INFECTION AND IMMUNITY, Aug. 1991, p. 2515–2521
0019-9567/91/082515-07$02.00/0

Vol. 59, No. 8

Cloning, Genetic Analysis, and Nucleotide Sequence of a Determinant Coding for a 19-Kilodalton Peptidoglycan-Associated Protein (Ppl) of *Legionella pneumophila*

BIRGIT LUDWIG,[1] ANGELA SCHMID,[2] REINHARD MARRE,[3] AND JÖRG HACKER[1]*

Lehrstuhl für Mikrobiologie[1] and Lehrstuhl für Biotechnologie,[2] Universität Würzburg, Röntgenring 11, W-8700 Würzburg, and Institut für Medizinische Mikrobiologie, Universität Lübeck, Ratzeburger Allee 160, W-2400 Lübeck,[3] Germany

Received 26 December 1990/Accepted 29 April 1991

A genomic library of *Legionella pneumophila*, the causative agent of Legionnaires disease in humans, was constructed in *Escherichia coli* K-12, and the recombinant clones were screened by immuno-colony blots with an antiserum raised against heat-killed *L. pneumophila*. Twenty-three clones coding for a *Legionella*-specific protein of 19 kDa were isolated. The 19-kDa protein, which represents an outer membrane protein, was found to be associated with the peptidoglycan layer both in *L. pneumophila* and in the recombinant *E. coli* clones. This was shown by electrophoresis and Western immunoblot analysis of bacterial cell membrane fractions with a monospecific polyclonal 19-kDa protein-specific antiserum. The protein was termed peptidoglycan-associated protein of *L. pneumophila* (Ppl). The corresponding genetic determinant, *ppl*, was subcloned on a 1.8-kb *ClaI* fragment. DNA sequence studies revealed that two open reading frames, *pplA* and *pplB*, coding for putative proteins of 18.9 and 16.8 kDa, respectively, were located on the *ClaI* fragment. Exonuclease III digestion studies confirmed that *pplA* is the gene coding for the peptidoglycan-associated 19-kDa protein of *L. pneumophila*. The amino acid sequence of PplA exhibits a high degree of homology to the sequences of the Pal lipoproteins of *E. coli* K-12 and *Haemophilus influenzae*.

Legionella pneumophila, the etiologic agent of Legionnaires disease, is an aerobic, gram-negative bacillus (36, 50). The bacterium multiplies intracellularly in human blood monocytes, human alveolar macrophages, and tissue culture cells. *L. pneumophila* is phagocytosed by an unusual mechanism, coiling phagocytosis, and is able to inhibit phagosome-lysosome fusion and phagosome acidification of the phagocytes (30, 31). Members of the genus *Legionella*, including *L. pneumophila*, normally inhabit natural aquatic environments, where they survive as intracellular parasites of freshwater protozoans (50).

The cell envelope of *L. pneumophila* has several interesting properties that may be related to the ability of these organisms to parasitize macrophages and free-living protozoa. The outer membrane contains a single major outer membrane protein (MOMP) that exists as a large aggregate stabilized by disulfide linkages (22). The MOMP, which exhibits channel-forming properties, is able to bind to the C3b receptor of phagocytic cells. This binding process may be a prerequisite for the uptake of the bacteria by the coiled phagocytosis mechanism (3, 40). Recently, another determinant encoding a protein of 24 kDa, which is reported to be associated with the outer membrane of *L. pneumophila*, was cloned and sequenced (18). This protein plays a role in the initiation of the uptake of *Legionella* organisms by macrophages, and it was therefore termed Mip (for macrophage infectivity potentiator [11]). As a strong antigen, another protein of 58 to 60 kDa was characterized. This protein, termed HtpB, has common features with the heat shock proteins of the bacterial GroEL family from different species, and it may therefore play an important role in the pathogenesis or survival processes of *Legionella* spp. (27, 42).

Here we describe the cloning and characterization of an *L. pneumophila*-specific determinant encoding an outer membrane protein of 19 kDa. This protein, termed PplA, is associated with the peptidoglycan layer. Analysis of the corresponding nucleotide sequence revealed a high degree of homology to the sequences of the genes coding for the peptidoglycan-associated Pal lipoproteins of *Escherichia coli* K-12 and *Haemophilus influenzae*.

MATERIALS AND METHODS

Media, enzymes, and chemicals. *Legionella* strains were cultivated on buffered charcoal-yeast extract (BCYE) agar plates supplemented with 0.025% ferric pyrophosphate and 0.04% L-cysteine (Oxoid, Wesel, Germany) (15) at 37°C in a 5% CO_2 atmosphere for 48 h prior to use. *E. coli* K-12 strains were grown in L broth. Radiochemicals were purchased from NEN Research Products, Dreieich, Germany. Restriction enzymes, exonuclease III, S1 nuclease, Klenow fragment, T4 polynucleotidekinase, and T4 ligase were obtained from Pharmacia, Freiburg, Germany; Boehringer, Mannheim, Germany; Biolabs, Schwalbach, Germany; and Gibco BRL, Karlsruhe, Germany. All other chemicals were obtained from Merck, Darmstadt, Germany; Difco, Detroit, Mich.; BRL, Neu-Isenburg, Germany; Oxoid, Wesel, Germany; Roth, Karlsruhe, Germany; and Serva, Heidelberg, Germany. Antibiotics were from Serva or Sigma (Deisenhofen, Germany).

