**Legionella pneumophila NudA Is a Nudix Hydrolase and Virulence Factor**

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**Legionella pneumophila**, the most common cause of Legionnaires’ disease, is a facultative gram-negative intracellular parasite. Legionnaires’ disease is a type of pneumonia that affects mainly adults, especially those who have altered local lung defenses or who have cellular immune system-suppressing diseases (20). A multitude of *L. pneumophila* virulence factors have been described, the majority of which affect the ability of the bacterium to grow and survive within blood monocytes and alveolar macrophages or within free-living ameoba (14). To attempt to find *L. pneumophila* virulence factors responsible for growth or survival in vivo, but not necessarily in macrophages, some of us previously performed signature-tagged mutagenesis of *L. pneumophila* using a guinea pig pneumonia model (21). Several different genes encoding new putative virulence factors were found, including ptsP, mutation of which results in decreased growth in macrophages and loss of virulence for guinea pigs (32). Immediately upstream of *ptsP* was an open reading frame (ORF) 528 bp in length that was in the same reading frame and appeared to be in the same operon. This reading frame, termed ORF2, shared significant homology with Nudix hydrolases from many different bacteria, including invA, from *Rickettsia prowazekii* and *Pseudomonas aeruginosa* and mutT of *Escherichia coli*. Nudix hydrolases are nucleoside diphosphate pyrophosphatases thought to be important in degrading toxic intracellular compounds and in the virulence of several different bacteria (4, 5, 28, 40). Nudix is an acronym for nucleoside diphosphate linked to another moiety, X, and these enzymes have a characteristic motif, GX5EX7RE, where X is any amino acid and U is I, L, or V (4). We sought to determine whether ORF2 (renamed *nudA*) was a *L. pneumophila* virulence factor and whether it functioned as a Nudix hydrolase.

**MATERIALS AND METHODS**

Bacteria, plasmids, and growth conditions. *L. pneumophila* serogroup 1 strain F2310, also known as AA100jm, is a spontaneous streptomycin-resistant mutant of strain 130b (ATCC BAA-74), originally isolated from a patient with combined Legionnaires’ disease and pneumococcal and meningococcal pneumonia (21, 43). AA100jm is virulent in guinea pigs, macrophages, and ameoba (21, 45). *L. pneumophila* bacteria were grown at 35 to 37°C in ambient air on 3-(N-morpholino)-propanesulfonic acid (MOPS)-buffered charcoal yeast extract agar medium supplemented with α-ketoglutarate (BCYEα) (22), in N-(2-acetamido)-2-aminooctanesulfonic acid (ACES)-buffered yeast extract broth supplemented with α-ketoglutarate (BYEα), or to resuscitate frozen bacteria in ACES-buffered charcoal yeast extract broth supplemented with α-ketoglutaric acid (BCYEα broth) (22). BYEα and BCYEα broth-grown bacteria were shaken (150 to 300 rpm) during growth; log-phase broth growth was defined as a bacterial density optical density at 660 nm (OD660) of 0.2, and late-stationary-phase growth was defined as a bacterial density OD660 of >3.0 (measured after 1:10 dilution). Of note, the highly motile coccobacillary form of *L. pneumophila* is not observed for the late-stationary-phase F2310 strain (unpublished observation).

We studied the identity and function of the 528-bp gene immediately upstream of *Legionella pneumophila* F2310 *ptsP* (enzyme I Ntr). This gene, *nudA*, encoded for a Nudix hydrolase based on the inferred protein sequence. NudA had hydrolytic activity typical of other Nudix hydrolases, such as *Escherichia coli* YgdP, in that Ap,N’s, in particular diadenosine pentaphosphate (Ap,A), were the preferred substrates. NudA hydrolyzed Ap,A to ATP plus ADP. Both *ptsP* and *nudA* were cotranscribed. Bacterial two-hybrid analysis showed no PtsP-NudA interactions. Gene *nudA* was present in 19 of 20 different *L. pneumophila* strains tested and in 5 of 10 different *Legionella* spp. other than *L. pneumophila*. An in-frame *nudA* mutation was made in *L. pneumophila* F2310 to determine the phenotype. The *nudA* mutant was an auxotroph that grew slowly in liquid and on solid media and had a smaller colony size than its parent. In addition, the mutant was more salt resistant than its parent and grew very poorly at 25°C; all of these characteristics, as well as auxotrophy and slow-growth rate, were reversed by transcomplementation with *nudA*. The *nudA* mutant was outcompeted by about fourfold by the parent in competition studies in macrophages; transcomplementation almost completely restored this defect. Competition studies in guinea pigs with *L. pneumophila* pneumonia showed that the *nudA* mutant was outcompeted by its parent in both lung and spleen. NudA is of major importance for resisting stress in *L. pneumophila* and is a virulence factor.
TABLE 1. Unique strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant properties</th>
<th>Reference or source</th>
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<tr>
<td>Strains</td>
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<tr>
<td>F2310</td>
<td>L. pneumophila serogroup 1, strain AA 100m</td>
<td>This study</td>
</tr>
<tr>
<td>F2527</td>
<td>F2310 Δmut4::kan (in-frame)</td>
<td>This study</td>
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<tr>
<td>F2529</td>
<td>F2527 containing pMMB2002</td>
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<tr>
<td>F2530</td>
<td>F2527 containing pBH17</td>
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</tr>
<tr>
<td>F2531</td>
<td>F2521 containing pMMB2002</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pMMB2002</td>
<td>pmMB207 (ΔnudA)</td>
<td>N. Ciancioatto (46)</td>
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<tr>
<td>pBH3</td>
<td>pUC18 (nudA+)</td>
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<tr>
<td>pBH17</td>
<td>pMMB2002 (nudA+)</td>
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Nucleic acid manipulation. All nucleic acid manipulations were accomplished according to standard molecular biology techniques unless otherwise stated (2). PCR was performed by using Taq polymerase (Promega) unless otherwise stated. All reagents used for DNA manipulations were purchased from New England Biolabs unless stated otherwise.

