

Purification and Characterization of a UDP-Glucosyltransferase Produced by *Legionella pneumophila*

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Legionella pneumophila is the agent of Legionnaires' disease. It invades and replicates within eukaryotic cells, including aquatic protozoans, mammalian macrophages, and epithelial cells. The molecular mechanisms of the *Legionella* interaction with target cells are not fully defined. In an attempt to discover novel virulence factors of *L. pneumophila*, we searched for bacterial enzymes with transferase activity. Upon screening ultrasonic extracts of virulent legionellae, we identified a uridine diphospho (UDP)-glucosyltransferase activity, which was capable of modifying a 45-kDa substrate in host cells. An approximately 60-kDa UDP-glucosyltransferase was purified from *L. pneumophila* and subjected to microsequencing. An N-terminal amino acid sequence, as well as the sequence of an internal peptide, allowed us to identify the gene for the enzyme within the unfinished *L. pneumophila* genome database. The intact gene was cloned and expressed in *Escherichia coli*, and the recombinant protein was purified and confirmed to possess an enzymatic activity similar to that of the native UDP-glucosyltransferase. We designated this gene *ugt* (UDP-glucosyltransferase). The *Legionella* enzyme did not exhibit significant homology with any known protein, suggesting that it is novel in structure and, perhaps, in function. Based on PCR data, an enzyme assay, and an immunoblot analysis, the glucosyltransferase appeared to be conserved in *L. pneumophila* strains but was absent from the other *Legionella* species. This study represents the first identification of a UDP-glucosyltransferase in an intracellular parasite, and therefore modification of a eukaryotic target(s) by this enzyme may influence host cell function and promote *L. pneumophila* proliferation.

Legionella pneumophila is the principal agent of Legionnaires' disease, a pneumonic illness of humans. This bacterium is a facultative intracellular parasite that invades and proliferates in both professional and nonprofessional phagocytes (54). The host cells for *L. pneumophila* include freshwater protozoans and human alveolar macrophages and epithelial cells. Bacterial factors that have been identified as crucial for intracellular infection include, among others, the major outer membrane protein (25), Mip (13), Hsp60 (17, 18), PilD and type II protein secretion (4, 43), type IV pili (29, 51), flagella (41), the Dot/Icm type IV protein secretion system, and the products of the *enh*, *lvh*, *mil*, and *pmi* loci (2, 11, 14, 16, 35, 49). In addition to these virulence factors, a variety of enzymes have been identified in *Legionella* cultures, including a low-molecular-mass cytotoxin (30), a metalloprotease cytolysin (8, 9), phospholipases (3, 4, 6), lipases (3), phosphatases (5, 44) and phosphokinases (45). For many of these activities, the role in virulence is unclear and/or the eukaryotic substrate(s) is unknown. Thus, further studies aimed at identifying and characterizing novel *L. pneumophila* enzymes should be important for understanding both Legionnaires' disease and bacterial interactions with eukaryotic cells.

It is well known that certain types of transferases are major virulence factors in some bacteria and represent the enzymatic components of toxins. For example, whereas diphtheria toxin

of *Corynebacterium diphtheriae*, cholera toxin of *Vibrio cholerae*, and exotoxin A of *Pseudomonas aeruginosa* are ADP-ribosyltransferases, toxins A and B of *Clostridium difficile* and the lethal toxin of *Clostridium sordellii* are uridine diphospho (UDP)-glucosyltransferases (48). Interestingly, in the gram-negative pathogens, these toxic transferases can be dependent upon type II protein secretion, a system that, as noted above, is also critical for *L. pneumophila* pathogenesis (4, 43, 56). Whereas ADP-ribosyltransferases have been found in both extra- and intracellular pathogens, UDP-glucosyltransferase toxins have been studied only in extracellular pathogens (1, 37, 39). For these various reasons, our research has been aimed at identification of similar types of enzymes in *Legionella* cultures. In the present study, we characterized a novel product of *L. pneumophila* that possesses UDP-glucosyltransferase activity and modifies a distinct component of eukaryotic host cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following *Legionella* strains were used in this study: *L. pneumophila* Philadelphia I (= ATCC 33217) (serogroup 1), 130b (= ATCC BAA-74) (serogroup 1), ATCC 33823 (serogroup 7), and ATCC 35096 (serogroup 8), *L. longbeachae* ATCC 33462, *L. gormanii* ATCC 33297, and *L. steigerwaltii* ATCC 35302. Bacteria were grown on buffered charcoal yeast extract (BCYE) agar for 2 days at 37°C (15). *Escherichia coli* strain BL21(DE3) and the expression vector pET28b (Novagen, Madison, Wis.) were used for cloning and the production of recombinant protein. *E. coli* was maintained on Luria-Bertani agar supplemented with 50 µg of kanamycin per ml when necessary (46).

