L. pneumophila* led us to investigate the contribution of NTPDase activity to *L. pneumophila* infection in a mouse model of Legionnaires disease. In addition, we characterized the enzyme kinetics of Lpg1905 hydrolysis of ATP, ADP, GTP, and GDP to examine functional differences between microbial and mammalian NTPDases that may reflect structural differences.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Bacterial strains and plasmids used in this study are listed in Table 1. *L. pneumophilia* strains were cultured in *N*-(2-acetamido)-2-aminoethanesulfonic acid-buffered yeast extract broth or on buffered charcoal yeast extract plates (33). When required, kanamycin and chloramphenicol were added to the medium at 25 and 6 μg/ml, respectively.

Purification of Recombinant His_6-tagged Lpg1905—As described previously (12), recombinant His_6-tagged Lpg1905 (rLpg1905) was expressed using pRSET:*lpg1905* in *Escherichia coli* BL21 (DE3) C41 strain and purified using nickel column chromatography. Protein was also purified from *E. coli* BL21 carrying the empty pRSET vector to provide a control for con-

TABLE 2
Oligonucleotide sequences used in site-directed mutagenesis of Lpg1905
The codon encoding the relevant amino acid is underlined with base changes in bold.

Sequences	Use
5′-CGCAACAGCTGGTATG**GC**ACTACTACCCCAATCAC-3′ 5′-GTGATTGGGGTAGTAG**TGC**CATACCAGCTGTTGCG-3′	Sense and anti-sense oligonucleotides to mutate Lpg1905 Arg^{122} to Ala
5′-GACTGGCTTGCTGTA**GC**TTATAAACTTGATAC-3′ 5′-GTATCAAGTTTATAA**GC**TACAGCAAGCCAGTC-3′	Sense and anti-sense oligonucleotides to mutate Lpg1905 Asn^{168} to Ala
5′-GGGCGGCGCTTCCGTT**GC**AATTGTCTTTCCAATG-3′ 5′-CATTGGAAAGACAATT**GC**AACGGAAGCGCCGCCC-3′	Sense and anti-sense oligonucleotides to mutate Lpg1905 Gln^{193} to Ala

taminating NTPases and NDPases in some experiments (mock purification). For detailed analysis of the enzyme kinetics of rLpg1905, further purification was performed. Pooled fractions were dialyzed overnight against 1 liter of 20 mM Tris, pH 7.9, 50 mM NaCl at 4 °C. Dialyzed sample was concentrated using Amicon 10,000-Da molecular mass cut-off concentrators (Millipore) to a volume of 1 ml and filtered using a centrifugal filter (Ultrafree-MC; Millipore). The sample was loaded onto an S200 S16/60 column (GE Healthcare) using the buffer 20 mM Tris, pH 7.9, 150 mM NaCl at a flow rate of 1 ml/min. 2-ml fractions were collected and analyzed using Coomassie-stained SDS-PAGE. The fractions containing rLpg1905 were pooled and concentrated using Amicon 10,000-Da molecular mass cut-off concentrators before aliquots were stored at −80 °C. Protein concentration was measured using the Bio-Rad protein assay.

Assays for Enzymatic Activity of rLpg1905 and Mutant Derivatives—To determine the divalent cation preference of rLpg1905, incubation buffer containing 30 mM Tris-HCl, pH 7.4, 2 mM ATP, or 2 mM ADP and between 0 mM and 5 mM Mg^{2+}, Ca^{2+}, or Zn^{2+} ions was used. rLpg1905 was added to the reaction at 0.2 ng/μl, and the reaction was incubated at 37 °C for 10 min. Where indicated 0.1 mM EDTA or EGTA was added to the reaction. The reaction was stopped by the addition of malachite green dye reagent (1.5% w/v ammonium molybdate, 0.122% w/v malachite green hydrochloride, 6 N sulfuric acid, 0.18% v/v Tween). Incubation buffer containing 30 mM Tris-HCl and 2 mM Mg^{2+} and a variety of substrate concentrations as indicated in the figure legends (31) was used to determine the pH preference of rLpg1905 and to analyze the kinetics of rLpg1905 and rLpg1905N168A as well as in the assessment of enzyme inhibition by POM-1 ($Na_6[H_2W_{12}O_{40}]$) and POM-6 ($(NH_4)18[NaSb_9W_{21}O_{86}]$) (34). To examine enzyme kinetics a linear production of phosphate is required, and the enzyme concentration was adjusted to achieve this. To assess ATPase activity 0.01 ng/μl of protein was used, and to assess ADPase activity 0.02 ng/μl of protein was used. The reactions were incubated at 37 °C for 10 min and stopped by the addition of 5% trichloroacetic acid. To analyze hydrolysis of non-adenine nucleotides, recombinant proteins were diluted into 100 μl of assay buffer (8 mM $CaCl_2$, 5 mM levamisole, 50 mM Tris-base, 50 mM imidazole, 150 mM NaCl, pH 7.5) at a concentration of 0.2 ng/μl (for ATPase and ADPase activity), 0.4 ng/μl (for GTPase and GDPase activity), and 2 ng/μl (for CTPase, CDPase, UTPase, and UDPase activity). 25 μl of 300 μM of substrate (ATP, ADP, GTP, GDP, CTP, CDP, UTP, or UDP) was added to the wells, and the plates were incubated at 37 °C for 5–60 min. 25 μl of malachite green dye reagent was added to each well to stop the reaction (12). For all experiments P_i release was measured with reference to an appropriate range of inorganic phosphate standards (35, 36). To compare the hydrolysis of different substrates, the results were normalized (where required) by conversion to μmol Pi/min/mg enzyme before determining the relative levels of activity.

Site-directed Mutagenesis of Lpg1905—Site-directed mutagenesis was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene) on pRSET:*lpg1905* to mutate Arg^{122} to Ala (R122A), Asn^{168} to Ala (N168A), and Gln^{193} to Ala, (Q193A), giving rise to pRSET:*lpg1905R122A*, pRSET:*lpg1905N168A*, and pRSET:*lpg1905Q193A*. Primer pairs used are listed in Table 2. To mutate Trp^{384} to Ala (W384A) primers 5′-CGGGATCCGACAACAAAATATAC-ACAATGTACCAATCG-3′ and 5′-CCCAAGCTTTCAAGC-GCGGTGAAGCACGACTCCAATTGT**CGC**ATCGAGATT-TTGTTC-3′ were used to amplify full-length *lpg1905* containing the W384A mutation (underlined, base changes in bold) and cloned into pGEM using the pGEM-T-Easy Kit (Promega) according to the manufacturer's specifications. This plasmid was then used as template to amplify *lpg1905* lacking the first 34 amino acids and cloned into pRSET as described previously (12) to generate pRSET:*lpg1905W384A*. All of the mutations were confirmed by nucleotide sequencing.