Complete sequencing of nudA gene. Subcloned DNA from a transposon-mutated and signature-tagged avirulent clone of L. pneumophila, clone mu3b (32) was sequenced in both directions 5'-to-3' by the pSP ORF, using primers 5'-walkout. Whole sequence data were analyzed and aligned by using SeqMan II software, version 5.03 (DNASTAR, Inc., Madison, WI). EdiSeq and Protein software (both DNASTAR, version 5.03) were used to translate DNA and to predict protein physical characteristics. GenBank sequence database searching was performed with the BLASTX and BLASTN search algorithms. The TIGR CMR BLAST site was also used for some sequence analyses (http://tigrblast.tigr.org/cmrbLAST/). Multiple sequence alignments were performed by using Megalign, version 5.03, with the Jotun Hein method (DNASTAR). The Berkeley Drosophila Genome Project promoter prediction program was used to predict promoter sites (http://www.fruitfly.org/seq_tools/promoter.html). Comparison of sequences to published L. pneumophila genome sequences used sequences and search software posted in GenBank, the Pasteur Institute (http://genolist.pasteur.fr/Legionella), and the Columbia University Genome Center (http://legionella.cu-genome.org/). Detection of Nudix hydrolase orthologs in the published L. pneumophila genomes was performed by searching the PEDANT database (http://pedant.gsf.de/) of each strain with a text search for “MutT.”

Macrophages. Guinea pig alveolar macrophages were prepared as previously described (21) and cultured in M199 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), and 2% L-glutamine. Walker-256, MDs, were a gift from Dr. F. Good of the University of Minnesota. They were cultured in DMEM supplemented with 10% FBS, 2% L-glutamine, and 2% penicillin and streptomycin.

Determination of flagellation. Plate-grown Legionella bacteria were suspended in sterile distilled water and stained for the presence of flagellae using the Ryu staining protocol (5). Macrophages were prepared as described above and infected with L. pneumophila (multiplicity of infection of 0.1) and then incubated in 5% CO2 in air at 37°C. Culture supernatants were harvested at the indicated times, diluted appropriately with Mueller-Hinton broth, and then plated onto BCYE agar plates. Bacterial competition studies took advantage of the Kan resistance of L. pneumophila F2527 (Δmut4) and the Kan susceptibility of its parent. In these competition studies, the concentration of the parent strain was determined by subtracting the total bacterial concentration determined by plating on nonselective BCYE medium from the bacterial concentration determined by plating on BCYE medium containing Kan.

The relative abilities of L. pneumophila strains F2310 (parent) and F2527 (Δmut4) to invade explanted guinea pig alveolar macrophages were determined by using a gentamicin protection assay as previously described (23).

Detection of nudA in other Legionella spp. strains. The presence of nudA in 20 different strains of L. pneumophila (14 serogroups) was detected by PCR with the nudA primers mu3b and 6568 EDELSTEIN ET AL. INFECT. IMMUN.

It was labeled as specified by the kit manufacturer (DIG High Prime DNA Labeling and Detection Kit; Roche Applied Science, Indianapolis, IN). Tested were one strain each of L. birminghamensis ATCC 43702), L. dumoffi (ATCC 33343), L. feeleii (ATCC 35072), L. jordanis (ATCC 33623), L. longbeachae serogroup 1 (F850), L. maccachernii (ATCC 35300), L. micdadei (F774), L. quateriones (ATCC 45907), L. rubrilucens (ATCC 35304), and L. steigerwalti (ATCC 35302). Genomic DNA from each strain was made by using the CTAB (cetyltrimethylammonium bromide) method, and 1 μg of EcoR1-digested DNA was electrophoresed in 0.8% agarose and then transferred to Hybond N+ transfer membranes (Amersham Biosciences). Hybridization was performed at 42°C using the supplied hybridization buffer; the first two washes were carried out at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS), and the final two washes were done at 42°C in 0.5× SSC-0.1% SDS. Probe detection was performed according to the kit instructions.

Phenotypic characterization of nudA mutant. Growth rates of the L. pneumophila bacteria in BYE broth were determined by monitoring OD600 at the specified times. The starting inoculum for these growth studies was obtained by inoculating BCYE plates with frozen bacteria and passing the bacteria twice on the plates (35°C). The bacteria were then suspended (Mueller-Hinton broth) and resuspended to a density equivalent to 0.5 McFarland unit in BHI broth (2% NaCl and 1 μg of E. coli-λ DNA) and grown electrophoresed in 0.8% agarose and then transferred to Hybrid N+ transfer membranes (Amersham Biosciences). Hybridization was performed at 42°C using the supplied hybridization buffer; the first two washes were carried out at room temperature with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate (SDS), and the final two washes were done at 42°C in 0.5× SSC-0.1% SDS. Probe detection was performed according to the kit instructions.

Because salt resistance is a common nonspecific marker of reduced virulence in L. pneumophila (11, 35, 38), we sought to determine whether the nudA mutant was more salt resistant than its parent. Bacterial NaCl resistance was determined by quantitative plating of serial decimal dilutions of late-stationary-phase or early-log-phase broth suspensions of the bacteria on BCYE medium made with or without NaCl (100 mM). The broth suspensions were obtained by passing the bacteria twice on BCYE plates as described above and then inoculating BCYE broth (10 ml) from the plates and growing the bacteria to late stationary phase or early log phase in a shaking incubator.

Colony sizes of bacteria were determined by inoculating BCYE plates with decimal dilutions of bacterial suspensions made from twice-passed growth on BCYE plates (35°C). After incubation at 37°C for 4 days, dilution plates with well-isolated colonies were selected for colony size measurement of randomly selected colonies by using a dissecting microscope equipped with a calibrated optical micrometer.

