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Characterization of a *Legionella pneumophila* gene encoding a lipoprotein antigen

N. C. Engleberg,[1,2]* D. C. Howe,[2] J. E. Rogers,[2] J. Arroyo[2] and B. I. Eisenstein[1,2]
[1]Department of Internal Medicine, and [2]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0620, USA.

Summary

A prominent 19 kDa surface antigen of *Legionella pneumophila*, cloned in *Escherichia coli*, was found to be intimately associated with peptidoglycan. The DNA region encoding this antigen was mapped on an 11.9 kb plasmid by means of deletion analysis and transposon mutagenesis. PhoA$^+$ gene fusions, generated by Tn*phoA* insertions into this region, confirmed the presence of a gene encoding a secreted protein. PhoA$^+$ transposon insertions were also associated with loss of the 19 kDa antigen in immunoassays using a monoclonal antibody (mAb1E9) and the replacement of the 19 kDa antigen with larger fusion proteins in immunoblots using *Legionella* immune serum. A 1540 bp *Pst*I fragment carrying the gene was sequenced, and the open reading frame encoding the antigen was identified. The gene encodes a polypeptide 176 amino acid residues long and 18913 Da in size. The presence of a signal sequence of 22 amino acids with a consensus sequence for cleavage by signal peptidase II indicates that the antigen is a lipoprotein, and striking similarity with peptidoglycan-associated lipoproteins (PALs) from *E. coli* (51% amino acid homology) and *Haemophilus influenzae* (55% homology) is noted. We conclude that the 19 kDa antigen of *L. pneumophila* is the structural equivalent of the PAL found in other Gram-negative species and suggest that its post-translational acylation may explain its potency as an immunogen.

Introduction

We previously described the cloning of a 19000 Da (19 kDa) antigen of *Legionella pneumophila* and subsequently showed that this protein is accessible to serum antibodies on the surface of both *L. pneumophila* and *Escherichia coli* clones carrying the gene for this antigen on a multicopy plasmid, pSMJ11 (Engleberg *et al.*, 1984a,b). All *L. pneumophila* strains that we have tested express this antigen, and strains of several other *Legionella* species express a smaller, cross-reactive protein of ~18 kDa (Engleberg *et al.*, 1986). In polyacrylamide gels, the 19 kDa *L. pneumophila* antigen nearly comigrates with the *E. coli* peptidoglycan-associated lipoprotein (PAL). Both the *L. pneumophila* protein and the comigrating *E. coli* PAL produce a 'tailing' phenomenon in polyacrylamide gels that is typically associated with lipoproteins. Peptidoglycan-associated lipoproteins similar to the *E. coli* PAL have been identified in several Gram-negative bacterial species (Mizuno, 1981; Deich *et al.*, 1990), and we hypothesized that the 18–19 kDa *Legionella* antigens might be homologues of these lipoproteins.

Although the functional role of the small, peptidoglycan-associated lipoproteins remains unproven, it is assumed that these molecules have a structural role, providing anchorage between the cell wall and the outer membrane. These molecules are of interest in pathogenic bacteria, because they may be potent B-cell mitogens and immunoadjuvants (Melchers *et al.*, 1975; Lex *et al.*, 1986). Moreover, because they are primarily localized to the outer membrane, they may be accessible to specific antibodies on the bacterial surface. The *Haemophilus influenzae* PAL has been shown to be a target for bactericidal antibodies and may be regarded as a potential vaccine candidate (Green *et al.*, 1987).

In this communication we present the DNA sequence of the 19 kDa *L. pneumophila* antigen gene. Its inferred amino acid sequence data confirm the presence of a signal for acylation and a high degree of amino acid homology with other known PAL sequences. We conclude that this protein is a homologue of the previously sequenced *E. coli* and *H. influenzae pal* genes (Chen and Henning, 1987; Deich *et al.*, 1990).