Bacterial strains and plasmids. The genomic library was constructed from *L. pneumophila* strain Philadelphia I (36). For cloning experiments, the *E. coli* K-12 strains HB101 (6) and DH5α (24) were used. Plasmid pLAFR2 is a cosmid vector described previously (21, 33). For subcloning, the

* Corresponding author.

vectors pBR322 (5), pUC8 (49), and pBluescriptIIKS (Stratagene, La Jolla, Calif.) were used. Subclones containing recombinant plasmids with pUC8 and pBluescriptIIKS were assayed on plates containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and IPTG (isopropyl-β-D-thiogalactopyranoside). Subclones are indicated in Fig. 4 and 7. Antibiotics were used at concentration of 50 μg/ml (ampicillin) or 10 μg/ml (tetracycline).

Cosmid cloning procedure. *Legionella*-specific chromosomal DNA was isolated as described before (32, 39). Chromosomal *Sau*3A fragments of 20 to 22 kb were ligated into the *Bam*HI site of cosmid pLAFR2 and transduced into *E. coli* HB101 with the help of the cosmid packaging system (28, 51).

Recombinant DNA techniques. Plasmid DNA was isolated as described previously (4, 23). For restriction enzyme analysis, DNA was treated with appropriate enzymes, and the resulting fragments were separated by gel electrophoresis on 0.8 to 1.0% agarose gels (41). DNA fragments were isolated from the agarose gel with the help of the GeneClean kit from Bio 101, La Jolla, Calif., as described by the manufacturer. For cloning, DNA fragments were ligated into suitable vector molecules after heat inactivation of the restriction endonucleases at 65°C for 10 min (41). Digestion of DNAs with exonuclease III was done by the method of Henikoff (25). *E. coli* K-12 strains were transformed by the $CaCl_2$ method (35).

Preparation of antisera. The antigen used for production of the *L. pneumophila*-specific antiserum was prepared from heat-killed cells of *L. pneumophila* Philadelphia I grown on agar plates as described previously (19). Antiserum was prepared by intravenous injection of the antigen into New Zealand rabbits. The final immunofluorescence titer of the antiserum was 1:1,024. Antiserum specific for the cloned 19-kDa protein of *L. pneumophila* was prepared from cell sonicates of *E. coli* K-12 clone HB101(pBLL2511). The final immunofluorescence titer was 1:512. To remove the *E. coli* K-12-specific antibodies, the antiserum was absorbed with a suspension of HB101(pLAFR2).

Immuno-colony blot. Immuno-colony blots were done as described by van Die et al. (48).

PAGE. For polyacrylamide gel electrophoresis (PAGE), recombinant *E. coli* clones were grown in L-broth to the stationary phase. *L. pneumophila* cells were harvested from BCYE agar plates. Cells were washed in 0.9% NaCl, suspended in 60 mM Tris-HCl (pH 6.8)–2% sodium dodecyl sulfate (SDS)–10% glycerol–5% mercaptoethanol–0.002% bromphenol blue and boiled for 5 min. The samples were run on 13% polyacrylamide slab gels as described by Laemmli (34).

Western immunoblots. Western blots were performed by the method of Towbin et al. (46).

Cellular location of proteins. A 150-ml amount of overnight cultures of the recombinant *E. coli* K-12 clone HB101 (pBLL2511) and an equal amount of *L. pneumophila* cells harvested from agar plates were pelleted by centrifugation at 4°C for 10 min and washed with 50 mM Tris-HCl buffer (pH 7.2). Cell pellets were suspended in 5 ml of 50 mM Tris-HCl buffer (pH 7.7). The suspensions of the *E. coli* K-12 clones were passed through a French press (three times, 900 lb/in^2). *L. pneumophila* cells were broken by sonication, and residual cells were removed by centrifugation. The suspensions were centrifuged at 50,000 rpm in a Beckman Ti70.1 rotor for 1 h. The supernatants contain the cytoplasmic and periplasmic proteins, and the pellets contain the components of the cell envelopes. The pellets were washed in a solution containing 2% SDS, 10 mM Tris-HCl buffer (pH 8.0), and 10 mM EDTA (pH 8.0) and centrifuged at 48,000 rpm in a Beckman Ti70.1 rotor for 1 h. The supernatants, which contained the SDS-soluble proteins, were collected. The pellets were resuspended in 2 ml of a suspension containing 2% Genapol X-80, a nonionic detergent similar to Triton X-100 (Fluka, Neu-Ulm, Germany), 20 mM Tris-HCl (pH 8.0), and 10 mM EDTA (pH 8.0) and centrifuged at 48,000 rpm in a Beckman Ti70.1 rotor for 1 h. The resulting supernatants contain peptidoglycan-associated proteins (37, 43).

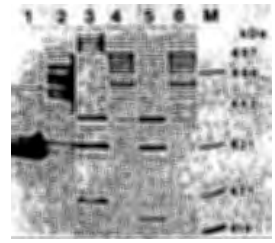

FIG. 1. SDS-PAGE of outer membrane (lanes 1, 3, and 5) and inner membrane (lanes 2, 4, and 6) preparations of *L. pneumophila* Philadelphia I (lanes 1 and 2), HB101(pBLL2511) (lanes 3 and 4), and HB101(pLAFR2) (control, lanes 5 and 6). Lane M, size markers.