Auxotrophy was characterized by study of growth of L. pneumophila bacteria on a minimal medium as described previously (21) with one exception. The exception was that inoculated minimal medium was incubated for up to 4 weeks to ensure accurate counting of bacterial colonies. Auxotrophs were retted with the same minimal medium supplemented with tryptophan (0.7 to 3.3 mM), ZnCl2 (15 to 74 μM), MgCl2 (150 to 740 μM), or KCi (7.6 to 40 mM) in various combinations; the concentrations of the supplements were based on the differential chemical compositions of Difco yeast extract and the components of the minimal medium (Difco Manual, 11th ed.; Becton Dickinson, Sparks, MD). Because hypersusceptibility to H2O2 has been described for other Nudix hydrolase mutants, killing of L. pneumophila bacteria by H2O2 (10 mM in sterile distilled water) was tested. This was performed by incubating late-stationary-
phase bacteria with the H2O2 for 20 min and then determining viable plate counts of the inoculum and peroxide-exposed bacteria. Late-stationary-phase growth was used for both these studies because pilot studies showed that the log-phase parent was completely killed by peroxide exposure, precluding any comparative studies.

Expression and purification of NudA. Gene nudA was amplified from pH3 by standard PCR procedures, subcloned into plasmids of lac promoter ET24a (+ NdeI, Madison, WI) to form plasmid pETnudA and transformed into the expression host HMS174(DE3) (Novagen). One colony of HMS174(DE3)/pETnudA was inoculated into LB medium (40 ml) containing Kan, incubated at 37°C on a rotary shaker overnight, and inoculated into prewarmed medium (2 liters). When the culture reached an OD600 of ~0.3, it was transferred to a lower temperature incubator (22°C) and grown to an OD600 of 0.8. IPTG (isopropyl-L-thiogalacto- pyranoside) at 100 mM was added to a final concentration of 0.5 mM, and growth was continued for an additional 3 h. The cells were harvested by centrifugation, washed by suspension in isotonic saline solution, centrifuged, dispersed in 2.5 volumes of buffer A (50 mM Tris-Cl [pH 7.5], 1 mM EDTA, 0.1 mM dithiothreitol), and disrupted in a French pressure cell at 110 MPa. The insoluble material was removed by centrifugation, and the supernatant containing NudA was adjusted to a protein concentration of 10 mg/ml with buffer A (fraction I). Protein was determined by the method of Bradford (7) with bovine serum albumin as the standard. In the following purification procedure, fractions containing NudA were identified by enzymatic activity, polyacrylamide gel electrophoresis, and spectrophotometry at 260 and 280 nm.

Streptomycin sulfate (10% [wt/vol] in buffer A) was added to fraction I to a final concentration of 1.5%, and the precipitate containing NudA was suspended in buffer A containing 1 M (NH4)2SO4 (fraction II). This was loaded onto a DEAE Sephadex column, eluted with buffer A, and the fractions containing NudA were pooled. Solid ammonium sulfate was added to a concentration of 70% saturation (0.5 g/ml), and the precipitate was dissolved in a minimal volume of buffer A (fraction III), loaded onto a Sephadex G-100 gel-filtration column, and eluted with a gradient of 0.1 to 0.5 M NaCl in buffer A. The fractions containing NudA were pooled (fraction V) and stored at −80°C. This fraction was used for characterization of the enzyme.

Enzyme assays. Standard reaction mixtures contained (in 50 μl): 50 mM Tris-Cl (pH 8.0), 5 mM MgCl2, 2 mM substrate, 0.1 to 2 μM of NudA, and either 0.5 μg of yeast inorganic pyrophosphatase for nucleoside triphosphates and their derivatives or 4 U of alkaline phosphatase for all other substrates. The mixture was incubated at 37°C for 15 min and terminated by adding 250 μl of 4 M EDTA. The liberated orthophosphate was assayed by the colorimetric procedure described, except for the use of EcorV, rather than Smal, cloning sites for the Kan insertion (42). The accuracy of the sequence of the 3′ end of the Kan insertion and of most of the mutated nudA ORF 3′ to the Kan insertion was confirmed by single-strand sequencing. The Kan insertion resulted in the termination of the putative promoter in pUT18c and also with nudA in pKT25 and ptsP in pUT18c, as previously described (36) and by personal communication (D. Ladant). MacConkey agar containing maltose (1%) was used as the indicator medium, and interactions were tested at both 30 and 37°C.

Cloning and mutation of nudA. The chromosomal DNA region surrounding the nudA gene was cloned into pUC19 by using ligation into the vector pUC19 and its derivatives, or by electroporation into E. coli (21). This plasmid DNA was cloned into pLAW344 for use as a heterologous expression vector in L. pneumophila strain F2310, by using Vent DNA polymerase and primers ali-64U (5′-CTTCTGGCAATCTTCTTCTCTC-3′) and ali-1538L (5′-CGCAGGGAATGAAATTGTCTAAGTG-3′); this yielded pH3. The sequence of this entire PCR product was confirmed by two-strand sequencing over its entire length. A mutation in nudA was made by using standard methods, which resulted in a truncated nudA, 376 bp in length, containing only the terminal 8 bp of the 69-bp predicted NudX hydrolase box (4). An insertion was made for the nudA insertion, which annealed to nudA 441 bp 5′ to its terminus and 442 bp 3′ to the ptsP start site and oriented toward the start of ptsP, using Moloney murine leukemia virus reverse transcriptase and its supplied buffer (Invitrogen). To exclude DNA contamination of the bacterial RNA preparation, a reverse transcription reaction was performed without adding reverse transcriptase. The CDNA, or total RNA, was then amplified by the PCR using Taq polymerase (Promega) and primers pTS23U191 and pTS24L4529 (5′-TTAACGCCCTTTACCTCGTG-3′); pTS24L4529 binds to ptsP 547 bp 3′ of its start. The DNA transcript length was determined by agarose gel electrophoresis.