Protein purification and microsequencing. The *L. pneumophila* UDP-glucosyltransferase was purified from strain Philadelphia I. Bacterial cells from 100 BCYE agar plates were suspended in 20 ml of 20 mM Tris-HCl (pH 7.4)–150 mM NaCl (TBS), washed once by centrifugation (Sorvall, Newtown, Conn.) at

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12,000 × *g* for 15 min (all centrifugation steps were performed at 4°C), and resuspended in 100 ml of TBS. The cell suspension was subjected to ultrasonication with five 1-min pulses with 2-min intervals between pulses at the high power setting (Techpan, Warsaw, Poland) on ice. Unbroken cells and cell wall fragments were pelleted by centrifugation at 18,000 × *g* for 60 min and discarded. The supernatant was treated with protamine sulfate (Sigma, St. Louis, Mo.) at a concentration of 2 mg/ml to sediment the majority of the bacterial DNA. After the sonicate was dialyzed overnight against 20 mM Tris-HCl (pH 7.4) (TB), it was loaded onto a Mono Q 10/10 anion-exchange column equilibrated with TB. The column was washed sequentially with TB and 0.05 M NaCl in TB, and fractions were tested for UDP-glucosyltransferase activity (see below). The enzymatically active preparation was then eluted with 20 ml of 0.1 M NaCl in TB. The eluate was dialyzed against 20 mM morpholineethanesulfonic acid (MES)-Na buffer (pH 6.25) (MB) and loaded onto a Mono S 5/5 cation-exchange column equilibrated with MB. The Mono S 5/5 column was then washed sequentially with MB, 0.05 M NaCl in MB, and 0.1 M NaCl in MB. The enzymatically active material was eluted with 3 ml of 0.15 M NaCl in MB. After the active samples were dialyzed against TB, they were loaded onto a Mono Q 5/5 anion-exchange column equilibrated with TB. Elution was performed with a linear 0.0 to 0.1 M NaCl gradient in TB. Active fractions, which eluted between 60 and 80 mM NaCl, were concentrated by ultrafiltration with a YM10 filter (Amicon, Danvers, Mass.) and subjected to gel chromatography on a TBS-equilibrated Superose 6 10/30 column, which was able to separate proteins having molecular masses ranging from 5 to 5,000 kDa. Fractions (0.4 ml) were collected, and the fractions with enzymatic activity, which eluted in a peak retention volume of 16.5 ml, were pooled and stored at -20°C as purified UDP-glucosyltransferase. All matrices used for liquid chromatography were obtained from Amersham Biosciences, Vienna, Austria. All chromatographic steps were done with the Amersham fast protein liquid chromatography system. Microsequencing of purified protein was performed at the Laboratory of Protein Microsequencing at the Institut Pasteur by standard procedures. In order to assay for UDP-glucosyltransferase activity in other strains of *L. pneumophila* or in other species of *Legionella*, crude ultrasonic extracts were prepared as described above for *L. pneumophila* Philadelphia I. Finally, in order to assay for a secreted UDP-glucosyltransferase activity, *L. pneumophila* Philadelphia I was grown in Proteose Peptone No. 3 (Difco, Detroit, Mich.) broth for 18 h at 37°C with constant shaking (200 rpm) (59). After centrifugation of the culture at 12,000 × *g* for 15 min, the resultant supernatant was filter sterilized and concentrated 10-fold by using an Amicon YM10 filter.