Purification of Site-directed Mutant Derivatives of Lpg1905—rLpg1905E159A was expressed and purified as described previously (12). rLpg1905R122A, rLpg1905N168A, rLpg1905Q193A, and rLpg1905W384A were all expressed in derivatives of *E. coli* BL21 (DE3) and purified using nickel column chromatography, as described for rLpg1905E159A (12). Assays to assess hydrolysis of nucleoside tri- and diphosphates were performed as described for rLpg1905.

Complementation of lpg1905::km Mutant with Plasmids Expressing Site-directed Mutant Derivatives of Lpg1905—Genes for the expression of site-directed Lpg1905 mutant proteins in *L. pneumophila lpg1905::km* were reconstructed to include the native ribosome-binding site and signal peptide and cloned into the *Legionella* expression vector pMIP (37). Because the large size of pMIP precluded use of the QuikChange® II site-directed mutagenesis kit (Stratagene), primers 5′-CGGGATCCGACAACAAAATATACACAATG-TACCATTCG-3′ and 5′-CCCAAGCTTTCAAGCGCGGTG-AAGCACGACTC-3′ were used to amplify full-length *lpg1905* including the ribosome-binding site for cloning into pGEM-T-Easy (Promega). To mutate Arg^{122} to Ala, Asn^{168} to Ala, and Gln^{193} to Ala, the QuikChange® II site-directed mutagenesis kit was then used as described for mutagenesis of pRSET:*lpg1905* to generate pGEM:*lpg1905R122A*, pGEM:*lpg1905N168A*, and

pGEM:*lpg1905Q193A*. Following BamHI/HindIII digestion, each mutant derivative was then cloned into pMIP, generating pMIP:*lpg1905R122A*, pMIP:*lpg1905N168A*, pMIP:*lpg1905Q193A*, and pMIP:*lpg1905W384A*, which were then electroporated into *L. pneumophila* 130b *lpg1905*::*km* as described previously (38). All of the mutations and insertions were confirmed by nucleotide sequencing. To confirm secretion of the mutant proteins by *L. pneumophila*, supernatants of overnight broth cultures were precipitated with trichloroacetic acid, and immunoblotting was performed using monospecific anti-Lpg1905 antibodies, as described previously (12).

Intracellular Replication of L. pneumophila in THP-1 Macrophages—The ability of *L. pneumophila* 130b and recombinant derivatives to replicate over a 72-h time period in the monocytic cell line THP-1 was assayed as described previously (37). Briefly, THP-1 cells were seeded into 24-well tissue culture trays (Sarstedt, Leicestershire, UK) at a density of 5×10^5 cells/well and pretreated with 10^{-8} M phorbol 12-myristate 13-acetate for 36–48 h to induce differentiation into adherent macrophage-like cells. Stationary phase *L. pneumophila* strains were resuspended in tissue culture medium and added to THP-1 cells at a multiplicity of infection of 5. After incubation for 2 h, the cells were treated with 100 μg/ml gentamicin for 1 h to kill extracellular bacteria. Infected macrophages were then washed three times with phosphate-buffered saline before incubation with tissue culture maintenance medium. At 3, 24, 48, and 72 h THP-1 cells were lysed with 0.05% digitonin. Viable numbers of *L. pneumophila* were enumerated by serial dilution and plating onto buffered charcoal yeast extract agar.

Competitive Pulmonary Infections of A/J Mice—Intratracheal inoculations of A/J mice with *L. pneumophila* were performed as described previously except that bacteria were introduced by intubation rather than incision (39, 40). All of the animal infections were approved by the Monash University Animal Ethics Committee, application number SOBSB/B/2006/21. 6–8-week-old female A/J mice (Jackson Laboratory) were anesthetized with ketamine (75 mg/kg) and xylazine (15 mg/kg), and the mice were intubated with a 20-gauge intravenous catheter over guide wire, facilitated by transillumination of the larynx using a fiber optic light. Approximately 10^5 colony-forming units of each *L. pneumophila* strain under investigation were then instilled in 25 μl of phosphate-buffered saline followed by 25 μl of air through the catheter using a 100-μl microsyringe attached to a blunt 20 gauge needle. Mutant derivatives of *L. pneumophila* were introduced into the lung in a 1:1 ratio with the wild type strain or the wild type strain carrying pMIP as indicated. At 72 h following infection mice were euthanized, and their lung tissue was isolated. The tissue was homogenized in 5 ml of phosphate-buffered saline, and complete host cell lysis was achieved by incubation in 0.1% saponin (Sigma) for 10 min at 37 °C. Serial dilutions of the homogenate were plated onto both plain and antibiotic selective buffered charcoal yeast extract agar to determine the number of viable bacteria and the ratio of wild type to mutant bacteria colonizing the lung. The competitive index (CI) was calculated as the proportion of mutant to wild type bacteria recovered from mice divided by the proportion of mutant to wild type bacteria in the inoculum (41).

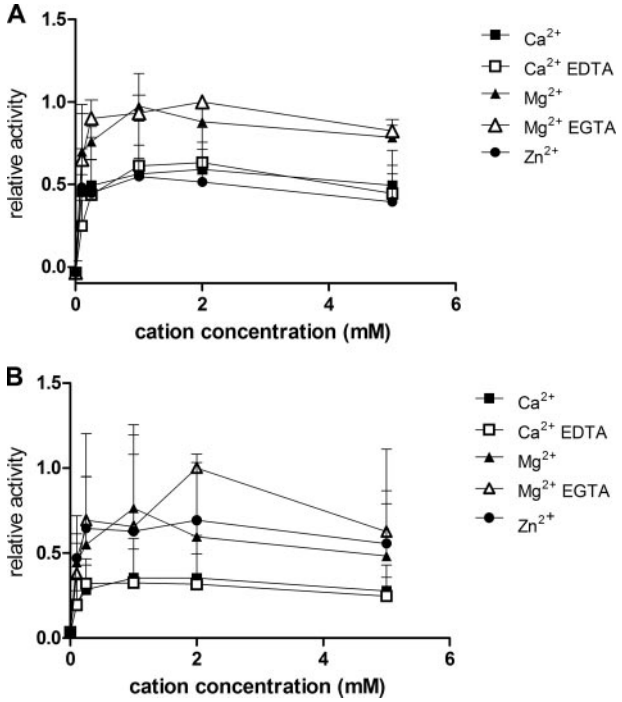

FIGURE 1. **Divalent cation preference of rLpg1905.** Shown is the relative ATPase (*A*) and ADPase (*B*) activity of rLpg1905 in the presence of Mg^{2+} ions (▲), Mg^{2+} ions and 0.1 mM EGTA (△), Ca^{2+} ions (■), Ca^{2+} ions and 0.1 mM EDTA (□), and Zn^{2+} ions (●). 2 mM ATP or ADP was used as the substrate. The results are expressed as relative activity of the highest value and are the means ± standard deviations of three experiments performed in triplicate.