Interaction between NudA and PtsP. To determine whether NudA and PtsP interacted, a two-bacteria hybrid system was used (36). This utilized test plasmids pKT25 and pUT18c, positive control plasmids pKT25-zip and pUT18c-zip, and E. coli indicator strains DHM1 and BTH101, all kindly provided by Daniel Ladant. The test genes were cloned in frame into both test plasmids, and the stability of the insertion junctions of the insertion junctions determined to confirm that the test genes were inserted correctly. The interaction of PtsP with NudA was determined with ptsP in pKT25 and nudA in pUT18c and also with nudA in pKT25 and ptsP in pUT18c, as previously described (36) and by personal communication (D. Ladant). MacConkey agar containing maltose (1%) was used as the indicator medium, and interactions were tested at both 30 and 37°C.
FIG. 1. Schematic drawings of the genetic organization of nudA (top) and of the nudA mutation used to study its function (bottom). Both drawings are drawn to scale. The top drawing shows the relationship of nudA to ptsP and orf1. Genes nudA and ptsP are in the same reading frame, which is different from the reading frame of orf1. The bottom drawing shows the mutation and insertion used to make the in-frame mutation nudA; the gray box (69 bp in length) indicates the region coding for the active Nudix site (Nudix box). The mutation was made by deleting 143 bp of nudA, including most of the Nudix box, and inserting an in-frame Kan cassette. The numbers above or below the drawings indicate the size in base pairs.

Graphical presentation of the quantitative data was prepared by using GraphPad Prism.

Nucleotide sequence accession number. The nudA sequence has been deposited in the GenBank database at the National Center for Biotechnology Information under accession number AY521221.

RESULTS

Sequence and genetic organization of nudA and the adjacent region. The region surrounding ptsP was sequenced on both strands 753 bp 5′ and 664 bp 3′; an additional 149 bp of single-strand sequence was obtained in the 3′ region, making a total of 813 bp of single and double-strand sequence 3′ to ptsP. A 528-bp ORF was 5′ to ptsP, and in the same reading frame; initially termed ORF2, this was finally named nudA. The termination of nudA was immediately upstream of the ATG start of ptsP, with no intervening bases. A predicted promoter (AC TTTGTTGAAAAATCAAAGTTATTAACGGTTTATTCTA AGCAGTACACATCAGT) (score 1.0) was identified starting 50 bp 5′ of the nudA start site, with a predicted transcriptional start (underlined) 10 bp 5′ to the ATG start (italic). A second ORF (orf1) was identified 3′ of ptsP, 807 bp in length; orf1 was in a different reading frame and began 1 bp 5′ to the TGA termination of ptsP. A promoter was predicted to begin 91 bp 5′ of the start of orf1, with a transcriptional start 50 bp upstream of the start site (score 0.99). A schematic of the genetic organization of this region is shown in Fig. 1.

The GC content of nudA was 43.4%, compared to a total genome GC content of 38% (12, 13). For comparison, the GC contents of ptsP and orf1 were 42.8 and 38.5%, respectively. NudA was predicted to have a mass of 20,577 Da and an isoelectric point of 9.8 for this 174-amino-acid protein. Immunoblot analysis of purified expressed NudA and NudA present in boiled bacterial cells showed both bands to have an approximate mass of 20,600 Da.

Sequence similarity searching for the nudA sequence of the GenBank database, using the blastx program, revealed that nudA was homologous to a large number of Nudix hydrolases, with the closest matches being Pseudomonas putida or P. fluorescens putative (di)nucleoside polyphosphate hydrolase (60% identities [89 of 147], 77% positives [114 of 147], expect 7e – 49), P. syringae MutT (59% identities [87 of 147], 77% positives [114 of 147], 4e – 48), and Vibrio parahaemolyticus MutT (49% identities [85 of 173], 67% positives [116 of 173], 8e – 46). Other matches of note included P. aeruginosa YgdP (8e – 46), V. cholerae MutT (2e – 45), C. burnetii MutT-nudix family (5e – 40), Rickettsia conorii InvA (3e – 21), and P. w listOfekziew InvA (5e – 20).

The predicted sequence of NudA contained a 23-amino-acid sequence, GGLAPGETAMQAMYRELHEEVGL, which conforms to the conserved motif of the putative Nudix active site, GX3EX,REUXEXEXGU (where X is any amino acid and U is L, or V) (4). The Nudix conserved domain was found in the predicted sequence of NudA when it was searched in GenBank using a conserved domain search, with high scoring matches for the pfam00293, COG0494, COG1051, and COG2816 conserved domains with respective scores and E values of 69.1, 3e-13; 52.7, 2e-8; 45.8, 3e-6; and 39.9, 2e-4. These conserved domains correspond to Nudix, MutT, ADP-ribose pyrophosphatase, and NPY1 domains, respectively.

Text searches of the PEDANT database for three published L. pneumophila genomes showed that the Philadelphia 1, Paris, and Lens strains contained five, six, and five different Nudix homologs, respectively (http://pedant.gs.f.de). All of these contained a stereotypical Nudix motif, had widely varying ionization constants ranging from 5.2 to 9.7, and widely varying GC contents. One Nudix hydrolase from each strain was nearly identical to the predicted sequence of nudA of strain F2310. No genome appeared to have more than one copy of each different Nudix hydrolase.