PCR amplification and cloning of the UDP-glucosyltransferase gene. Based on data from the *L. pneumophila* Philadelphia I genome project at Columbia University (http://genome3.cpmc.columbia.edu/~legion/ngn1033_033), two DNA primers were designed for amplification of the putative UDP-glucosyltransferase gene (IDT, Coralville, Iowa). The sequence of the sense primer was 5'-ATGGGAGACGAGTATGAATTCAGCAAGAAG-3' (an engineered *Eco*RI site is underlined), and the sequence of the antisense primer was 5'-TTCAAAAATTTGTGTGTCGACATTAAGCTAC-3' (an engineered *Sal*I site is underlined). The PCR was performed under standard conditions (i.e., denaturation at 94°C, annealing at 55°C, and elongation at 72°C) with a Sprint thermocycler (Hybaid, Ashford, England). *Legionella* genomic DNA for the PCR was extracted by the phenolic method (46). For cloning of the UDP-glucosyltransferase gene, the PCR product that was amplified from *L. pneumophila* Philadelphia I DNA was digested with *Eco*RI and *Sal*I and ligated into similarly digested pET28b. *E. coli* BL21(DE3) was transformed with the resulting plasmid by electroporation by using an Electroporator 2510 (Eppendorf, Hamburg, Germany).

For purification of a recombinant enzyme, the *E. coli* clone was grown in Luria-Bertani broth supplemented with kanamycin for 3 h on a shaker at 37°C. Hyperexpression of the cloned protein was then induced by supplementing the culture with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma) and incubating it for an additional 2 h. The bacterial cells from 100 ml of culture were harvested by centrifugation at 8,000 × *g* for 15 min, resuspended in 6 ml of TBS, and then lysed by ultrasonic treatment (three 20-s pulses with 1-min intervals between pulses at the medium power setting). Following clarification by centrifugation, the bacterial extract was subjected to chromatography on nickel-equilibrated chelating Sepharose Fast Flow (Amersham Biosciences) used according to the manufacturer's instructions. Purified recombinant UDP-glucosyltransferase was eluted with 0.5 M imidazole and desalted by using an FD10 column (Amersham Biosciences). Pure protein was stored at -20°C.

UDP-glucosyltransferase assay. Protein samples derived from *Legionella* strains were tested for the ability to UDP-glucosylate substrates contained in HeLa, THP-1, CHO, and Vero cells, which were obtained from the Pasteur Institut and the Gamaleya Research Institute. The various cell lines were grown in 250-ml culture flasks (Becton Dickinson, Oxnard, Calif.) containing RPMI

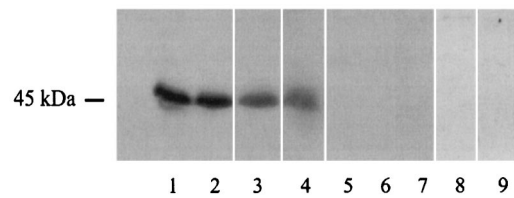


FIG. 1. Identification of a new UDP-glucosyltransferase activity. Ultrasonic extracts from different *Legionella* species were incubated in the presence of HeLa cell extract and [¹⁴C]UDP-glucose. After this, the samples were subjected to SDS-PAGE, dried, and exposed to X-ray film. Lane 1, *L. pneumophila* Philadelphia I; lane 2, *L. pneumophila* 130b; lane 3, *L. pneumophila* ATCC 33823; lane 4, *L. pneumophila* ATCC 35096; lane 5, *L. longbeachae* ATCC 33462; lane 6, *L. gormanii* ATCC 33297; lane 7, *L. steigerwaltii* ATCC 35302; lane 8, *L. pneumophila* Philadelphia I without eukaryotic substrate; lane 9, HeLa cell extract without bacterial extract. The data represent data obtained in several experiments.