RESULTS

Hydrolysis of ATP and ADP by Lpg1905 Is Dependent on Divalent Metal Cations—All of the mammalian surface-located NTPDases are inactive in the absence of Mg^{2+} or Ca^{2+} cations (13). The majority of parasitic enzymes are stimulated by Mg^{2+} or Ca^{2+} (28, 42–44), but Zn^{2+} has also been shown to stimulate ecto-NTPDase activity of *Trypanosoma brucei* (27). In contrast, ecto-NTPDase activity of *Leishmania* spp. is only stimulated by Mg^{2+} and not Ca^{2+} (24, 45). To characterize the co-factor requirements of Lpg1905 further, we determined the divalent cation preference of the enzyme and tested whether a range of cations could be utilized. We demonstrated that rLpg1905 required divalent metal cations for activity, which was stimulated by Zn^{2+}, Mg^{2+}, or Ca^{2+} (Fig. 1). Maximal ATPase and ADPase activity was obtained with 1–2 mM Mg^{2+}, although Zn^{2+} was able to stimulate ADPase activity to the same extent as Mg^{2+}. The presence of chelating agents 0.1 mM EGTA (which has low affinity for Mg^{2+}) and 0.1 mM EDTA (which has low affinity for Ca^{2+}) were also used with buffers containing either Mg^{2+} or Ca^{2+}, as appropriate, to demonstrate that the observed activity was not due to contamination of the assay buffer by other cations.

Lpg1905 Is Highly Active Only at a Near Neutral pH—The effect of pH on both ATPase and ADPase activity of Lpg1905 was evaluated over a pH range of 5–9. Optimal activity was observed at pH 7.4 for both substrates (Fig. 2, *A* and *B*). Significant hydrolysis of both ATP and ADP was still observed at pH

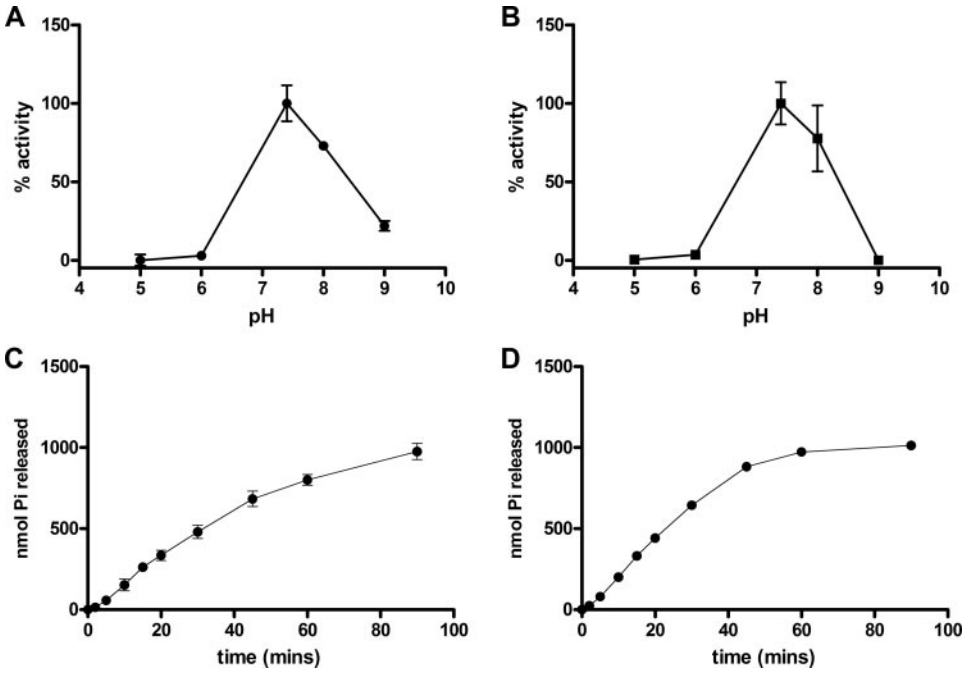

FIGURE 2. *A* and *B*, effect of pH on activity of rLpg1905. Relative hydrolysis of 1 mM ATP (*A*) or 1 mM ADP (*B*) in buffer containing 30 mM Tris-HCl and 2 mM Mg^{2+} over the indicated pH range is shown. The results are expressed as the percentages of activity and are the means ± standard deviations of three experiments performed in triplicate. *C* and *D*, activity of rLpg1905 over time. *C*, hydrolysis of 1 mM ATP by 0.01 ng/μl Lpg1905, measured as nmol P_i released over time. *D*, hydrolysis of 1 mM ADP by 0.02 ng/μl Lpg1905, measured as nmol P_i released over time. The results are expressed as nmol P_i released over time and are the means ± standard deviations of three experiments performed in triplicate.

8, but negligible hydrolysis of either substrate occurred at pH 9 or at pH 6 or lower. This suggests that the physiological site of activity is a pH-neutral environment such as the early *Legionella* replicative vacuole, the macrophage cytosol, or the extracellular environment.