There was only one base difference between the nudA sequence of strain F2310 (parent) and that of Philadelphia 1, Paris, and Lens strains contained five, six, and five different Nudix homologs, respectively (http://pedant.gs.f.de). All of these contained a stereotypical Nudix motif, had widely varying ionization constants ranging from 5.2 to 9.7, and widely varying GC contents. One Nudix hydrolase from each strain was nearly identical to the nudA of strain F2310. No genome appeared to have more than one copy of each different Nudix hydrolase.

Examination of the genetic organization of nudA and ptsP homologs in other bacteria was determined by using the TIGR CMR BLAST search and region view module. This database search showed that homologs of nudA immediately preceded ptsP homologs in P. aeruginosa PA01, Y. pestis C092, E. coli MG1655 and EDL933, C. burnetii RSA493, and V. cholerae N16961. However, a nudA homolog was not found to be adjacent to a ptsP homolog in Rickettsia conorii Malish7, Salmonella enterica serovar Typhimurium SGSC1412, Haemophilus influenzae KW20, or Neisseria meningitidis 2492. A smaller survey of the relation of orf1 to ptsP found the same organization in V. vulnificus CMCP6 and V. cholerae N16961 as was seen in F2310 but not in P. aeruginosa PA01, E. coli MG1655, Y. pestis...
C. multocida PM70, H. influenzae KW20, C. burnetii RSA493, or N. meningitidis MC58.

Multiple homologs of ORF1 were also found in the GenBank or TIGR databases, all of which were predicted or hypothetical permeases, with the closest matches being from Desulfovibrio desulfuricans (28% identities [64 of 222], 50% positives [111 of 222], 2e-25) and Geobacter metallireducens (28% identities [62 of 218], 50% positives [109 of 218], 7e-23) but also including P. aeruginosa (2e-19), V. cholerae (2e-19), and P. multocida (3e-17). All three sequenced L. pneumophila strains, Philadelphia 1, Paris, and Lens, had 98 to 99% nucleotide identity to orf1 over the entire 807-bp region.

Presence of nuda in other Legionella strains. The presence of nuda in other L. pneumophila strains, including strains from serogroups 1 to 14, was studied by PCR. A PCR product of the correct size was found in 19 of 20 strains tested. The one negative strain was one of two serogroup 5 strains tested. Southern blotting for nuda gave positive bands in one strain each of L. dumoffii, L. jordanis, L. longbeachae serogroup 1, L. micdadi, and L. quateriorensis; no bands were detected in single strains of L. birminghamensis, L. feeleii, L. maceachernii, L. rubrilucens, or L. steigerwaltii. All of the positive Southern blots for the non-L. pneumophila species revealed bands that were different from one another by at least several thousand base pairs and from the reference band of strain F2310 (parent).

Determination of nuda operon. The reverse transcriptase reaction product of total bacterial RNA, using a primer binding to nuda, and directed 3’ to 5’, was 1,000 bp in length, as measured by gel electrophoresis, and the same size as the PCR product of this reaction when bacterial DNA was used as the template. If ptsP is transcribed on an operon independent of nuda, no PCR product would be expected, since the PCR primers used included one that bound to nuda and one to ptsP. The negative control, F2310 (parent) total RNA used in the reverse transcriptase reaction without reverse transcriptase, had no product in the PCR, indicating the lack of contamination by bacterial DNA. These results indicate that nuda and ptsP are cotranscribed.

Interaction between NudA and PtsP. To determine whether the adjacent and highly conserved relationship of nuda to ptsP on the same operon had a correlate with interactions with their respective encoded proteins, the physical interactions of these two proteins were studied using two bacterial hybrid interaction studies. These studies showed no interaction of NudA and PtsP, regardless of which expression vector was used to express either protein, at both 30 and 37°C, using host strain DHM1. Positive control plasmids pKT25-zip and pUT18c-zip showed interaction, as expected, and a variety of negative controls showed no interaction, all with host strain DHM1. Use of host strain BTH101 gave unreliable results with the control plasmids and no evidence of interaction of NudA and PtsP.

NudA growth-phase dependence. Immunoblot analysis of F2310 (parent) fractions reacted with NudA antibody showed that NudA production appeared to be independent of bacterial growth phase, since approximately equal amounts of NudA were observed in immunoblots of log-phase and late-stationary-phase cultures.

Expression and purification of NudA. NudA expressed well from pETnudA in HMS174(DE3), and the purification procedure yielded approximately 10 mg, with an estimated purity by gel electrophoresis of >95% (data not shown). It is noteworthy that NudA precipitated with the nucleic acid fraction upon addition of streptomycin at the first step in the purification scheme.

Substrate specificity of NudA. The Nudix hydrolase superfamily of enzymes, catalyzing the hydrolysis of a variety of nucleoside diphosphate derivatives, may be divided into subfamilies depending on their preferred substrates. Since the “Nudix box” containing the amino acid signature sequence characteristic of the superfamily is highly conserved among all its members, the specificities for different substrates must be determined in regions of the protein outside of the Nudix motif. By comparing amino acid sequences of different members of the superfamily with the same or similar specificities, certain landmark amino acids outside of the Nudix box have been identified that are predictive of likely substrates for newly discovered proteins (7). The following is part of the sequence of NudA containing amino acids 30 to 80 (from the N terminus): 30AWQFPQGLAPGETAMQAMYRELHEEVDGLKD GDVEILGSTRRWLKYRLPK50. The amino acids indicated in boldface italics represent the highly conserved Nudix signature sequence. Seventeen amino acids downstream of the Nudix box is a tyrosine (boldface, underlined) that is predictive of a subfamily of Nudix hydrolases active on diadenosine polyphosphates (17). Accordingly, we tested a number of these compounds with purified NudA and found that Ap5A was the preferred substrate. In Table 2, the relative rates of hydrolysis of the related compounds from Ap3A to Ap5A are compared. A chain length of five phosphates appears to be optimal with Ap5A hydrolyzed at 60% of the rate of Ap3A. The tetra- and tripolyphosphate derivatives were practically not hydrolyzed. Also listed in the table are known substrates of other Nudix hydrolases. The inactivity toward these compounds indicates that NudA is specific for the higher diadenosine polyphosphates and may therefore be considered a member of the diadenosine polyphosphate subfamily.