1640 medium supplemented with 10% fetal calf serum (Gibco Laboratories, Grand Island, N.Y.). After 3 to 4 days of growth at 37°C in the presence of 5% CO₂, cells were scrapped from the plastic surfaces, washed, and resuspended in 2 ml of ice-cold TBS per culture flask. Cell extracts were prepared by ultrasonication, which involved a 5- to 7-s pulse at medium power. UDP-glucosyltransferase reactions were carried out by using mixtures consisting of 5 μl of reaction buffer (150 mM triethanolamine [pH 7.5], 6 mM MgCl₂, 0.9 mM GDP, 3 mM dithiothreitol), 2 μl of [¹⁴C]UDP-glucose (code CFB102-10μCi; Amersham Biosciences), 5 μl of bacterial preparation at a protein concentration of 0.05 to 1.5 mg/ml, and 5 μl of eukaryotic cell ultrasonic extract at a protein concentration of 1 mg/ml. Each mixture was incubated at 37°C for 1 h, after which the reaction was stopped by adding Laemmli sample buffer and heating the mixture at 100°C for 5 min. The samples were then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by using 7% polyacrylamide stacking and 12% polyacrylamide resolving gels (28). After this, each gel was washed in 7% acetic acid, dried, and exposed to Hyperfilm (Amersham Biosciences) for 3 days at -70°C.

Immunoblot analysis and protein determination. Following SDS-PAGE (28), an immunoblot analysis was performed by using standard procedures (57), a 1/5,000 dilution of primary rabbit serum, and a 1/4,000 dilution of anti-rabbit horseradish conjugate (Bio-Rad, Moscow, Russia). For production of primary antiserum, 2- to 3-kg rabbits, obtained from the animal facility at the Gamaleya Research Institute, were immunized three times with 100 μg of native, purified UDP-glucosyltransferase in TBS at 2-week intervals. Ten days after the last injection, the rabbits were exsanguinated by sublethal cardiac puncture, and the resultant serum was stored. Protein concentrations were determined by a Coomassie brilliant blue R250 assay (Serva, Heidelberg, Germany) by using bovine serum albumin as a standard (10).

RESULTS

Identification of a novel UDP-glucosyltransferase activity.

As part of our work aimed at identifying virulence factors of the legionellae, we sought a UDP-glucosyltransferase in cultures of *L. pneumophila*. To do this, culture supernatant from the *L. pneumophila* Philadelphia I strain and ultrasonic lysates of cells from several strains were tested in reaction mixtures consisting of HeLa cells extracts and [¹⁴C]UDP-glucose. The HeLa cell line is one of a number of human epithelial cell lines that are susceptible to *L. pneumophila* invasion and intracellular replication (18). When sonicates were used, but not when supernatants were used, we observed incorporation of radioactive label into a ca. 45-kDa protein band (Fig. 1 and data not shown). The activity was observed for all four *L. pneumophila* strains tested, including representatives of serogroups 1, 7, and 8. There was no protein labeling if the bacterial lysate or HeLa cell extract was omitted from the mixture (Fig. 1), indicating

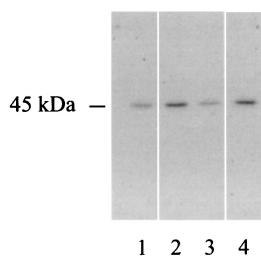


FIG. 2. Identification of a substrate for *L. pneumophila* UDP-glucosyltransferase in different eukaryotic cell extracts. Ultrasonic extracts from the *L. pneumophila* Philadelphia I strain were incubated in the presence of HeLa (lane 1), Vero (lane 2), CHO (lane 3), and THP-1 (lane 4) cell preparations and [14 C]UDP-glucose. After this, the samples were subjected to SDS-PAGE, dried, and exposed to X-ray film.

that the reaction resulted from the interaction of prokaryotic and eukaryotic factors. Interestingly, no reaction was observed when sonicates from other *Legionella* species were used (Fig. 1). To determine whether the labeling reaction required factors peculiar to HeLa or epithelial cells, the experiment was repeated with extracts from human monocytic THP-1 cells as well as from Vero and CHO cells. Like HeLa cells, THP-1, Vero, and CHO cells have been shown to support *L. pneumophila* intracellular growth (27, 33, 38). In all cases, similar UDP-glucosylation reactions were observed (Fig. 2). Based on these observations, as well as the fact that other bacterial enzymes (i.e., clostridial toxins) can transfer UDP-glucose to mammalian cell substrates (23, 24, 40), we hypothesized that *L. pneumophila* possesses a UDP-glucosyltransferase that is active against a conserved 45-kDa component of eukaryotic cells.