Lpg1905 Efficiently Hydrolyzes Both ATP and ADP but Displays a Slight Preference for ATP—Substrate preference for nucleoside triphosphates and diphosphates varies among members of the ecto-NTPDase family of enzymes. For example, CD39/NTPDase1 hydrolyzes both ATP and ADP with similar efficiency, whereas NTPDase2 preferentially exhibits ATPase activity (13). We performed detailed enzyme kinetics of Lpg1905 using ATP and ADP as substrates, at pH 7.4 in the presence of 2 mM Mg^{2+}. At a concentration of 0.01 and 0.02 ng/μl of protein, respectively, hydrolysis of ATP and ADP was initially linear (Fig. 2, *C* and *D*), with linear regression giving an R^2 value of 0.95 (ATP hydrolysis) and 0.88 (ADP hydrolysis) at 45 min. We then analyzed the effects of varying substrate concentration when production of phosphate was linear to determine kinetic parameters for both ATP and ADP hydrolysis. Plotting of the initial velocities as a function of substrate concentration for both ATP and ADP hydrolysis revealed normal Michaelis-Menten kinetics for both substrates (Fig. 3). GraphPad Prism5 software was used to perform nonlinear regression analysis, determining a K_m value of 0.4 mM for ATP and 1.0 mM for ADP, suggesting a higher affinity of Lpg1905 for ATP, although the V_{max} was similar for both substrates (1182 μmol Pi/min/mg for ATP hydrolysis and 996 μmol Pi/min/mg for ADP hydrolysis). The K_{cat}/K_m ratio was then calculated to measure substrate specificity. For ATP hydrolysis a K_{cat}/K_m ratio of 1.2×10^8/M/min was obtained, whereas for ADP hydrolysis a K_{cat}/K_m ratio of 7.9×10^7/M/min was obtained, demonstrating the preference of Lpg1905 for ATP. However, the K_{cat}/K_m ratios for both substrates are in the upper range of observed values for enzyme-substrate reactions (46), indicating that Lpg1905 is very efficient at hydrolyzing both ATP and ADP.

ATPase Activity of Lpg1905 Is Inhibited by POM-1 and POM-6 but with Differing Efficacies—Polyoxometalate compounds have recently been identified as potent inhibitors of ecto-NTPDases (34). POM-1 ($Na_6[H_2W_{12}O_{40}]$) inhibits NTPDase1 and NTPDase3 with similar efficacy, with K_i values of 2.58 and 3.26 μM, respectively (34). POM-1 is also able to inhibit NTPDase2, albeit less efficiently, with a K_i value of 28.8 μM (34). In contrast, POM-6 ($(NH_4)18[NaSb_9W_{21}O_{86}]$) inhibits NTPDase2 and NTPDase3, with K_i values of 3.94 and 3.77 μM but is a very weak inhibitor of NTPDase1, with an IC_{50} greater than 1 mM (34). Here we tested the ability of these particular polyoxometalates to inhibit rLpg1905 activity to determine whether a microbial ecto-NTPDase exhibited increased or decreased susceptibility to inhibition compared with mammalian NTPDases. To achieve 100% inhibition of rLpg1905 activity with POM-1, an ATP concentration of 0.025 mM was used in the presence of 0.001–1 mM POM-1. Enzyme velocity was plotted against $\log_{10}$ of the inhibitor concentration, and nonlinear curve fitting was performed using GraphPad Prism5 software to determine the K_i (Fig. 4*A*). This revealed that POM-1 is a relatively weak inhibitor of rLpg1905, exhibiting a K_i value of 267 μM, compared with the ability of this compound to inhibit the three mammalian NTPDases (34). To achieve 100% inhibition with POM-6, an ATP concentration of 0.1 mM was used, in the presence of 0.001–0.4 mM of POM-6 (Fig. 4*B*). In contrast to POM-1, POM-6 was more effective at inhibiting rLpg1905 with a K_i value of 67 μM, demonstrating that it also possessed greater inhibitory activity against rLpg1905 than against CD39/NTPDase1. POM-6 was more effective, however, at inhibiting rat NTPDase2 and NTPDase3 than rLpg1905. Although the differences between POM-1 and POM-6 inhibition compared with mammalian NTPDases were minor, these results nevertheless suggested functional differences between rLpg1905 and mammalian ecto-NTPDases.

Lpg1905 Efficiently Hydrolyzes Guanosine Nucleoside Tri- and Diphosphates but Has Low Activity against Uridine or Cytidine Nucleotides—Numerous NTPDases from parasites have been shown to hydrolyze non-adenine nucleotides as well as adenine nucleotides (21, 27, 42, 43, 47–50). Therefore, we

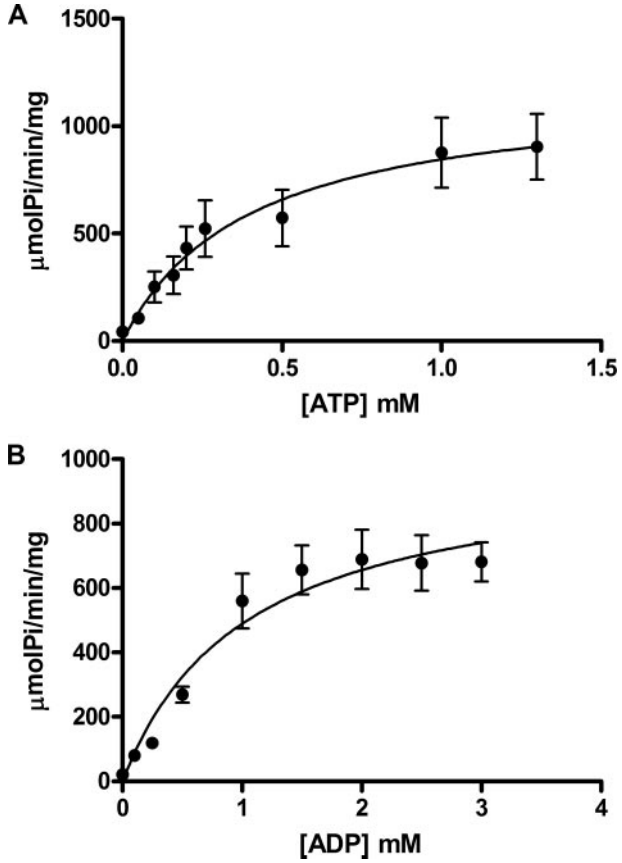

FIGURE 3. **Kinetics of purified rLpg1905.** Michaelis-Menten plots of enzyme velocity demonstrating the effect of varying ATP (*A*) or ADP (*B*) concentration are shown. Curve fitting using nonlinear regression was performed using GraphPad Prism5 software. The results are expressed as μmol P_i released per min per mg protein and are the means ± standard deviations of three experiments performed in triplicate.