Isolation and separation of inner and outer membranes. Membranes of the recombinant *E. coli* clones were prepared and separated into inner and outer membrane fractions by the method of Osborn et al. (38). *Legionella* membranes were isolated by the following procedure. Cells were harvested from agar plates in 10 mM cold HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4) and passed through a French press (three times at 900 lb/in^2). Whole cells were removed by centrifugation at 1,000 × *g* for 20 min. Total membranes were recovered by ultracentrifugation at 50,000 rpm in a Beckman Ti60 rotor for 1 h. The pellet containing the inner and outer membranes was suspended in 1 ml of 25% (wt/wt) sucrose containing 5 mM EDTA (pH 7.5) and layered on top of a sucrose step gradient as described previously (38). After centrifugation at 36,000 rpm in a Beckman SW40 rotor for 18 h, gradient fractions were collected and analyzed by SDS-PAGE. The distribution of outer membrane proteins showed a successful separation of the membranes (Fig. 1).

Gene probe and radioactive labeling. As a *ppl*-specific gene probe, the 1.8-kb *Cla*I fragment of pBLL30 (see Fig. 4) was used. After elution from agarose gels, the DNA fragment was labeled with [α-^{32}P]dATP by the method of Feinberg and Vogelstein (20), with a random priming kit purchased from Boehringer.

Southern hybridization. The transfer of DNA from agarose gels to nitrocellulose paper and the washing and autoradiography of the filters were performed as described earlier (44). The filters were hybridized in 50% formamide for 24 h at 42°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filters were washed three times with 0.1× SSC–0.1% SDS at 56°C (high-stringency conditions [37]). These conditions allow approximately 10% mismatches, estimated by the method of Davis et al. (12).

Oligonucleotide synthesis. Oligonucleotides used for DNA sequencing were synthesized with an Applied Biosystems 380A DNA synthesizer by the phosphoramidite method of Beaucage and Caruthers (2). Oligonucleotides were purified with oligonucleotide purification cartridges from Applied Biosystems, Foster City, Calif.

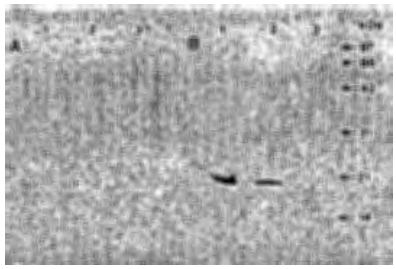

FIG. 2. Western blots of inner membrane (blot A) and outer membrane (blot B) preparations of *L. pneumophila* Philadelphia I (lanes 1), HB101(pBLL2511) (lanes 2), and HB101(pLAFR2) (control, lanes 3). The samples were probed with antiserum raised against clone HB101(pBLL2511), producing the 19-kDa protein.

DNA sequencing. The sequence of the *ppl*-specific DNA region was determined with the help of a sequencing kit from Boehringer as described by the manufacturer.

Computer analysis. The programs used for compiling the nucleotide sequence data were from J. Devereux (University of Wisconsin Genetics Computer Group) (14).

RESULTS

Cloning of the *Legionella*-specific 19-kDa outer membrane protein in *E. coli* K-12. A genomic library of *L. pneumophila* Philadelphia I was constructed (51). The 2,885 *E. coli* clones harboring recombinant plasmids with *Legionella*-specific DNA inserts of 20 to 22 kb were screened by immuno-colony blots with anti-*Legionella*-specific antiserum. Seventy-six clones reacted strongly, and cell lysates were analyzed by SDS-PAGE and Western blotting. Proteins of 19 kDa were produced by 23 recombinant clones. Antibodies raised against the recombinant *E. coli* K-12 clone HB101 (pBLL2511), coding for the 19-kDa *Legionella* antigen, recognized the corresponding proteins produced by the recombinant *E. coli* clones and by *L. pneumophila* Philadelphia I. Furthermore, the bacterial envelopes of *L. pneumophila* Philadelphia I and *E. coli* HB101(pBLL2511) were separated into inner and outer membranes by the method of Osborn et al. (38). As indicated by the SDS-PAGE gel and the Western blot shown in Fig. 1 and 2, and 19-kDa protein of *L. pneumophila* is associated with the bacterial outer membrane in both *L. pneumophila* and *E. coli*. The inner membrane preparations did not react with the 19-kDa protein-specific antibodies.

Peptidoglycan association of the 19-kDa protein. The fractions containing the cytoplasmic and the periplasmic proteins and the membrane protein fractions of *L. pneumophila* and *E. coli* clone HB101(pBLL2511) expressing the 19-kDa protein were isolated as well as the SDS-soluble and Genapol-soluble components of the membrane fractions. The latter contains peptidoglycan-associated proteins (37, 43). The different protein fractions were probed with the monospecific polyclonal antiserum raised against the 19-kDa protein-producing *E. coli* clone. As shown in Fig. 1 and 2, the 19-kDa protein of *L. pneumophila* is membrane associated. This is also demonstrated in Fig. 3B and F. A very weak signal which appeared after blotting of the cytoplasmic/periplasmic fraction of HB101(pBLL2511) may result from overproduction of this protein in the recombinant strain (Fig. 3E). A small amount of protein can be dissolved from the membrane fractions after washing with SDS (Fig. 3C and G).