Results

Peptidoglycan association of the 19 kDa antigen

In a previous study, we showed that the *L. pneumophila* major outer membrane protein (MOMP) is peptidoglycan-bound by first heating bacteria in 2% sodium dodecyl

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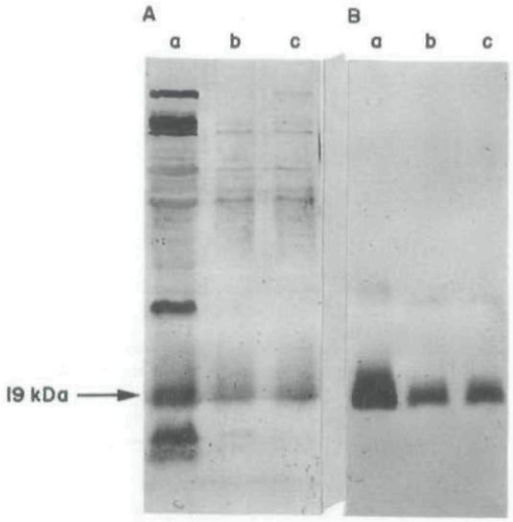

Fig. 1. Immunoblots of solubilized proteins from *L. pneumophila* AA100. Identical immunoblots were probed with either *L. micdadei* immune serum (panel A) or mAb1E9 (panel B). *L. micdadei* antiserum was used in this experiment to minimize background and to limit the number of visualized antigens. Immunoblots of bacterial lysates with homologous antisera produce a background reactivity with lipopolysaccharide (LPS). *L. micdadei* antiserum has no detectable LPS cross-reactivity, but it has strong cross-reactivity with the 19kDa antigen and several other *L. pneumophila* proteins. Lanes: a, supernatant from bacteria boiled in 2% SDS for 2 min; b, insoluble pellet from lane a samples washed and reboiled in 2% SDS for 5 min; c, insoluble pellet washed and reboiled in 4% SDS for 5 min.

sulphate (SDS) for 2 min, recovering the insoluble pellet by centrifugation, and subsequently releasing the MOMP from the pellet by reheating to 100°C for 10 min in 2% SDS with 5% 2-mercaptoethanol (Engelberg *et al.*, 1989). In that experiment, immunoblots of the peptidoglycan-associated proteins with *Legionella* immune serum showed that the predominant antigen released from the insoluble pellet was 19kDa in size. To confirm that this peptidoglycan-associated antigen is identical to the one encoded on pSMJ11, we repeated this experiment and immunoblotted the peptidoglycan-associated fraction with immune sera and with a monoclonal antibody (mAb1E9) that reacts with the cloned antigen. The immunoblot shown in Fig. 1 confirms that much of the 19kDa antigen is associated with peptidoglycan, since a significant proportion is retained with the insoluble pellet after most other bacterial proteins are solubilized. The reactivity of the antigen with mAb1E9 in both soluble and insoluble fractions confirms the identity of this protein.

Subcloning of the 19kDa antigen gene

The 19kDa antigen was originally cloned on plasmid pSMJ11 (Engleberg *et al.*, 1984a). Various restriction fragments were deleted from the insert in pSMJ11, and the resulting clones were tested for expression of the 19kDa antigen (Fig. 2A). The ~3.75kb insert of pSMJ11.4 was the smallest of the fragments that still expressed the antigen. Either of two deletions within the pSMJ11.4 insert (made at unique *Sph*I and *Ava*I sites using S1 nuclease digestion and blunt-end ligation) abrogated expression of the 19kDa antigen, suggesting that these mutations may disrupt a reading frame that is critical for antigen expression.

To be able, eventually, to mobilize the cloned gene from *E. coli* into *L. pneumophila*, the insert expressing the 19kDa antigen was cloned into a vector with an antibiotic resistance marker that is selectable in *L. pneumophila*, i.e. pTLP5 (chloramphenicol-resistant, Cm^R; streptomycin-sensitive, Sm^S). Accordingly, the *Eco*RI–*Pvu*II fragment of pSMJ11.4 was first ligated to pUC18 digested with *Eco*RI and *Sma*I. This manipulation allowed for the positioning of the *Bam*HI site of the pUC18 multiple cloning site next to the *Pvu*II–*Sma*I junction at which the insert and vector are joined. The large *Bam*HI–*Bcl*I fragment of this intermediate plasmid was then isolated and cloned into the unique *Bam*HI site of pTLP5 to yield pDH11.2. Immunoblotting experiments confirmed that the 19kDa antigen is expressed from pDH11.2 to the same degree as from pSMJ11.4 (data not shown).