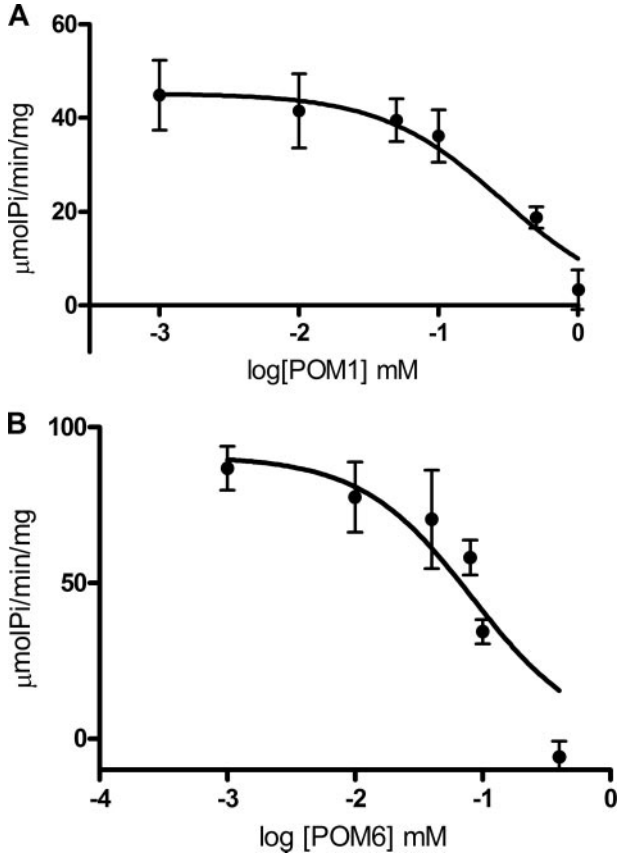

FIGURE 4. **Inhibition of rLpg1905 by POM inhibitors.** *A*, hydrolysis of 25 μM ATP by rLpg1905 in the presence of POM-1 ($Na_6[H_2W_{12}O_{40}]$) concentrations ranging between 0.001 and 1 mM. *B*, hydrolysis of 100 μM ATP by rLpg1905 in the presence of POM-6 ($(NH_4)_{18}[NaSb_9W_{21}O_{86}]$) ranging between 0.001 mM and 0.4 mM. Curve fitting using nonlinear regression was performed using GraphPad Prism5 software. The results are expressed as μmol P_i released per min per mg protein and are the means ± standard deviations of at least three experiments performed in duplicate.

tested the ability of rLpg1905 to hydrolyze GTP, GDP, UTP, UDP, CTP, and CDP. rLpg1905 efficiently hydrolyzed both GTP and GDP (Fig. 5), and whereas GTP hydrolysis occurred at a similar level to ATP, hydrolysis of GDP was approximately twice that observed for ADP (Fig. 5). In contrast, despite utilizing a higher concentration of enzyme in an attempt to demonstrate consistent activity against CTP, CDP, UTP, and UDP, only low levels of hydrolysis of these substrates were observed (Fig. 5). From these results, we concluded that the likely role of Lpg1905 in *L. pneumophila* virulence relates to the hydrolysis of adenine and/or guanine nucleotides.

Mutation of Conserved Residues Present in the ACRs Affects the Level of Catalytic Activity but Not Substrate Specificity—Extensive site-directed mutagenesis studies have been carried out on both CD39/NTPDase1 and NTPDase3, examining the importance of various conserved residues to catalytic activity (51–58). Previously we showed that, similar to CD39/NTPDase1 and NTPDase3, mutation to alanine of Glu^{159} in ACR3 (E159A) of rLpg1905 abolishes both ATP and ADP hydrolysis (12, 53, 56). As expected, rLpg1905E159A was also unable to catalyze the hydrolysis of GTP, GDP, UTP, UDP, CTP, or CDP (data not shown). To examine the contribution of other conserved amino acids to catalytic function, we generated four amino acid changes within ACR2–5 and examined the effect of the mutations on overall levels of catalytic activity and substrate specificity. In contrast to similar mutations in NTPDase3 (56), mutation of any one of Arg^{122} (ACR2), Gln^{226} (ACR4), and Trp^{384} (ACR5) to alanine abolished NTPDase activity against all substrates (data not shown). Only mutation of Asn^{168} (ACR3) to alanine resulted in residual enzyme activity. The activity of rLpg1905N168A toward all substrates, except CDP was decreased to ~35–50% of wild type activity (ATP, 49 ± 6%; ADP, 52 ± 15%; GTP, 49 ± 16%; GDP, 40 ± 12%; CTP, 44 ± 11%; CDP, 76 ± 14%; UTP, 37 ± 5%; UDP, 33 ± 8%). Therefore this mutation had a different effect on Lpg1905 activity compared with NTPDase3, where mutation of the corresponding asparagine residue to alanine resulted in a decrease of NTPase activity but not NDPase activity for all substrates (56). Collectively these results reinforce the importance of conserved residues in ACR2–5 to NTPDase activity. Furthermore, it highlights important enzymatic differences between microbial enzymes and mammalian enzymes because the mutations introduced into Lpg1905 had different effects on enzyme activity compared with the same mutations in NTPDase3.

Because significant catalytic activity was retained only for rLpg1905N168A, we compared the kinetics of this enzyme to rLpg1905. A time course analysis revealed that rLpg1905N168A hydrolyzes 1 mM ATP and ADP at a much