Purification and partial sequence of an *L. pneumophila* UDP-glucosyltransferase. To investigate the existence of a *Legionella* UDP-glucosyltransferase, we tested various fractions from ultrasonic extracts of *L. pneumophila* Philadelphia I for the ability to generate, when coupled with HeLa cell extracts, the radiolabeled, 45-kDa protein band. The biochemical procedure developed consisted of an initial combination of anion- and cation-exchange chromatography and a final gel chromatography step (see Materials and Methods). As shown by SDS-PAGE, the preparation from the last step appeared to consist of a single major protein with a molecular mass of approximately 60 kDa (Fig. 3). The purified protein yielded the same result as the sonicate; i.e., it facilitated radiolabeling of a 45-kDa protein band (Fig. 4). The yield obtained with this preparative scheme was 0.7 mg of pure product per 150 mg of starting crude protein. Since the molecular mass of the reactive material purified from the bacteria was 60 kDa instead of 45 kDa, we concluded that *L. pneumophila* encodes the UDP-glucosyltransferase and not the substrate for a eukaryotic enzyme. As a first step toward understanding the molecular nature of the *Legionella* UDP-glucosyltransferase, we subjected the purified protein to a microsequencing analysis. The N-terminal peptide sequence was found to be DQQLSXLRRFFSAL, while an internal peptide sequence was (F/Q)NISLIDIDSVR. BLASTP searches (cost to open gap, 11; expect value, 10) of the National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) failed to detect any relationship between these peptide se-

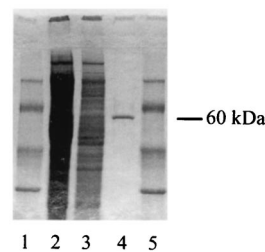


FIG. 3. Purification of a UDP-glucosyltransferase from *L. pneumophila*. Samples were subjected to SDS-PAGE and stained with Coomassie brilliant blue R250. Lanes 1 and 5, molecular mass markers; (from top to bottom, 94, 67, 43, and 30 kDa; Amersham Biosciences); lanes 2 and 3, ultrasonic lysate of *L. pneumophila* Philadelphia I containing 30 and 15 μ g of protein, respectively; lane 4, purified, 60-kDa UDP-glucosyltransferase (ca. 0.5 μ g).

quences and known proteins, indicating that the *Legionella* UDP-glucosyltransferase is a previously unrecognized protein.

Cloning and PCR detection of the *L. pneumophila* UDP-glucosyltransferase gene. In an attempt to identify the gene encoding the *Legionella* UDP-glucosyltransferase, we used our protein sequence data to search the incomplete genomic database of *L. pneumophila* Philadelphia I (<http://genome3.cpmc.columbia.edu/~legion/ngnp1033033>). An open reading frame whose translated sequences matched the sequences of isolated peptides was found on contig CTG.BC.5E37D10.01.44.07280. The 1.57-kb gene, which had not yet been assigned a possible function, was predicted to encode a protein product with a molecular mass of 57.8 kDa, a size that was compatible with the size of the purified UDP-glucosyltransferase (i.e., ca. 60 kDa). There was an apparent discrepancy between the N-terminal sequences of the purified protein (DQQLSXLRRFFSAL) and the translated gene product (MKARRDQQLSKLRMRFFSAL). The absence of the amino acid string MKARR in the sequenced protein might have resulted from posttranslational modification of the enzyme or from proteolytic degradation during storage or purification. BLASTX searches (cost to open gap, 11; expect value, 10) with the entire open reading frame sequence only retrieved hits possessing scores of less than 36 bits and E values of more than 1.1. These results demonstrated that there was a lack of overall homology between the *Legionella* protein and known proteins in the National Center for Biotechnology Information database

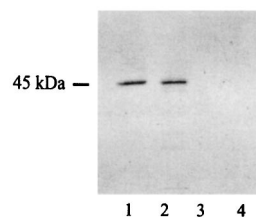


FIG. 4. Enzymatic activity of UDP-glucosyltransferase purified from *L. pneumophila* and recombinant *E. coli*. Approximately 0.5 μ g of purified native protein (lanes 1 and 3) or recombinant protein (lanes 2 and 4) was incubated in the presence of [14 C]UDP-glucose with (lanes 1 and 2) or without (lanes 3 and 4) HeLa cell extract. After this, the samples were subjected to SDS-PAGE, dried, and exposed to X-ray film.