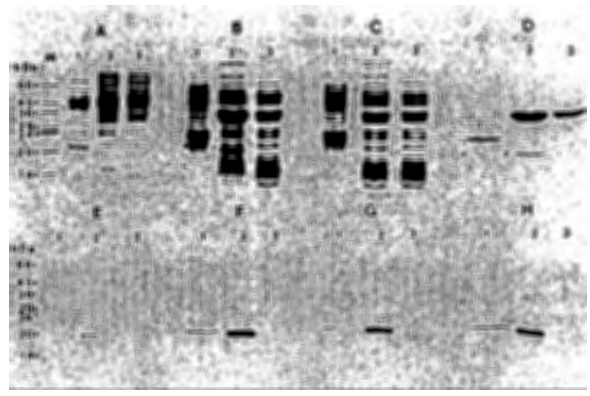

FIG. 3. SDS-PAGE (panels A to D) and Western blots (panels E to H) of cytoplasmic and periplasmic protein fractions (panels A and E), whole-cell membrane fractions (panels B and F), the SDS-soluble membrane protein fractions (panels C and G), and the Genapol-soluble peptidoglycan-associated protein fractions (panels D and H) of *L. pneumophila* Philadelphia I (lanes 1), HB101 (pBLL2511) (lanes 2), and HB101(pLAFR2) (control, lanes 3). The samples were probed with antiserum raised against clone HB101(pBLL2511), producing the 19-kDa protein. PplA is marked by arrowheads.

This was also shown for other proteins associated with the murein layer (3a, 37, 43). The majority of the 19-kDa protein, however, was isolated from the cell envelopes following resuspension of the membrane fractions with the detergent Genapol (Fig. 3D and H). These data point to an association of the 19-kDa protein with the peptidoglycan layer in *L. pneumophila* and also in the recombinant *E. coli* strain. We therefore termed the 19-kDa protein Ppl (peptidoglycan-associated protein of *L. pneumophila*).

Subcloning of the *ppl* determinant. Four *Hin*dIII fragments of the cosmid pBLL2511 were cloned into vector pUC8 (49). A *Hin*dIII fragment of 5.0 kb which represents the insert DNA of plasmid pBLL25-1 conferred production of the 19-kDa protein (Fig. 4). Since production of the 19-kDa protein could be detected after subcloning of the *Hin*dIII fragment opposite to the direction of transcription of the vector-specific promoter, the *ppl* determinant seems to be transcribed from its own promoter.

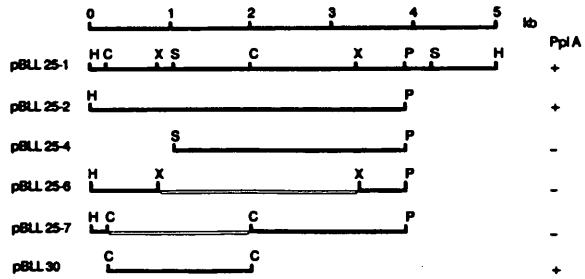

FIG. 4. Physical map of the *L. pneumophila*-specific 5.0-kb *Hin*dIII fragment of plasmid pBLL25-1, which was isolated from the DNA of cosmid pBLL2511, coding for the 19-kDa peptidoglycan-associated protein. Subclones and deletion mutants which were derived from pBLL25-1 are shown. The open lines represent deleted DNA regions. Expression of the 19-kDa protein, detected by Western blots, is indicated by +. Abbreviations: H, *Hin*dIII; C, *Cla*I; X, *Xho*I; S, *Sph*I; P, *Pst*I.

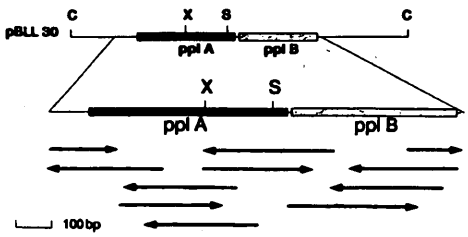

FIG. 5. Sequencing strategy for the region specific for *pplA* and *pplB* on the 1.8-kb *Cla*I fragment of pBLL30. The direction of sequenced DNA fragments is shown by arrows. Abbreviations: C, *Cla*I; X, *Xho*I; S, *Sph*I.

In order to localize the *ppl* determinant precisely, a set of deletion mutants and subclones were constructed. The smallest insert which was able to mediate Ppl production was a 1.8-kb *Cla*I fragment cloned into the vector pBluescriptIIKS. The corresponding plasmid was termed pBLL30. In a Southern hybridization experiment, chromosomal DNA isolated from strain *L. pneumophila* Philadelphia I was hybridized with the radioactively labeled 1.8-kb *Cla*I fragment of pBLL30. Strong signals appeared after hybridization, which clearly showed the identity of the cloned *ppl*-specific sequence and the *ppl* determinant on the *Legionella* genome (data not shown).

DNA sequence of the *ppl* determinant. It was shown that the *ppl* determinant is located on the *Cla*I fragment of 1.8 kb (Fig. 4), and therefore the nucleotide sequence of this DNA region was established (for sequence strategy, see Fig. 5). Two open reading frames (ORFs) were found, and the sequences of these are given in Fig. 6. One ORF starts at an ATG codon at positions 110 to 112 and ends at a TGA at positions 637 to 639. The exact start position of the second ORF is not established yet, but it may extend from position 637, 682, or 742 to position 1068. The two ORFs represent genes which have been designated *pplA* and *pplB*. The gene *pplA* codes for a protein of 176 amino acids (aa), with a calculated molecular mass of 18.9 kDa. The gene has an upstream consensus ribosome-binding site (45). The protein PplA contains a potential lipoprotein signal sequence-processing site at aa 21 and 22. Cleavage between these amino acids generates a predicted mature protein of 155 aa and a molecular mass of 16.8 kDa (52). The N-terminal region of PplA agrees with reported membrane protein signal sequences and contains a hydrophilic region followed by a strong hydrophobic region. While no homology was found between the putative protein PplB of 16.8 kDa and any other gene products sequenced, PplA shows homology to the peptidoglycan-associated lipoproteins (Pal) of *E. coli* K-12 (59.6% similarity, 36.3% identity [10]) and *H. influenzae* (60.8% similarity, 41.2% identity [13]).