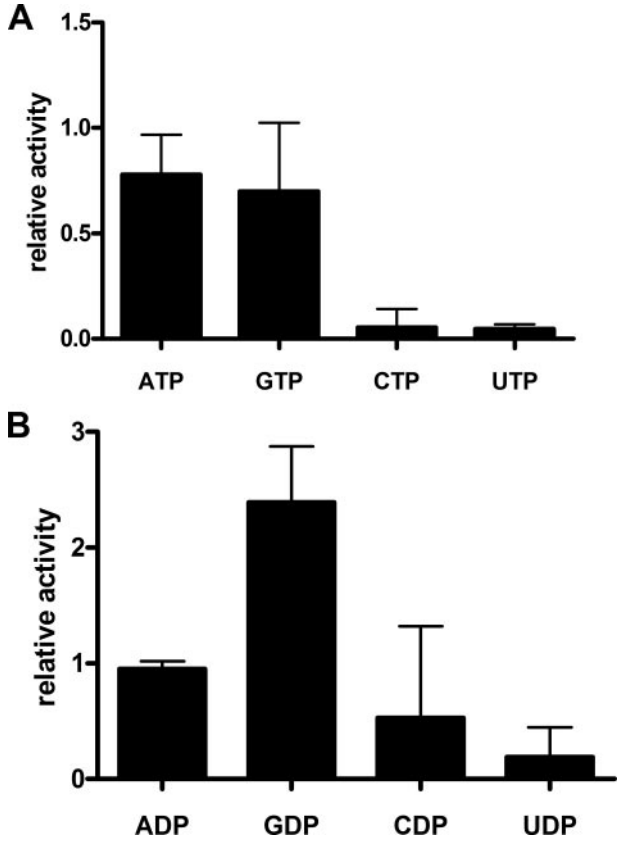

FIGURE 5. **Substrate specificity of rLpg1905.** The ability of the rLpg1905 to hydrolyze nucleoside tri- or diphosphates was evaluated. *A*, results are expressed as the means ± standard deviations relative to the highest activity reported for ATP, which is 1. *B*, results are expressed as the means ± standard deviations relative to the highest activity reported for ADP, which is 1. The results are the means of at least three experiments performed in triplicate.

lower rate than rLpg1905 (Fig. 6, *A* and *B*). Plotting of the initial velocities as a function of substrate concentration for both ATP and ADP hydrolysis by rLpg1905N168A revealed normal Michaelis-Menten kinetics for both substrates but with a relatively increased K_m for the mutant enzyme (Fig. 6, *C* and *D*). The K_m was increased 1.3-fold for ATP hydrolysis and 3-fold for ADP hydrolysis, demonstrating a lower affinity of rLpg1905N168A for both ATP and ADP. This suggested decreased substrate affinity was the reason for the observed decrease in activity and may indicate that Asn^{168} is located in the substrate-binding site of Lpg1905.

The Catalytic Activity of Lpg1905 Is Important for Bacterial Replication in Tissue Culture Models of L. pneumophila Infection—Previously, we established that an *lpg1905* mutant of *L. pneumophila* suffers an intracellular replication defect in THP-1 macrophages (12). Furthermore, we showed that the catalytic activity of Lpg1905 is important for bacterial replication, because complementation of the *lpg1905* mutant with a plasmid, pMIP, expressing wild type Lpg1905, but not inactive Lpg1905E159A, restored intracellular replication to wild type levels (12). In this study, we confirmed the necessity of catalytic activity for replication of *L. pneumophila* in THP-1 macrophages. The *lpg1905* mutant was complemented with pMIP expressing Lpg1905R122A, Q193A, N168A, or W384A, and we confirmed that the mutated forms of Lpg1905 were produced and secreted by *L. pneumophila* (Fig. 7*A*). Complemented strains were then tested for their ability to replicate in THP-1 macrophages. Complementation of the *lpg1905* mutant with pMIP expressing Lpg1905R122A, Q193A, or W384A resulted in an intracellular replication defect similar to that seen for the *lpg1905* mutant carrying pMIP alone or expressing Lpg1905E159A (Fig. 7*B*). In contrast, the introduction of pMIP expressing Lpg1905N168A into the *lpg1905* mutant resulted in partial complementation (Fig. 7*B*). The intermediate level of replication of *L. pneumophila* expressing Lpg1905N168A suggested that the quantity of catalytic activity was important for intracellular replication of *L. pneumophila*.

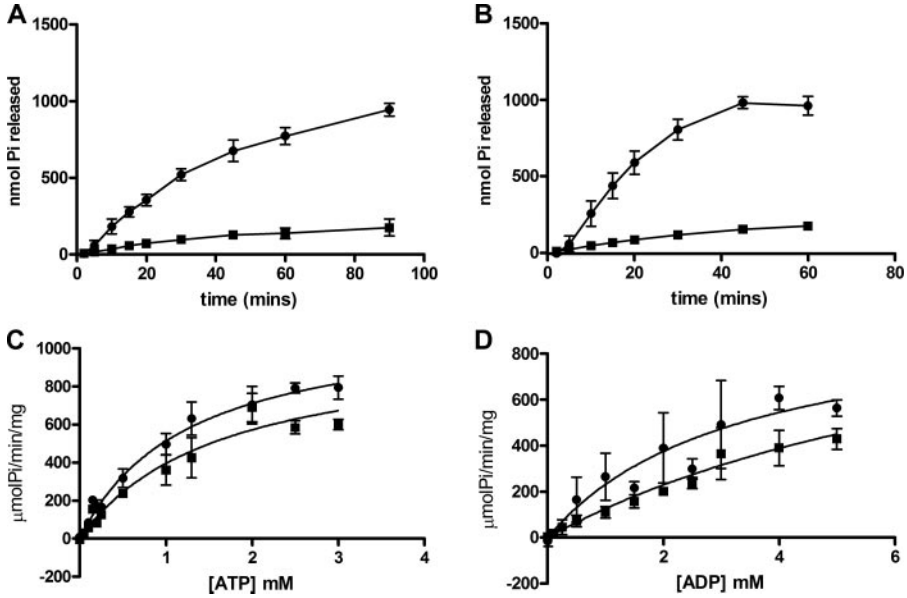

FIGURE 6. **Kinetics of rLpg1905N168A.** *A*, hydrolysis of 1 mM ATP by 0.01 ng/µl rLpg1905 (●) and rLpg1905N168A (■), measured as nmol P_i released over time. *B*, hydrolysis of 1 mM ADP by 0.02 ng/µl rLpg1905 (●) and rLpg1905N168A (■), measured as nmol P_i released over time. The results are expressed as the means ± standard deviations of three experiments performed in triplicate. *C* and *D*, Michaelis-Menten plot of enzyme velocity for rLpg1905 (●) and rLpg1905N168A (■), demonstrating the effect of varying ATP (*C*) or ADP (*D*) concentration. Curve fitting using nonlinear regression was performed using GraphPad Prism5 software. The results are expressed as nmol P_i released (*A* and *B*) and µmol P_i released per min per mg protein (*C* and *D*) and are the means ± standard deviations of at least three experiments performed in triplicate.