Products of the reaction. The course of hydrolysis of Ap5A, analyzed by high-pressure liquid chromatography at various time points revealed a decrease in Ap3A and a commensurate increase in ATP and ADP. No other peaks appeared, and no P was formed during the incubation. Thus, the hydrolysis of Ap5A may be described by the following equation:

$$\text{Ap5A} + \text{H}_2\text{O} \rightarrow \text{ATP} + \text{ADP} + \text{Mg}^{2+}$$

Other properties of the enzyme. As with all of the orthogonal invasion-associated Nudix hydrolases studied thus far (5,
NudA had an alkaline pH optimum. The maximum rate of Ap5A hydrolysis was observed at pH 8.0, with activities falling off to 35 and 50% at pH 7 and 9.5, respectively. A divalent cation was absolutely required and was best satisfied by Mg\(^{2+}\). Mn\(^{2+}\) was ca. 15% as effective, whereas Zn\(^{2+}\) and Ca\(^{2+}\) were inactive. Analysis of the kinetic parameters of the hydrolysis of Ap5A by NudA showed the following: \(V_{\text{max}}\), 4.3 U/mg; \(K_{\text{cat}}\), 1.49 s\(^{-1}\); \(K_{\text{m}}\), 0.3 \(\mu\)M; and \(K_{\text{cat}}/K_{\text{m}}\), 4.96 \(10^3\) s\(^{-1}\) M\(^{-1}\).

Production of NudA and PtsP in the nudA mutant and its complement. Immunoblot analysis of the boiled nudA mutant (F2527) with either NudA or PtsP antibody showed that the mutant produced no detectable NudA but that PtsP was present in normal amounts and of expected size. A transposon-insertion mutant of ptsP, F2345 (32), originating from parent strain F2310, produced normal amounts of NudA and no detectable PtsP. In contrast, the parent strain (F2310) was shown to contain both NudA and PtsP under the same conditions. In addition, the transcomplemented nudA mutant (F2530) was shown to contain NudA, although the density of the NudA band was slightly less than that of the parent strain when equivalent amounts of total cells were used in the immunoblot study (data not shown). These data showed that the nudA mutant was defective in the production of NudA and that it had no polar effect on the production of PtsP. Also, the normal production of NudA by the ptsP mutant demonstrated that ptsP has no role in the regulation of nudA.

Phenotype of nudA mutant and effect of transcomplementation. L. pneumophila strain F2527 (ΔnudA) grew more slowly than did F2310 (parent) on both solid media and in broth and with a smaller colony size (Fig. 2). When both the parent and mutant were inoculated in equal amounts and grown (37°C) in the same tube of BYE broth at 25°C as measured by spectroscopy in duplicate. The growth curves of F2310 and F2527 were indistinguishable from those of F2531 and F2529, respectively, and are not shown for graphical clarity. Symbols: ○, F2529; ▲, F2527; ●, F2531. (C and D) Same as panel B, including symbols, but at 37 and 42°C, respectively. A second study of broth growth gave results nearly identical to those shown in panels B to D.

10, 15, 28), NudA had an alkaline pH optimum. The maximum rate of Ap5A hydrolysis was observed at pH 8.0, with activities falling off to 35 and 50% at pH 7 and 9.5, respectively. A divalent cation was absolutely required and was best satisfied by Mg\(^{2+}\). Mn\(^{2+}\) was ca. 15% as effective, whereas Zn\(^{2+}\) and Ca\(^{2+}\) were inactive. Analysis of the kinetic parameters of the hydrolysis of Ap5A by NudA showed the following: \(V_{\text{max}}\), 4.3 U/mg; \(K_{\text{cat}}\), 1.49 s\(^{-1}\); \(K_{\text{m}}\), 0.3 \(\mu\)M; and \(K_{\text{cat}}/K_{\text{m}}\), 4.96 \(10^3\) s\(^{-1}\) M\(^{-1}\).

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The nudA mutant was an auxotroph in that only $2 \times 10^{-4}$% of its inoculum grew on minimal medium, in contrast to ca. 40% of the parental inoculum. Prototrophy was not restored to the auxotrophic nudA mutant bacteria by the supplementation of the minimal medium by tryptophan or the mineral salts tested.

Transcomplementation of the nudA mutant (F2530) completely restored the growth rate of the bacterium in broth at 25, 37, and 42°C (Fig. 2B to D). The colony size of the complemented mutant was also almost completely restored to that of the parent (Fig. 2A).

Salt resistance of the nudA mutant was lost with transcomplementation with nudA. In a comparison of log phase grown bacteria, an average of 0.6 ± 0.2% (standard error of the mean, $n = 4$, with pooled data from two independent experiments) of the nudA mutant bacteria complemented with an empty plasmid (F2529) were salt resistant. Transcomplementation of the mutant with nudA (F2530) resulted in 0.07 ± 0.03% ($n = 4$, pooled data) salt-resistant bacteria versus 0.02 ± 0.007% ($n = 4$, pooled data) salt resistance of the parent with an empty plasmid (F2531). ANOVA showed a significant between-group salt resistance difference ($P = 0.01$), and post hoc analysis showed no significant difference between the salt resistance of F2530 and F2531, whereas the salt resistance of F2529 was significantly greater than that of F2530 or F2531 ($P < 0.05$).

Transcomplementation with nudA of the nudA mutant almost completely restored prototrophy. Only $5 \times 10^{-5}$ % of an inoculum of F2529 ($\Delta nudA + pMMB2002$) grew on minimal medium. In contrast, 0.5 and 21% of the inocula of F2530 ($\Delta nudA + nudA$ in pMMB2002) and F2531 (parent + pMMB2002), respectively, grew on the same media. Transcomplementation thus increased the prototrophy of the mutant by ca. 10,000-fold.