Characterization of clones carrying mutations in *pplA* and *pplB*. In order to confirm the identity of PplA with the 19-kDa protein of *L. pneumophila*, plasmid pBLL30, which consists of the 1.8-kb *Cla*I fragment of *L. pneumophila* and the vector pBluescriptIIKS, was used for exonuclease III digestion studies. It is obvious from Fig. 7 that the clones HB101(pBLL30-2), HB101(pBLL30-9), and HB101 (pBLL30-3), which carry deletions in the *pplA*-specific DNA region still carrying *pplB*, did not produce the 19-kDa protein. In contrast, the clone pBLL30-6, which has lost the *pplB*-specific sequences but carries the gene *pplA*, gave a strong reaction in a Western blot with the 19-kDa protein-specific antiserum. It is therefore concluded that the gene *pplA* codes for the *L. pneumophila*-specific peptidoglycan-associated protein of 19 kDa.

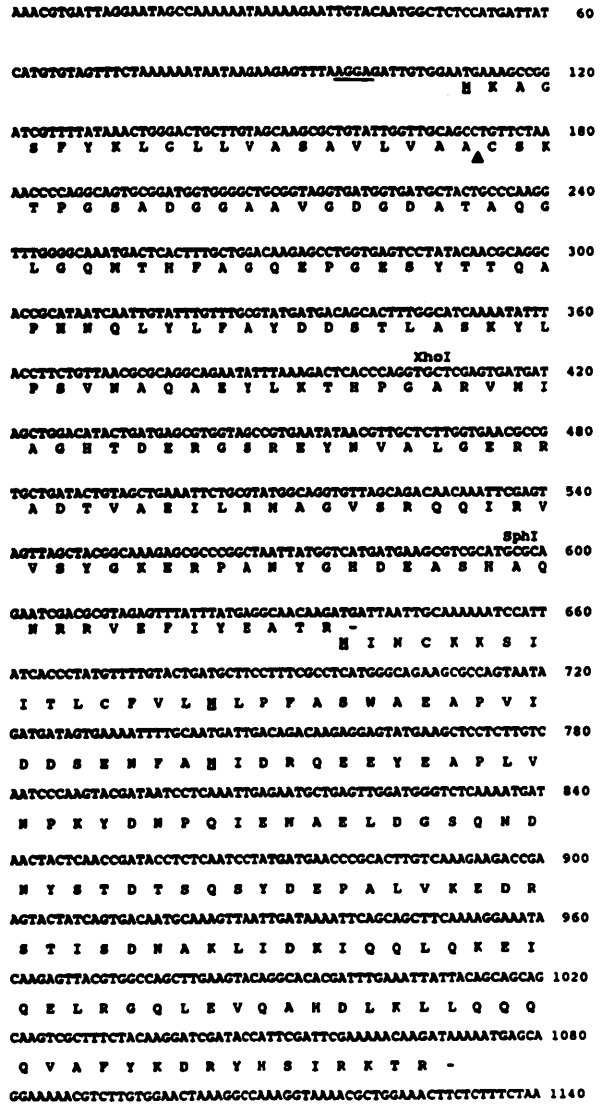

FIG. 6. Nucleotide and deduced amino acid sequences of the genes *pplA* and *pplB* of *L. pneumophila* and the corresponding proteins. The putative processing site of PplA is indicated by an arrowhead. Important restriction sites are shown. The putative ribosome-binding site is underlined. Possible initiator methionines are underlined.

DISCUSSION

We report on the expression and characterization of an *L. pneumophila*-specific peptidoglycan-associated protein of 19 kDa, termed PplA. The corresponding determinant was cloned in *E. coli*, and its nucleotide sequence was established. Recently, two other *Legionella*-specific antigens were expressed in *E. coli* and sequenced (18, 27, 42). One of these, the Mip protein, plays a role in the uptake of *Legion-*

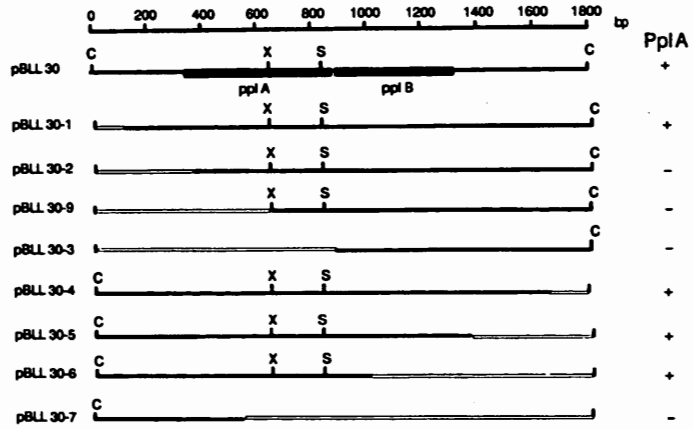

FIG. 7. Deletion mutants generated by exonuclease III digestion of plasmid pBLL30. The open lines represent deleted DNA regions. Production of the 19-kDa protein, detected by Western blots, is indicated by +. Abbreviations: C, *Cla*I; X, *Xho*I; S, *Sph*I.

ella organisms by phagocytic cells (11). The other antigen, which represents a heat shock-like protein of 58 kDa termed HtpB, may also be involved in the pathogenicity of *L. pneumophila*.