*Mutagenesis of a DNA fragment encoding the 19kDa antigen with Tn*phoA

In initial experiments with transpositional mutagenesis, Tn*phoA*-1 was inserted into the fragment of interest on pSMJ11.4. In later experiments, Tn*phoA–oriT* was inserted into the slightly shorter fragment on pDH11.2. This second set of transposon insertions was generated in anticipation of future experiments (now in progress) in which the mutated genes on these plasmids will be transferred to *L. pneumophila* and exchanged for the native genes. In all transposition experiments, *E. coli* CC118 carrying the relevant plasmid was infected with either λTn*phoA*-1 or λ::Tn*phoA–oriT* and plated on LB/XP media containing kanamycin (200 or 300 μg ml^{-1}). Plasmid minipreps were prepared from individual transductants that appeared blue (i.e. $PhoA^+$) and were used to retransform strain CC118. If the transconjugants were all $PhoA^+$ on media with XP, then the plasmid was assumed to have acquired a phosphatase function and was analysed using restriction endonucleases. Each transposition yielding a PhoA fusion was designated by the letter B (for 'blue') followed by an individual number (given in Fig. 2B). Several kanamycin-resistant transductants that were $PhoA^-$ were likewise analysed to identify random Tn*phoA–oriT* insertions in pDH11.2. These transpositions were designated by the letter W (for 'white') followed by an individual number.