Virulence of nudA mutant in guinea pig alveolar macrophages and guinea pigs. The nudA mutant (F2527) grew slightly less well in guinea pig alveolar macrophages than did its parent (F2310) (Fig. 3A). Only on day 2 postinfection was there a significant difference between the mean bacterial concentrations, with the ratio of F2310 to F2527 on that day being equal to 4.1 ($P = 0.001$ [nonpaired $t$ test]). To help determine whether F2527 was truly less virulent than F2310 in macrophages, bacterial competition studies were undertaken; this method can demonstrate significant L. pneumophila virulence defects when single bacterial studies show none (23). When F2310 and F2527 were mixed together in similar amounts before infecting macrophages, the parent outcompeted the mutant by 4.0-fold and 4.8-fold after 2 and 3 days, respectively ($P < 0.001$ for day 0 or 1 versus day 2 or 3 [unpaired $t$ tests with Welch’s correction for unequal variances]) (Fig. 3B). The parent bacterium also outcompeted the nudA mutant when guinea pigs were infected with a mixture of both strains by intratracheal inoculation. At 50 h after infection F2310 outcompeted F2527 by 4.3- and 3.8-fold in the lung and spleen, respectively (Fig. 4).

**FIG. 3.** Growth of F2527 ($\Delta nudA$) and F2310 (parent) in guinea pig alveolar macrophages. (A) Comparison of growth of F2310 and F2527 when each was independently used to infect macrophages. Error bars show the standard error of the mean ($n = 3$). Two other independent experiments gave the same results. Symbols: ■, F2310; ▲, F2527. (B) Competition between F2310 and F2527, when the bacteria were mixed together before infection of the macrophages; data are normalized and pooled from three independent experiments and are presented as the mean ratios of F2310 to F2527. Error bars show standard error of the mean ($n = 9$). Three other independent experiments gave the same results.

**FIG. 4.** Multiplication of a mixture of F2310 (parent) and F2527 ($\Delta nudA$) in guinea pigs infected by the intratracheal route. The datum points represent mean values of the ratio of F2310 to F2527; error bars represent the standard errors of the mean of three independent experiments (total $n = 17$ animals). The F2310:F2527 ratios at 50 h postinfection for both lung and spleen differ significantly from 1.0 (ratio at time zero) by one-sample $t$ tests ($P < 0.0001$). Symbols: □, lung; ▲ (dotted line), spleen.
Transcomplementation of the nudA mutant partially restored its decreased virulence for guinea pig alveolar macrophages (Fig. 5). In competition studies with the parent, the transcomplemented mutant was significantly more virulent than the mutant containing an empty plasmid (P ≤ 0.002 for F2531/F2530 versus F2531/F2529; P = 0.3 for day 1, all by nonpaired t tests). The inset shows the growth of F2531 alone in macrophages. Note that a lower ratio indicates greater virulence of the strain in reference to that of F2531 and that a ratio of 1 would mean virulence equivalent to F2531. One other set of experiments gave similar results. Symbols: ■, F2531:F2529; ▽, F2531:F2530.

DISCUSSION

Nudix hydrolases are a family of ubiquitous enzymes found in viruses, bacteria, and eukaryotes, including humans, with pleiotropic functions and multiple substrate specificities (4, 34). Although originally described solely as a mutation prevention mechanism, as MutT in E. coli, it is now apparent that Nudix hydrolases have different functions in multiple hosts, including bacterial pathogenesis (3, 5, 15, 44), mRNA decapping in mammals and vertebrates (29, 50), and viral pathogenesis (10, 49). Most bacteria studied contain one or more different Nudix hydrolases, unrelated to genome size (32). For example, E. coli K-12 contains 12, Neisseria meningitidis contains 1 to 2, Mycobacterium tuberculosis H37RV contains 7, Bacillus anthracis contains 30, and Mycoplasma pneumoniae M129 contains no Nudix hydrolases (52). Two bacteria, Bartonella bacilliformis, and E. coli K1, have been shown to have Nudix hydrolases, ialA and ygdP, respectively, that contribute to erythrocyte and human brain microvascular endothelial cell invasion, respectively (3, 5, 15, 44). In addition, Rickettsia prowazekii has been shown to have a Nudix hydrolase invA homolog that is of unknown function but could be a virulence gene for this organism (28).

Our studies show that L. pneumophila nudA is a Nudix hydrolase that is a virulence factor for intracellular and guinea pig infection. A polar effect of the nudA mutation on a co-transcribed gene that would account for either the virulence defect or stress-related defects is very unlikely. The mutation insertion was by design nonpolar, and functional evidence for readthrough of the mutation into the downstream ptsP gene was shown by the expression of normal amounts of PtsP in the nudA mutant. In addition, transcomplementation of the nudA mutant restored significant, albeit not complete, virulence for macrophage multiplication. We attribute incomplete complementation to the complexity of the mixed infection assay and to the reduced expression of NudA in the transcomplemented mutant, as shown by immunoblot. It is probable that sufficient levels of NudA were produced by the transcomplemented strain to reverse the mutation-associated changes in metabolism and growth but that they were insufficient for complete restoration of virulence.