Together with the 19-kDa protein described here, only these three proteins could be detected following screening of *Legionella*-specific genomic libraries of *E. coli* with *Legionella*-specific antiserum (17, 26). Thus, we and others were unable to clone the gene coding for the major outer membrane protein of *L. pneumophila*, termed MOMP, which is responsible for the interaction of the bacteria with the C3b receptors on phagocytic cells (3, 16, 22). Differences in the promoter regions of *E. coli* and *L. pneumophila* as a consequence of the different A+T contents of the two species may be one reason for the limited number of *L. pneumophila* antigens cloned and expressed in *E. coli*.

By using sucrose density gradient centrifugation, it was demonstrated that the 19-kDa protein of *L. pneumophila* is part of the bacterial outer membrane. Large amounts of the PplA protein were detected on SDS-PAGE and in Western blots (Fig. 3) with Genapol suspensions of cell envelope fractions. The detergent Genapol is used for the isolation of porins from peptidoglycan layers of different organisms (3a, 43). It is concluded from these data that the 19-kDa protein is noncovalently but tightly bound to the peptidoglycan layer of *L. pneumophila* and the recombinant *E. coli* clones. A trace of PplA protein was also detected in Western blots of SDS-soluble protein fractions of the cell envelopes. These data, however, do not contradict our suggestion that PplA is associated with the peptidoglycan layer, because only a very small amount of the 19-kDa protein was isolated after washing of the bacterial membrane with SDS which was not detectable on SDS-PAGE (Fig. 3C). It was also shown for other peptidoglycan-associated proteins that a minority of these can be dissolved from the cell envelope by treatment with SDS (3a, 37, 43).

The 19-kDa protein represents a less abundant protein in SDS-PAGE than another peptidoglycan-associated protein of about 31 kDa described recently (9; see also reference 1), but it is present and highly immunogenic. The 19-kDa protein of *L. pneumophila* Philadelphia I described here may be the analog antigen to outer membrane proteins of 19 to 20 kDa which were previously selected from recombinant clones of cosmid genomic libraries of *L. pneumophila* strains Knoxville and Wardworth (17, 26) and which are also poorly expressed in *E. coli* and in *Legionella* spp.

The sequence studies with the *ppl*-specific determinant reveal that two ORFs, *pplA* and *pplB*, exist on the DNA fragment coding for the peptidoglycan-associated protein of 19 kDa. The isolation of plasmids with exonuclease III-generated deletions in the *ppl* region, however, clearly showed that the gene *pplA* codes for the 19-kDa protein. We presently have no further information as to whether the second gene, *pplB*, plays any role in expression or processing of the peptidoglycan-associated protein PplA.

The protein sequence of PplA shows a remarkable degree of homology to the amino acid sequences of the Pal lipoproteins of *E. coli* K-12 and *H. influenzae* (10, 13) (Fig. 8). We have no functional evidence that we have cloned a lipoprotein, but the amino acid sequence of PplA exhibits common features with the protein sequences of the lipoproteins. Thus, the N-terminal part of PplA includes the amino acid motif Ala-Ala-Cys-Ser, with a hypothetic cleavage site between the alanine and cysteine residues, which is in total agreement with the cleavage sites specific for the signal peptidase II system responsible for the processing of lipoproteins (7, 52). Furthermore, a β-turn structure follows the cleavage site of PplA, which is also specific for lipoproteins (10, 29).

As shown in Fig. 8, the C-terminal part of PplA shows a high degree of homology to the carboxy-terminal regions of the lipoproteins of *E. coli* and *H. influenzae*. By using gene fusions between the gene *pal* of *H. influenzae* and the *phoA* determinant, coding for the enzyme alkaline phosphatase, Deich et al. (13) were able to show that the C-terminal part of the Pal protein interacts with the bacterial outer membrane. The homology between the C termini of Pal and PplA may argue for a strong selective pressure on these particular regions which may play an essential role in the membrane architecture of *L. pneumophila* and *H. influenzae*. It is interesting that the gene *pplA* shows an A+T content of 49%, which is considerably lower than the average A+T content of the *L. pneumophila* genome, suggesting that the gene *pplA* may originate from an organism other than *Legionella* spp.

Outer membrane proteins play a major role in the pathogenicity of various organisms (8, 47). *L. pneumophila* represents an intracellular pathogen which is able to cause severe

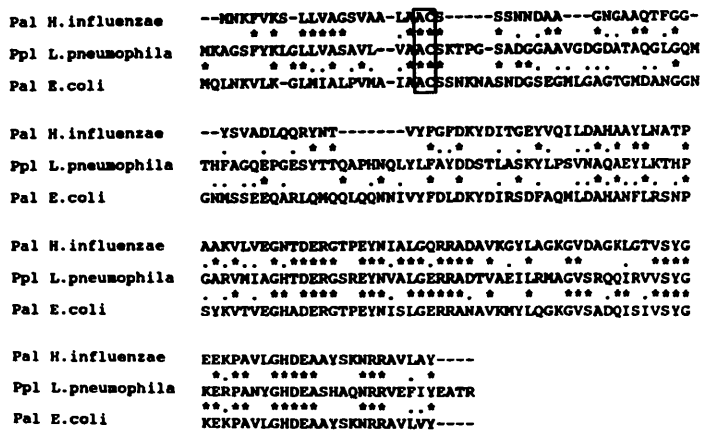

FIG. 8. Comparison of the primary amino acid sequences of the *L. pneumophila*-specific protein PplA with those of the peptidoglycan-associated outer membrane Pal lipoproteins of *H. influenzae* and *E. coli* K-12. Identical amino acid residues are indicated by asterisks, and amino acid residues that are functionally similar are indicated by dots. Dashes represent gaps introduced for optimal alignment. The putative signal-processing sites are indicated by a box.