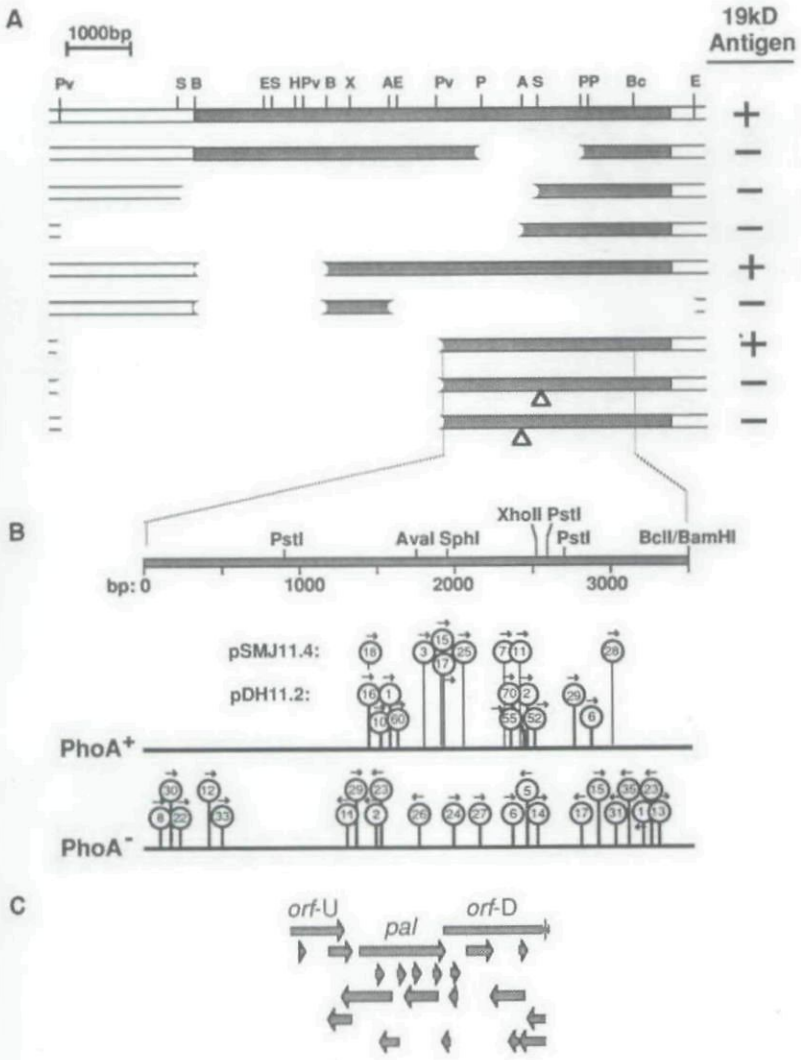

Fig. 2. Analysis of pSMJ11 subclones and their expression of the 19kDa antigen.
A. The shaded areas of the linearized map (above) represent the insert of *L. pneumophila* DNA; the unshaded portions represent relevant sections of the vector, pBR322. The triangles represent localized deletions made by endonuclease digestion at the specified site, S1 nuclease treatment, repair with Klenow fragment, and blunt-end ligation. The column at the right indicates whether a 19kDa antigen was seen on Western blots of the strains carrying the respective deletion.
B. A restriction map of the region cloned in pSMJ11.4 and pDH11.2 is shown with the position of Tn*phoA–oriT* insertions below. The direction of the *phoA* reading frame is indicated by an arrow over each transposition marker. For reference purposes, the numerical designation for each transposition is given (see text concerning nomenclature). Note that the region from position 600 to 1200 is entirely free of transpositions.
C. Potential open reading frames deduced from the sequence of the large *Pst*I fragment are indicated by the open arrows below the map.

Within the ~3500bp insert of pSMJ11.4 and pDH11.2, PhoA$^+$ gene fusions were limited to a ~1600bp region, and all transpositions giving productive fusions were oriented in the same direction (Fig. 2B). Several of these transpositions were clustered near the *Ava*I and *Sph*I sites, previously shown to be critical for the expression of the 19kDa antigen. Clones carrying transpositions B-16, B-17, and all transpositions between, failed to react with mAb1E9 by dot immunoblot analysis. In addition, Western blot analysis of some of these clones using *Legionella* immune serum showed that the 19kDa antigen was absent and that new antigenic bands of >47kb, presumably fusion proteins, were present instead (Fig. 3, panel A). Fusions downstream of B-17 did not affect expression of the 19kDa antigen. We inferred from these findings that the region defined by gene fusions B-16 to B-17 represents the gene encoding the 19kDa antigen.

An additional cluster of PhoA$^+$ fusions was mapped to a ~1000bp region immediately downstream from the 19kDa antigen gene (Fig. 2B). The clones carrying the fusions from B-25 to B-28 were reactive with mAb1E9 and all of those tested expressed the 19kDa antigen on Western blots with *Legionella* immune serum. Western blot analysis of several of these clones with alkaline phosphatase antiserum showed fusion products of increasing size (from ~69 to ~88kDa) corresponding to the point of the transposon insertion, i.e. as the transposon insertions mapped further downstream, the fusion products were larger (Fig. 3, panel B). This analysis suggests that the cloned region immediately downstream from the 19kDa antigen gene contains another gene encoding a single secreted protein.

Finally, 22 Tn*phoA–oriT* transpositions that did not produce gene fusions were mapped within the pDH11.2

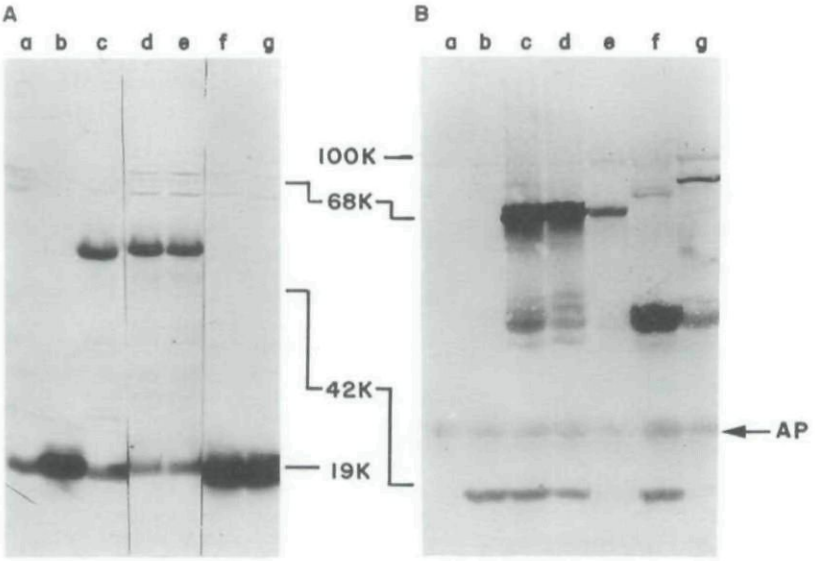

Fig. 3. Immunoblots of strains carrying Tn*phoA–oriT* gene fusions in the DNA fragment encoding the 19 kDa antigen.
A. Immunoblot with *L. pneumophila* immune serum. Lane a, *E. coli* control; lane b, *E. coli* (pSMJ11.4); lanes c–g, Tn*phoA* transpositions in pSMJ11.4, B-3, B-15, B-17, B-7, and B-11. Note the weak reactivity of the rabbit serum with a comigrating 19 kDa antigen in *E. coli*.
B. Immunoblot with alkaline phosphatase antiserum. Lane a, *E. coli* control; lane b, *E. coli* (pDH11.2::B-55); lanes c–g, Tn*phoA–oriT* transpositions in pDH11.2, B-70, B-2, B-52, B-29, and B-6. The mobilities of prestained molecular weight markers are indicated. AP, native *E. coli* alkaline phosphatase monomer.