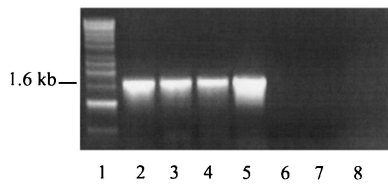


FIG. 5. PCR detection of the *L. pneumophila* UDP-glucosyltransferase gene. Based on nucleotide sequence data for *L. pneumophila* Philadelphia I, specific primers were used to PCR amplify DNA fragments from purified genomic preparations. Following amplification, the DNA mixtures were subjected to agarose gel electrophoresis and stained with ethidium bromide. Lane 1, molecular mass markers (from top to bottom, 10, 8, 6, 5, 4, 3, 2.5, 2, 1.5, 1 [threefold intensity], and 0.5 kb; Amersham Biosciences); lane 2, *L. pneumophila* Philadelphia I; lane 3, *L. pneumophila* 130b; lane 4, *L. pneumophila* ATCC 33823; lane 5, *L. pneumophila* ATCC 35096; lane 6, *L. longbeachae* ATCC 33462; lane 7, *L. gormanii* ATCC 33297; lane 8, *L. steigerwaltii* ATCC 35302.

(<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). In order to determine whether the gene which we examined in fact encodes the *L. pneumophila* UDP-glucosyltransferase, we sought to clone it and test its protein product for activity in our HeLa cell extract assay. To do this, we utilized the pET28 expression system, which allows cloning of recombinant proteins in frame with an upstream polyhistidine tag and thereby permits protein purification by nickel affinity chromatography. Using PCR and primers based on sequences in the *Legionella* database, we amplified a 1.6-kb fragment from Philadelphia I genomic DNA (Fig. 5, lane 2). The PCR fragment was expected to contain the entire UDP-glucosyltransferase open reading frame plus 16 bp upstream and 29 bp downstream. By incorporating convenient restriction sites into the primers, the DNA fragment was easily cloned into pET28b, resulting in plasmid p28b-13. *E. coli* BL21(p28b-13) was confirmed by SDS-PAGE to express a considerable amount of the cloned gene product (Fig. 6). When tested in the UDP-glucosylation assay, the recombinant protein modified a substrate in a HeLa cell extract with the same molecular mass (45 kDa) as the substrate modified by the native enzyme (Fig. 4). These results confirm that the *L. pneumophila* gene encodes UDP-glucosyltransferase activity. We designated this gene *ugt* (UDP-glucosyltransferase). The *ugt* gene mapped between a putative heat shock protein gene and a predicted deaminase gene in the unfinished *L. pneumophila*

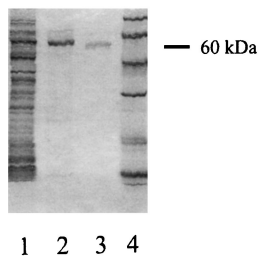


FIG. 6. SDS-PAGE analysis of recombinant *L. pneumophila* UDP-glucosyltransferase. Lane 1, crude lysate of *E. coli* BL21(p28b-13); lane 2, purified recombinant UDP-glucosyltransferase; lane 3, UDP-glucosyltransferase purified from *L. pneumophila*; lane 4, molecular mass markers (94, 67, 43, 30, 20, and 14 kDa). Because of its N-terminal polyhistidine tag, the recombinant enzyme migrated more slowly than the native enzyme.