Catalytic Activity of Lpg1905 Is Required for Virulence in the A/J Mouse Model of Legionnaires Disease—Intratracheal inoculation of A/J mice results in an acute pneumonia resembling *L. pneumophila* infection in immunocompetent humans (59). After 72 h, bacteria are gradually cleared from the lungs of A/J mice without overt signs of illness (59). This model has been used previously to test isogenic mutants of *L. pneumophila* for a role in virulence (40, 60). Here 6–8-week-old A/J mice were infected with a 1:1 ratio of wild type *L. pneumophila*

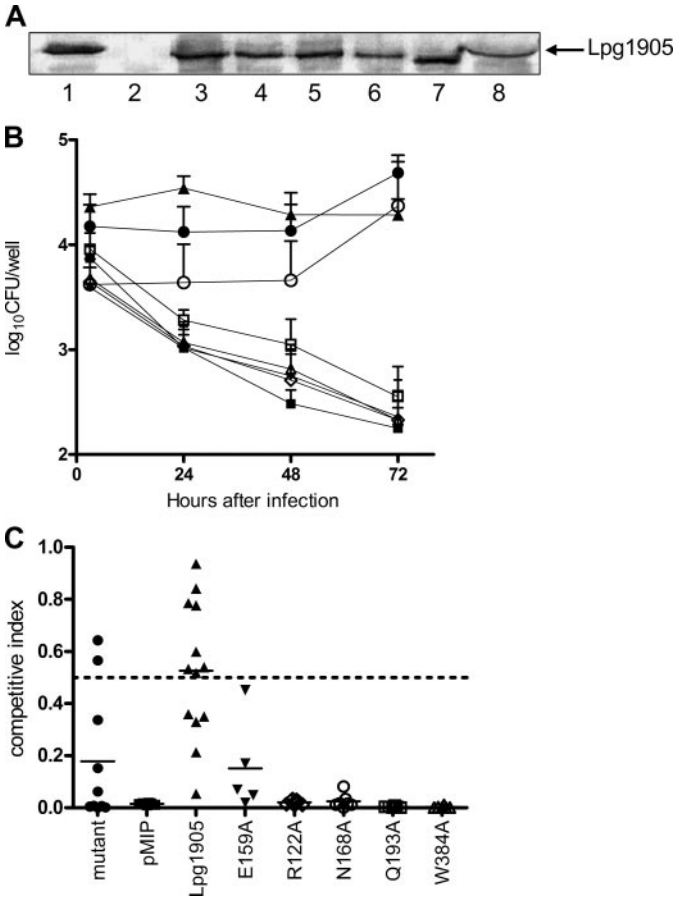

FIGURE 7. *A*, immunoblot of trichloroacetic acid-precipitated culture supernatants of derivatives of *L. pneumophila* 130b. A band corresponding to Lpg1905 was detected in all strains carrying pMIP expressing Lpg1905 and mutant derivatives. *Lane 1*, *L. pneumophila* 130b (pMIP). *Lane 2*, *L. pneumophila lpg1905*::*km* (pMIP). *Lane 3*, *L. pneumophila lpg1905*::*km* (pMIP:*lpg1905*). *Lane 4*, *L. pneumophila lpg1905*::*km* (pMIP:*lpg1905R122A*). *Lane 5*, *L. pneumophila lpg1905*::*km* (pMIP:*lpg1905N168A*). *Lane 6*, *L. pneumophila lpg1905*::*km* (pMIP:*lpg1905Q193A*). *Lane 7*, *L. pneumophila lpg1905*::*km* (pMIP:*lpg1905W384A*). *Lane 8*, *L. pneumophila lpg1905*::*km* (pMIP:*lpg1905E159A*). *B*, effect on bacterial replication in THP-1 macrophages of complementing the *lpg1905* mutant derivative of *L. pneumophila* 130b with an expression plasmid expressing Lpg1905 and mutated derivatives. The results are expressed as $\log_{10}$ colony-forming units present in the extracellular medium and associated with cells at specific time points after inoculation (means + standard deviation of at least three independent experiments from duplicate wells). The strains tested were *L. pneumophila* 130b (pMIP) (●), *lpg1905*::*km* (pMIP) (■), *lpg1905*::*km* (pMIP:*lpg1905*) (▲), *lpg1905*::*km* (pMIP:*lpg1905E159A*) (◇), *lpg1905*::*km* (pMIP:*lpg1905R122A*) (◆), *lpg1905*::*km* (pMIP:*lpg1905N168A*) (○), *lpg1905*::*km* (pMIP:*lpg1905Q193A*) (□), and *lpg1905*::*km* (pMIP:*lpg1905W384A*) (△). *C*, competitive pulmonary infection of A/J mice with *L. pneumophila* 130b and mutant derivatives at 72 h. The results are expressed as the CI of the mutant (test) strain for each mouse. The *horizontal bar* indicates the average CI. The *lpg1905*::*km* mutant derivative was tested in competition with *L. pneumophila* 130b (mutant, ●). Strains tested in a 1:1 mixed infection with *L. pneumophila* 130b (pMIP) were *lpg1905*::*km* (pMIP, ■), *lpg1905*::*km* (pMIP:*lpg1905*,) (Lpg1905, ▲), *lpg1905*::*km* (pMIP:*lpg1905E159A*) (E159A, ▼), *lpg1905*::*km* (pMIP:*lpg1905R122A*) (R122A, ◇), *lpg1905*::*km* (pMIP:*lpg1905N168A*) (N168A, ○), *lpg1905*::*km* (pMIP:*lpg1905Q193A*) (Q193A, □), and *lpg1905*::*km* (pMIP:*lpg1905W384A*) (W384A, △). The average CI for each strain was: CI = 0.18 (*lpg1905*::km mutant), CI = 0.014 (pMIP); CI = 0.526 (Lpg1905), CI = 0.152 (E159A); CI = 0.021 (R122A); CI = 0.024 (N168A); CI = 0.003 (Q193A); and CI = 0.003 (W384A). The CI values of all strains carrying mutant derivatives of Lpg1905 expressed from pMIP were significantly lower than that observed for *lpg1905*::*km* (pMIP:*lpg1905*) ($p < 0.05$, unpaired two-tailed *t* test).

and the *lpg1905* mutant. Viable numbers of each strain were determined 72 h after infection by plating serial dilutions of lung homogenate onto CYE agar and CYE agar containing kanamycin. Growth on nonselective media represented total colony-forming units isolated from the lung, and kanamycin-resistant colonies represented colony-forming units of the *lpg1905* mutant. The CI was calculated as the proportion of mutant to wild type bacteria recovered from mice divided by the proportion of mutant to wild type bacteria in the inoculum. A mutant with a CI of less than 0.5 is considered to be attenuated (61). The CI for the *lpg1905* mutant was 0.18, demonstrating that this mutant is attenuated for virulence *in vivo* (Fig. 7*C*). Similar to *in vitro* tissue culture models (12), introduction of the pMIP complementation vector into *L. pneumophila* exacerbated the virulence defect of the *lpg1905* mutant. The CI of the *lpg1905* mutant carrying pMIP in competition with wild type *L. pneumophila* carrying pMIP was 0.014 (Fig. 7*C*). Complementation of the *lpg1905* mutant with pMIP expressing Lpg1905 restored virulence to the mutant; however, complementation of the *lpg1905* mutant with pMIP expressing any of Lpg1905E159A, R122A, Q193A, N168A, or W384A was not able to complement the virulence defect *in vivo* (Fig. 7*C*). Therefore the catalytic activity of Lpg1905 is important for its function during infection. The lack of even partial complementation observed for *L. pneumophila* expressing Lpg1905N168A suggested that the quantity of Lpg1905 activity may also be important for its virulence function. This could indicate that Lpg1905 plays an additional role *in vivo* that is not evident in simple tissue culture models.