insert fragment (Fig. 2B). These transpositions were randomly distributed within the fragment, except for a region of ~750 bp immediately upstream of the 19 kDa antigen gene, into which no transpositions were isolated.

DNA sequence of the 19 kDa protein antigen

The two *Sph*I–*Pst*I fragments representing the upstream and downstream portions of the 19 kDa antigen gene, as well as a *Sau*3A fragment that spans the internal *Sph*I site, were isolated and cloned into the replicative form of both M13mp18 and M13mp19. Single-stranded templates of the resulting phages were purified and sequenced as described below. The first 140 bp of sequence are derived from a single strand and therefore should be considered tentative. All remaining portions of the sequence were derived from primer extension on both strands using overlapping templates.

The DNA sequence obtained from these M13 subclones is shown in Fig. 4. Three long open reading frames were identified, all of which would be transcribed in the same direction as the Tn*phoA* gene fusions. One of the open reading frames (528 bp) spans the region to which the 19 kDa antigen gene was mapped (Fig. 2C). An upstream open reading frame (*orfU*) of at least 309 bp begins upstream of the boundary of the sequenced fragment and terminates 101 bp before the beginning of the 19 kDa gene. A downstream open reading frame (*orfD*) of at least 613 bp begins 1 bp before the termination codon of the 19 kDa antigen gene (i.e. the sequence is . . . ATGA . . .) and extends beyond the boundary of the subcloned fragment.

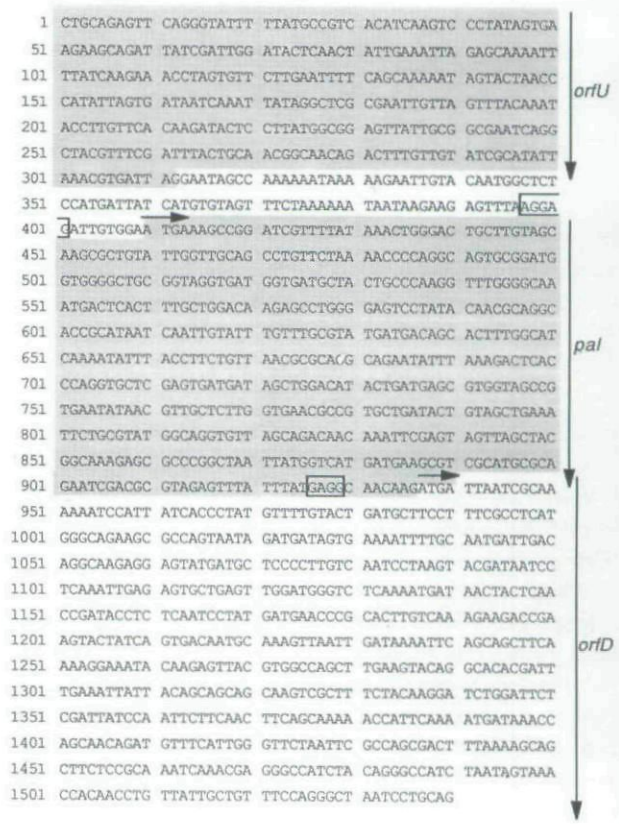

Fig. 4. DNA sequence of 1540 bp encoding the 19 kDa antigen of *L. pneumophila*. The positions of the three large open reading frames are indicated on the right, with arrows indicating the initiation codons of *pal* and *orfD*. Putative Shine-Dalgarno sequences associated with the translational starts are indicated by boxes. These sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession number X60543.