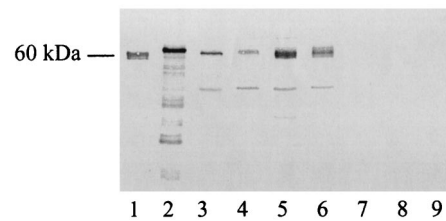


FIG. 7. Immunoblot analysis of different *Legionella* strains. Ultrasonic extracts were obtained from various legionellae grown on BCYE agar, and then samples containing approximately 5 μ g of protein were subjected to SDS-PAGE, transferred onto a nitrocellulose filter, and probed with an antiserum raised against purified *L. pneumophila* UDP-glucosyltransferase. Lane 1, UDP-glucosyltransferase purified from *L. pneumophila*; lane 2, purified recombinant UDP-glucosyltransferase; lane 3, *L. pneumophila* Philadelphia I; lane 4, *L. pneumophila* 130b; lane 5, *L. pneumophila* ATCC 33823; lane 6, *L. pneumophila* ATCC 35096; lane 7, *L. longbeachae* ATCC 33462; lane 8, *L. gormanii* ATCC 33297; lane 9, *L. steigerwaltii* ATCC 35302. Minor bands observed in recombinant protein and some *L. pneumophila* preparations probably resulted from proteolytic degradation of a major 60-kDa enzyme.

genome project sequence. The previously described *lspFGHIJK* genes, which are associated with type II protein secretion (19, 43), were approximately 4 kb downstream of *ugt*. PCR analysis with the primers described above revealed that all *L. pneumophila* strains tested possessed a 1.6-kb *ugt*-containing fragment (Fig. 5). In contrast, representatives of *L. longbeachae*, *L. gormanii*, and *L. steigerwaltii* were negative in the PCR (Fig. 5).

Immunoblot assay of the *L. pneumophila* UDP-glucosyltransferase. In the next stage of our investigation, polyclonal anti-UDP-glucosyltransferase antibodies were generated, and Western blotting was used to test a panel of bacterial extracts for the presence of the UDP-glucosyltransferase. All *L. pneumophila* strains tested contained a major ca. 60-kDa protein that reacted with the antiserum, while strains representing other *Legionella* species were negative in the immunoblot analysis (Fig. 7). Thus, our results demonstrated that there was a good correlation among UDP-glucosyltransferase activity (Fig. 1), PCR amplification (Fig. 5), and Ugt cross-reactive proteins. Furthermore, they indicated that the UDP-glucosyltransferase may be peculiar to *L. pneumophila*.

DISCUSSION

Based on the behavior of purified native and recombinant proteins, we discovered a new *L. pneumophila* enzyme, a UDP-glucosyltransferase. Enzymes capable of transferring the UDP-glucose moiety have been described previously in viruses, bacteria, plants, and animals (12, 36, 42, 53, 60). Importantly, though, the *L. pneumophila* Ugt enzyme is the first example of a glucosyltransferase that is produced by a facultative intracellular parasite. As a group, the UDP-glucosyltransferases have been linked with a variety of processes, including, in bacteria, biosynthesis of antibiotics and modification of substrates having different chemical structures (34, 36). Based on the overall lack of homology between the *L. pneumophila* enzyme and known proteins and the fact that the legionellae are intracellular parasites of protozoan and mammalian cells, Ugt is likely a new type of UDP-glucosyltransferase and may catalyze a

novel reaction. Indeed, the enzyme transferred UDP-glucose to an unidentified 45-kDa protein within eukaryotic cells. Since Ugt-containing *Legionella* extracts failed to undergo autoglucosylation, there do not appear to be additional substrates in the bacterial cell. Thus, the *L. pneumophila* enzyme may have evolved to interact specifically with a host cell target.