cases of pneumonia (50). One important feature in the pathogenesis of *L. pneumophila* is its ability to inhibit phagolysosomal fusion and acidification of the vacuole of phagocytic cells (30, 31). Cell surface components may be associated with these processes, which are necessary for the intracellular survival of *L. pneumophila*. Furthermore, membrane proteins may protect bacteria from destruction by exogenous enzymes. The cloning and characterization of the *ppl* determinant may enable us to construct specific mutants of *L. pneumophila* which do not produce the PplA antigen and therefore to determine whether this protein contributes to its pathogenicity.

ACKNOWLEDGMENTS

We thank W. Ehret (Munich), R. Benz (Würzburg), and L. Philipps (Würzburg) for helpful discussion and critical reading of the manuscript. We are also indebted to U. Rdest (Würzburg) for suggestions and to H. Kurz for editorial assistance.

The work was supported by the BMFT project "Infektionskrankheiten" (01 Ki 8829, 8812), by the Sonderforschungsbereich (SFB) 176, and by the Fonds der Chemischen Industrie.

REFERENCES

1. **Amano, K., and J. C. Williams.** 1983. Partial characterization of peptidoglycan-associated proteins of *Legionella pneumophila*. J. Biochem. **94**:601–606.
2. **Beaucage, S. L., and M. H. Caruthers.** 1981. Deoxynucleoside phosphoramidites: a new class of key intermediate for deoxypolynucleotide synthesis. Tetrahedron Lett. **22**:1859–1862.
3. **Bellinger-Kawahara, C., and M. A. Horwitz.** 1990. Complement component C3 fixes selectively to the major outer membrane protein (MOMP) of *Legionella pneumophila* and mediates phagocytosis of liposome-MOMP complexes by human monocytes. J. Exp. Med. **172**:1201–1210.

3a. **Benz, R.** Personal communication.

4. **Birnbiom, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1522.
5. **Bolivar, F., R. L. Rodrigues, P. Y. Greene, M. C. Betlach, H. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of a new cloning vehicle. II. A multipurpose cloning system. Gene **2**:95–113.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
7. **Braun, V.** 1975. Covalent lipoprotein from the outer membrane of *Escherichia coli*. Biochim. Biophys. Acta **415**:335–377.
8. **Buchanan, T. M., and W. A. Pearce.** 1979. Pathogenic aspects of outer membrane components of gram-negative bacteria, p. 475–514. *In* M. Inouye (ed.), Bacterial outer membranes. John Wiley & Sons, Inc., New York.
9. **Butler, C. A., and P. S. Hoffman.** 1990. Characterization of a major 31-kilodalton peptidoglycan-bound protein of *Legionella pneumophila*. J. Bacteriol. **172**:2401–2407.
10. **Chen, R., and U. Henning.** 1987. Nucleotide sequence of the gene for the peptidoglycan-associated lipoprotein of *Escherichia coli* K-12. Eur. J. Biochem. **163**:73–77.
11. **Cianciotto, N. P., B. J. Eisenstein, C. H. Mody, G. B. Toews, and N. C. Engleberg.** 1989. A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. Infect. Immun. **57**:1255–1262.
12. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering: advanced bacterial genetics, p. 220–221. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green.** 1988. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from *Haemophilus influenzae*. J. Bacteriol. **170**:489–498.
14. **Devereux, J.** 1984. UWGCG—computer programs. University of Wisconsin, Madison.
15. **Edelstein, P. H.** 1982. Comparative study of selective media for isolation of *Legionella pneumophila* from potable water. J. Clin. Microbiol. **16**:697–699.
16. **Ehret, W., G. Anding, and G. Ruckdeschel.** 1984. Characterization of membrane proteins from various strains and serogroups of *Legionella pneumophila* and other *Legionella* species, p. 265–268. *In* C. Thornsberry, A. Balows, J. C. Feeley, and W. Jakubowski (ed.), *Legionella*: Proceedings of the 2nd International Symposium. American Society for Microbiology, Washington, D.C.
17. **Engleberg, N. C., E. Pearlman, and B. J. Eisenstein.** 1984. *Legionella pneumophila* surface antigens cloned and expressed in *Escherichia coli* are translocated to the host cell surface and interact with specific anti-*Legionella* antibodies. J. Bacteriol. **160**:199–203.
18. **Engleberg, N. C., C. Carter, D. R. Weber, N. P. Cianciotto, and B. J. Eisenstein.** 1989. DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage infectivity. Infect. Immun. **57**:1263–1270.