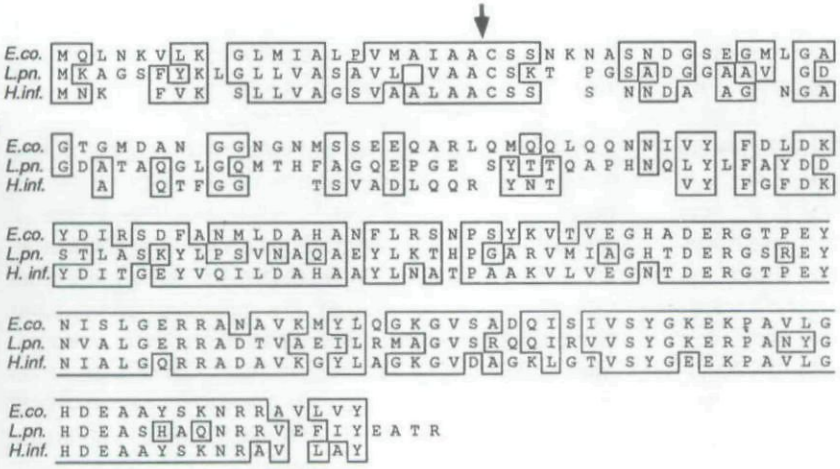

Fig. 5. Comparison of the inferred amino acid sequence of the *L. pneumophila* 19 kDa antigen with the *E. coli* and *H. influenzae* peptidoglycan-associated lipoprotein (*pal*). Boxed areas indicate regions of homology allowing for conservative substitutions. The arrow indicates the putative cleavage site for signal peptidase II.

The ATG codon that begins the 528 bp reading frame (at nucleotide 410 in Fig. 4) is preceded by a consensus Shine-Dalgarno sequence 8 bp upstream, strongly suggesting that this codon represents a translational initiation site (Stormo *et al.*, 1982). There is no obvious consensus promoter sequence upstream of this initiation site (Mulligan *et al.*, 1984). The reading frame encodes a polypeptide of 176 amino acid residues having a molecular weight of 18913 Da.

Because our characterization of the 19 kDa antigen suggested that it was peptidoglycan-bound and that it might be a lipoprotein, we compared the inferred amino acid sequence of this gene with that of the previously sequenced *E. coli* and *Haemophilus influenzae* peptidoglycan-bound lipoproteins (PAL) (Chen and Henning, 1987; Deich *et al.*, 1990). With inclusion of conservative substitutions, the comparison revealed 51% and 55% homology between the 19 kDa antigen and the *E. coli* and *H. influenzae pal* gene products, respectively (Fig. 5). The most striking similarity was observed in the carboxy-terminal half of the proteins, which includes the peptidoglycan-binding domain (66% and 61% homology, respectively). All three sequences have putative signal sequences terminating at a cysteine residue (at residue 22 in *E. coli* and *L. pneumophila* and at residue 20 in *H. influenzae*). At these positions, there is also strong conformity with a consensus sequence for signal peptidase II (Leu–Ala/Ser–Gly/Ala–Cys), which is found in about three-quarters of all bacterial lipoproteins studied (Hayashi and Wu, 1990). Both the *E. coli* and *L. pneumophila* sequences have conserative substitutions at the −3 position; however, Leu is strictly conserved at this position in only 75% of all sequenced lipoproteins. All three sequences have Ser at position +2, a feature of 38% of all bacterial lipoproteins (Hayashi and Wu, 1990). Given these striking sequence similarities, we conclude that the 19 kDa antigen of *L. pneumophila* is a homologue of the *E. coli* lipoprotein and from now on we will refer to this *L. pneumophila* gene as *pal*.

Partial analysis of the genes flanking pal

It was apparent from the analysis of PhoA gene fusions that the long open reading frame downstream of *pal*, *orfD*, also encodes a secreted protein. *orfD* has several in-frame methionine codons, but the only one preceded by a possible Shine-Dalgarno sequence is the ATG codon that overlaps with the termination codon of *pal* (at nucleotide 937). Using a partial amino acid sequence inferred from *orfD*, we searched the protein data bank for similarities with previously registered sequences. No extensive homology with a known protein sequence was uncovered. A similar analysis of the other partial amino acid sequence inferred from *orfU* also failed to yield any useful insights.