Given the different lifestyles of *L. pneumophila*, Ugt could serve any number of roles, including as a virulence determinant. In one scenario, the enzyme may promote intracellular infection of host cells. Such a hypothesis is supported by the fact the 45-kDa substrate was conserved within monocytes and epithelial cells, two types of cells that support *L. pneumophila* intracellular growth. In another scenario, Ugt may facilitate extracellular survival in the mammalian host and/or in natural aquatic environments. Two observations suggest that the *Legionella* UDP-glucosyltransferase may promote pathogenesis, participating in intracellular infection and/or extracellular survival. First, enzymatic activity and cross-reactive proteins appeared to be specific to *L. pneumophila*, the most pathogenic of the *Legionella* species. Second, UDP-glucosyltransferases can play critical roles in bacterial virulence. In the genus *Clostridium*, UDP-glucosyltransferases represent the enzymatic components of the so-called large clostridial toxins, which include toxins A and B of *C. difficile*, as well as the lethal and hemorrhagic toxins of *C. sordellii* (48). Another large clostridial toxin, the alpha-toxin of *Clostridium novyi*, uses UDP-N-acetylglucosamine instead of UDP-glucose as its cofactor (50). The 250- to 300-kDa clostridial toxins modify low-molecular-mass G proteins of the Rho and Ras subfamilies by attaching a sugar moiety to a threonine residue in the effector domain of the GTPase (1, 40, 50). The diseases caused by these clostridia have different clinical and epidemiological manifestations; *C. difficile* is an agent of antibiotic-associated diarrhea and pseudomembranous colitis in humans, *C. sordellii* produces gas gangrene, diarrhea, and enterotoxemia in humans and animals, and *C. novyi* causes gas gangrene in humans and animals (21, 31). However, a common feature of these diseases is that they all involve bacterial extracellular proliferation in the face of an inflammatory response (7). It has been speculated that inhibition of phagocytosis may result from the toxins' ability to target Ras-related proteins, which are important regulators of actin cytoskeleton rearrangements (1, 20, 32, 47). Indeed, the clostridial glucosyltransferases result in suppression of actin polymerization and rapid disorganization of the cytoskeleton network (1). Since the molecular masses of the Rho and Ras proteins are only 21 to 25 kDa (20), it is unlikely that the *Legionella* and *Clostridium* enzymes catalyze the same reaction. However, this does not preclude a role for Ugt in the cytoskeleton rearrangements or organelle trafficking alterations that are associated with *L. pneumophila* intracellular infection (55); e.g., there are multiple molecular mechanisms by which bacterial toxins influence the host cell cytoskeleton (40, 52). Thus, the *L. pneumophila* UDP-glucosyltransferase may promote pathogenesis by facilitating an intimate interaction with cells (e.g., macrophages) that is favorable for intracellular growth or by minimizing, as the clostridial toxins do, association with cells (e.g., polymorphonucleocytes) that are nonpermissive.

Clearly, future studies should include the generation of *L. pneumophila* mutants specifically defective for the glucosyltransferase and then an examination of their behavior in pro-

tozoan and mammalian models of intracellular infection, as well as in animal models of legionellosis. Any attenuation would indicate that the transferase enzyme has a role in virulence and definitely justify further identification of the eukaryotic substrates for the *L. pneumophila* transferase. Such investigations not only should shed light on the function of this enzyme in *Legionella* biology but also may increase our understanding of eukaryotic cell physiology. If the UDP-glucosyltransferase were implicated in infection, then we would also need to further investigate its locations in and/or processing by the bacterial cell. Although our data indicate that the enzyme is inside the *Legionella* cell, bacterial toxins can be cell associated and exhibit their pathogenic activity only after cell lysis (22, 26). Alternatively, it is conceivable that Ugt either is subject to type II secretion but only under certain growth conditions or is delivered into target cells by another specialized secretion apparatus, such as the *Legionella* type IV secretion system (19, 43, 49, 58). Another interesting question for possible future investigation is where and how Ugt is transported into host cells. Since the *Legionella* enzyme did not exhibit homology with the C-terminal, receptor-binding domain of the clostridial toxins, it may interact with host cell plasma membranes in a unique way. Alternately, it may be delivered into the host cell cytosol only after the bacteria have entered the replicative phagosome.

In summary, in this paper we describe the identification and cloning of a novel UDP-glucosyltransferase enzyme from *L. pneumophila*. Based on its apparently unique structure and the existence of a eukaryotic substrate, this enzyme may represent an important virulence factor of *L. pneumophila*. Additional study of this enzyme and its gene should yield new insights into the biochemistry of UDP-glucosyltransferases, the biology of a unique facultative intracellular parasite, and the pathogenesis of Legionnaires' disease.

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