DISCUSSION

Previously we showed that Lpg1905 is a novel prokaryotic ecto-NTPDase, similar to CD39/NTPDase1, which enhances the replication of *L. pneumophila* in eukaryotic cells (12). In this study, we defined the enzymatic properties of Lpg1905 and demonstrated that apart from ATP and ADP, Lpg1905 also cleaves GTP and GDP with similar efficiency to ATP and ADP, respectively. In contrast, the enzyme was not able to utilize CTP, CDP, UTP, or UDP to any great extent. These results suggest that it is the ATP/ADPase and/or GTP/GDPase activity of Lpg1905 that is most important for *L. pneumophila* virulence. Interestingly, amino acid changes that are known to affect the level of activity and substrate preference for NTPDase3 (56, 58) unexpectedly abolished activity of Lpg1905. Mutation of Asp^{168} to alanine was the only amino acid change that did not result in complete inactivation; however, this mutation had little effect on the NTPase:NDPase ratio or substrate specificity. Therefore we were not able to assess the relative contribution of ATP/ADPase and/or GTP/GDPase activity to virulence. Nevertheless, our mutational analysis of Lpg1905 demonstrated definitively that enzymatic activity is closely linked to *L. pneumophila* replication in macrophages and virulence in mice.

At this stage the major site of Lpg1905 activity during infection is unclear, although potentially the enzyme could exert an effect in both intracellular and extracellular environments. As an intracellular pathogen, *L. pneumophila* may utilize Lpg1905 to cleave ATP to AMP inside cells. A possible consequence of

this could be a rise in the AMP:ATP ratio and activation of AMP-activated protein kinase (62). Activation of AMP-activated protein kinase has numerous effects on cell cycle and mitochondrial function, all of which could provide a more beneficial environment for intracellular survival and replication of *L. pneumophila* (63, 64). In addition, there is evidence suggesting that activation of AMP-activated protein kinase results in inhibition of the production of proinflammatory cytokines such as interleukin-1β (65). Another potential role for Lpg1905 in virulence is modulation of extracellular levels of ATP. The importance of extracellular ATP as a "danger signal" is becoming increasingly evident (66). ATP stimulates signaling through purinergic type 2 (P2) receptors, which results in the modulation of host immune and inflammatory responses and apoptosis (13, 67). ATP triggers the release of proinflammatory cytokines including interleukin-1β, interleukin-2, and interferon γ (68, 69). Mammalian ecto-NTPDases modulate this response by controlling the level of extracellular ATP. For example, CD39 expressed on regulatory T cells mediates immune suppression through the hydrolysis of ATP (30, 70) and on endothelium is a crucial thromboregulatory factor (71). Extracellular ATP also stimulates phagolysosome fusion and killing of intracellular *Mycobacterium* spp. by human macrophages in a P2 receptor-dependent manner (72, 73). In addition, another intracellular bacterial pathogen *Porphyromonas gingivalis* inhibits P2 receptor mediated apoptosis by secreting an extracellular nucleoside diphosphate kinase, an ATP-consuming enzyme (74). Together these studies suggest that the control of extracellular ATP and P2 receptor signaling may benefit an intracellular pathogen such as *L. pneumophila* by delaying P2 receptor-dependent phagolysosome fusion, stimulation of inflammation, and/or apoptosis.

We also demonstrated that Lpg1905 cleaves GTP and GDP. Although *L. pneumophila* does not require the addition of purines for growth, the supplementation of medium with guanine can increase growth rate *in vitro* (75). However, *L. pneumophila* does not appear to possess a 5′-nucleotidase (9, 10), which is required for the hydrolysis of GMP to guanine, suggesting that the hydrolysis of GTP and GDP may be related to a role in the manipulation of host cell pathways rather than purine salvage. The conversion of GTP to GDP inside cells is critical to intracellular signaling and vesicle trafficking pathways (76). Indeed, *L. pneumophila* recruits the host cell GTPase Rab1 to the replicative vacuole where the Dot/Icm effector SidM/DrrA prevents recycling of GDP from the *Legionella* phagosome (77–79). Interfering with levels of intracellular GTP and GDP may be another way in which Lpg1905 from *L. pneumophila* modulates host cell signaling.

A number of parasites express surface-located or secreted NTPDases that have been implicated in virulence, and the external location of these enzymes makes them desirable drug targets (32). Recently, a new class of drugs, *N*-alkylaminoalkanethiosulfuric acids, was shown to partially inhibit a surface-located NTPDase from *S. mansoni*, which may form the basis of the anti-schistosomal efficacy of these compounds (80). However, because membrane-associated and surface-located mammalian NTPDases, such as CD39, play important roles in thromboregulation and modulation of the immune response (13, 30, 70), it is important that antimicrobial compounds designed to inhibit the NTPDases of pathogens do not adversely affect the function of human NTPDases. Although in this study we found differing effects of the promising NTPDase inhibitors, POM-1 and POM-6, on Lpg1905 activity compared with mammalian NTPDases, these effects were relatively minor and not therapeutically useful given that any residual activity against mammalian NTPDases would be pharmacologically unacceptable. Nevertheless, these functional differences provide some evidence that the rational design of novel anti-microbial agents based on structural information could be possible. Indeed the differing effects of altering corresponding amino acids in Lpg1905 and NTPDase3 on enzyme activity support the existence of significant structural differences. The structural resolution of a number of microbial and mammalian NTPDases would greatly advance our understanding of the catalytic mechanism of NTPDase enzymes and form the basis for a rational approach to the development of inhibitors with different specificities and targets.

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