19. **Farshy, C. E., and J. C. Feeley.** 1979. ELISA for Legionnaires' disease—antibody and antigen detection systems: an interim report, p. 124–129. *In* G. L. Jones and G. A. Hébert (ed.), Legionnaires': the disease, the bacterium and methodology. U.S. Department of Health, Education and Welfare, Atlanta, Ga.
20. **Feinberg, H. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specificity. Anal. Biochem. **132**:6–13.
21. **Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of mutants. Gene **18**:289–296.
22. **Gabay, J. E., M. Blake, W. D. Niles, and M. A. Horwitz.** 1985. Purification of *Legionella pneumophila* major outer membrane protein and demonstration that it is a porin. J. Bacteriol. **162**:85–91.
23. **Grinstedt, J., P. M. Bennett, S. Higginson, and M. H. Richmond.** 1978. Regional preference of insertion of Tn*501* and Tn*802* into RP1 and its derivatives. Mol. Gen. Genet. **166**:313–320.
24. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
25. **Henikoff, H.** 1987. Rapid methods of DNA sequence analysis. Methods Enzymol. **155**:156–165.
26. **Hindahl, M. S., and B. H. Iglewski.** 1987. Cloning and expression of a common *Legionella* outer membrane antigen in *Escherichia coli*. Microb. Pathog. **2**:91–99.
27. **Hoffman, P. S., L. Houston, and C. A. Butler.** 1990. *Legionella pneumophila htpAB* heat shock operon: nucleotide sequence and expression of the 60-kilodalton antigen in *L. pneumophila*-infected HeLa cells. Infect. Immun. **58**:3380–3387.
28. **Hohn, B., and J. Collins.** 1980. Small cosmid for efficient cloning of large DNA fragments. Gene **11**:291–298.
29. **Hopp, T. P., and K. P. Woods.** 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA **78**:3824–3828.
30. **Horwitz, M. A.** 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. J. Exp. Med. **158**:2108–2126.
31. **Horwitz, M. A.** 1988. Phagocytosis and intracellular biology of *Legionella pneumophila*, p. 231–237. *In* F. C. Cabello and C. Pruzzo (ed.), Bacteria, complement and the phagocytic cell. NATO ASI Series H, vol. 24. North Atlantic Treaty Organization, Berlin.
32. **Knapp, S., J. Hacker, I. Then, D. Müller, and W. Goebel.** 1984. Multiple copies of hemolysin genes and associated sequences in the chromosome of uropathogenic *Escherichia coli* strains. J. Bacteriol. **159**:1027–1033.
33. **Knapp, S., and J. Mekalanos.** 1988. Two *trans*-acting regulatory genes (*vir* and *mod*) control antigenic modulation in *Bordetella pertussis*. J. Bacteriol. **170**:5059–5066.
34. **Laemmli, U. K.** 1979. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
35. **Lederberg, E. M., and S. N. Cohen.** 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. **119**:1072–1074.
36. **McDade, J. E., C. C. Shepard, D. W. Fraser, T. R. Tsai, M. A. Redus, W. R. Dowdle, and the Laboratory Investigation Team.** 1977. Legionnaires' disease. Isolation of a bacterium and demonstration of its role in other respiratory disease. N. Engl. J. Med. **297**:1197–1203.
37. **Nikaido, H.** 1983. Proteins forming large channels from bacterial and mitochondrial outer membranes: porins and phage lambda receptor protein. Methods Enzymol. **97**:85–100.
38. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
39. **Ott, M., H. Hoschützky, K. Jann, I. van Die, and J. Hacker.** 1988. Gene clusters for the S fimbrial adhesin (*sfa*) and F1C fimbriae (*foc*) of *Escherichia coli*: comparative aspects of structure and function. J. Bacteriol. **170**:3983–3990.
40. **Payne, N. R., and M. A. Horwitz.** 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. J. Exp. Med. **166**:1377–1389.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Sampson, J. S., S. P. O'Connor, B. P. Holloway, B. B. Plikaytis, G. M. Carlone, and L. W. Mayer.** 1990. Nucleotide sequence of *htpB*, the *Legionella pneumophila* gene encoding the 58-kilodalton (kDa) common antigen, formerly designated the 60-kDa common antigen. Infect. Immun. **58**:3154–3157.
43. **Schülein, K., and R. Benz.** 1990. *lamB* (maltoporin) of *Salmonella typhimurium*: isolation, purification and comparison of sugar binding with *lamB* of *Escherichia coli*. Mol. Microbiol. **4**:625–632.
44. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
45. **Stormo, G. D., T. D. Schneider, and L. M. Gold.** 1982. Characterization of translational initiation sites in *Escherichia coli*. Nucleic Acids Res. **10**:2971–3011.
46. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
47. **Tufano, M. A., R. Janniello, M. Galdiero, L. De Mártino, and F. Galdiero.** 1989. Effect of *Salmonella typhimurium* porins on biological activities of human polymorphonuclear leukocytes. Microb. Pathog. **7**:337–346.
48. **van Die, I., B. van Geffen, W. Hoekstra, and H. Bergmans.** 1985. Type 1 C fimbriae of an uropathogenic *Escherichia coli* strain: cloning and characterization of the genes involved in the expression of the 1C antigen and nucleotide sequence of the subunit gene. Gene **34**:187–196.
49. **Vieira, J., and J. Messing.** 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**:259–268.
50. **Winn, W. C.** 1988. Legionnaires disease: historical perspective. Clin. Microbiol. Rev. **1**:60–81.
51. **Wintermeyer, E., U. Rdest, B. Ludwig, A. Debes, and J. Hacker.** Characterization of legiolysin (*lly*), responsible for haemolytic activity, colour production and fluorescence of *Legionella pneumophila*. Mol. Microbiol., in press.
52. **Wu, H. C., and M. Tokunaga.** 1986. Biogenesis of lipoproteins in bacteria. Curr. Top. Microbiol. Immunol. **125**:127–157.