The markedly reduced ability of F2527(ΔnudA) to grow at 25°C is similar to the reduced ability of E. coli ygdIP to grow at 18°C (31). Nudix hydrolase substrates such as Ap4A are hypothesized to be “alarmones” that are synthesized in high concentrations in the presence of heat shock or oxidative stress (6, 34, 37, 39). In E. coli these alarmones bind to heat shock and oxidative stress proteins (26, 34), and it is thought that alarmone synthesis may modulate the heat stress response (34). E. coli apaH is a non-Nudix dinucleoside hydrolase that normally hydrolyzes Ap4A to ADP and Ap5A to ADP + ATP (24, 33). E. coli apaH mutants accumulate Ap4A, have increased susceptibility to heat and UV light, are unable to grow at 43°C, and are nonmotile; the mechanism of these phenotypes is thought to be due to the modulation of RpoE by excess Ap4A and to alteration in cyclic AMP and by binding to heat shock and oxidative stress proteins (24, 34). Helicobacter pylori nudA, which preferentially hydrolyzes Ap4A, is important for survival to oxidative stress caused by H2O2 but not heat shock (40). We show that the L. pneumophila nudA mutation is responsible for delayed growth at 25, 37, and 42°C, reduced colony size, auxotrophy, and salt resistance but not increased susceptibility to H2O2 or decreased motility as has been reported for other Nudix hydrolase mutants. Salt resistance has not been previously studied in Nudix hydrolase mutants, and...
its presence in the *L. pneumophila nudA* mutant is difficult to explain in terms of the expected increased susceptibility to stress observed in Nudix hydrolase mutants of other bacteria (4). It is noteworthy that salt resistance can be a nonspecific marker of reduced virulence of *L. pneumophila* by unknown mechanisms (11, 35).

Another alarmone, ppGpp, has been shown to trigger the entry of the Lp02 strain of *L. pneumophila* into the late stationary phase under limited nutrient conditions via a RpoS-dependent mechanism and to enhance the virulence of the bacterium for macrophages (30). Late-stationary-phase growth of this strain also greatly enhances NaCl sensitivity and heat resistance (8). The *L. pneumophila nudA* mutant is reduced in virulence and is salt resistant and heat sensitive, which superficially suggests that *nudA* could play a role in ppGpp modulation. However, ppGpp is not a substrate of NudA, NudA expression is independent of bacterial growth phase, and the virulence defect in the *nudA* mutant is much less severe than that seen in log phase Lp02 bacteria in comparison to late-stationary-phase bacteria. It is more likely that the growth-phase-related characteristics of Lp02 and the effect of NudA on F2310 share similar phenotypes but not mechanisms. A caveat, however, is that *L. pneumophila* strain F2310 (parent) is unlike strain Lp02 in several ways, including its overall greater virulence and the lack of virulence enhancement in the postexponential phase (48).

The enzymatic characteristics of *L. pneumophila* NudA closely resemble the homologous Nudix hydrolase invasion-associated proteins InvA from *R. prowazekii* (9) and YgdP from *E. coli* (2). IaLA, the homologue from *B. bacilliformis*, also belongs in this group but prefers Ap4A instead of Ap5A (9, 16).

Our studies do not support a role for *L. pneumophila* NudA as an invasin, based on the similar invasion abilities of the *nudA* mutant and its parent, as well as on the lack of differences between these strains for macrophage multiplication on day 1 postinfection. This is in contrast to the invasion-like nature of the *E. coli* K-12 and *B. bacilliformis nudA* orthologs (3, 44).

Our findings on *nudA* can probably be generalized to other *L. pneumophila* strains based on the highly conserved NudA sequence in the three *L. pneumophila* strains that have been completely sequenced and on the results presented by us on the presence of *nudA* in 95% of *L. pneumophila* strains tested by us. The *nudA* gene also appears to be common in other *Legionella* spp. based on the identification of *nudA*-homologous sequences in 5 of 10 other *Legionella* spp. tested by Southern hybridization. It is possible, but unproven, that *nudA* is a virulence factor for these other species.

The genetic organization of *nudA* in relation to *ptsP* was conserved in the four *L. pneumophila* strains of known sequence and a number of other bacteria. The highly conserved nature of this genetic organization and cotranscription in the F2310 (parent) *L. pneumophila* strain suggests some interaction between the two genes. In addition, the GC contents of both genes are significantly higher than that of the total genome and different from the immediate downstream gene, *orf1*, suggesting that both *nudA* and *ptsP* originated from the same non-*L. pneumophila* organism. We were unable to find evidence of protein-protein interaction with a bacterial two-hybrid system. In addition, mutations in each gene had no apparent effect on expression of the other. These results do not exclude trans interactions between *nudA* and *ptsP*, especially ones related to production of energetic phosphate compounds by NudA hydrolisis of Ap2,A or degradation of Np3,N by the enzyme. In fact, Ismail et al. speculate that in *Salmonella enterica* serovar Typhimurium, NudA hydrolisis of Np3,N has a role in the regulation of PtsP by binding to the regulatory domain of PtsP (33).

The mechanism by which NudA exerts its pleiotropic phenotype in *L. pneumophila* is unknown. One of us has speculated that Nudix hydrolases perform a “housecleaning” role by hydrolyzing potentially toxic nucleotides and by preventing the unbalanced accumulation of normal metabolites (4). In the case of *L. pneumophila*, NudA appears to prevent inimical responses to external stress. The presence of this enzyme confers a growth advantage in macrophages and therefore appears to have a role in both extracellular and intracellular survival of the bacterium. Whether the slow-growth of the *nudA* mutant in macrophages is solely related to its slower growth in vitro, or to factors exclusive to the intracellular environment is unknown but possible. The intracellular environment of *L. pneumophila* is complex and dynamic, making extrapolation of extracellular growth characteristics to intracellular growth characteristics tenuous. Based on the profound effect of a *nudA* mutation on 25°C growth, it is reasonable to hypothesize that NudA plays a role in the survival of the intra-amebal bacterium at the same or lower temperature in the environment. Future studies are needed to determine the role of NudA in the environmental persistence of *L. pneumophila*, its interactions with other bacterial genes, and its effect on bacterial persistence and relapse in mammalian infected with *L. pneumophila*.

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