Further subcloning of the 19 kDa protein antigen gene

Because the region upstream of *pal* was devoid of transpositions from a point approximately 400 bp upstream from the 5′ end of our sequenced region to the 3′ end of *orfU* (Fig. 2), we speculated that *orfU* may be an essential gene when *pal* and/or the downstream genes are cloned in multiple copies. If so, *orfU* might encode either a transcriptional repressor of *pal* (and other downstream genes) or a protein that interacts post-translationally with the products of *pal* and/or other genes to assure their appropriate expression. All the subclones we had generated, including the M13 derivatives prepared for sequencing, carried either fragments of *pal* or an intact copy of *pal* with the transpositionally silent, upstream region.

To determine whether *pal* could be expressed independently of *orfU*, we attempted to subclone the 1333 bp *Pst*I–*Xho*II fragment of pDH11.2 that carries the entire *pal*

gene and the 3′ end of *orfU* (Fig. 2B) by ligating it into the pBR322 derivative, pJA1, after digestion with *Bam*HI and *Nsi*I. After several attempts to do this had failed, we repeated the ligation reaction and then transformed competent *E. coli* HB101 and HB101(pDH11.2::Tn*phoA*–*oriT*.B-16) in parallel. Phenotypically, the latter strain is Km^R (kanamycin-resistant), Cm^R, $PhoA^+$, and PAL^-, and the plasmid (which we will abbreviate in further references as 'pDH11.2::B-16') includes an intact *orfU* region. As expected, there were no ampicillin-resistant (Ap^R) transformants of HB101; however, using the same amount of the same ligation mixture, there were 53 Ap^R transformants of HB101(pDH11.2::B-16), two of which were also PAL^+. One of the PAL^+ isolates contained two plasmids: one was the size of pDH11.2::B-16, and the other was the size predicted for the pJA1-*pal* hybrid plasmid. When this isolate was passed on streptomycin-containing media to counterselect pDH11.2::B-16 (which carries *rpsL* (Sm^S) allele), 30 colonies were screened and found to be Cm^S, Km^R, and Ap^R; all 30 were PAL^+. The original PAL^+ isolate and the Sm^R isolates were unstable and became PAL^- with passage, in spite of continued selective pressure with ampicillin, and they lost all detectable plasmid DNA. The second of the two PAL^+ transformants carried a single plasmid intermediate in size between pDH11.2::B-16 and the pJA1-*pal* hybrid. Streptomycin-resistant derivatives from this isolate were Cm^S, Km^S, Ap^R, and PAL^+. This isolate and its derivatives were also highly unstable with respect to PAL reactivity and continued presence of plasmid DNA.

Discussion

These studies demonstrate that the 19kDa surface antigen is the peptidoglycan-associated lipoprotein of *L. pneumophila*. The label 'peptidoglycan-associated lipoprotein' was first applied to certain matrix proteins of *Pseudomonas aeruginosa* and *E. coli* (Mizuno, 1979). These molecules were characterized by their retention on the cell wall in 2% SDS at 35°C, by labelling with [^{14}C]-palmitic acid and 2-[^{3}H]-glycerol, and by biochemical separation from the covalently bound murein-lipoprotein described by Braun (Mizuno, 1979; Braun, 1975). Subsequently, similar lipoproteins were found in *Proteus mirabilis* and a wide variety of Gram-negative organisms (Mizuno, 1981). Even before DNA sequencing, we suspected that this was also the identify of the *L. pneumophila* 19kDa antigen because of its comigration of the *E. coli* PAL, the non-covalent but strong association with the cell wall, and the tailing phenomenon associated with this protein band in Western blots.

DNA sequencing was directed at a small region of the cloned insert of pSMJ11 that had been identified as the gene encoding the antigen by deletion analysis and transposon mutagenesis. The isolation of $PhoA^+$ fusions within this gene again confirmed that its product is a secreted polypeptide. An open reading frame, beginning with a consensus initiation codon, was found in the appropriate map position and correct orientation.

The sequence of the putative open reading frame encodes a protein with extensive homology to the *pal* genes of both *E. coli* and *H. influenzae*. The degree of homology is roughly comparable between any of the three sequences, particularly in the *C*-terminal half of the protein that is known to include the peptidoglycan-binding domain (Mizuno *et al.*, 1982). In addition, the *L. pneumophila* protein begins with a signal sequence of 22 residues that ends with a consensus cleavage site for signal peptidase II. The presence of these features in an amino acid sequence has been generally considered sufficient evidence to characterize the sequence as a lipoprotein (Hayashi and Wu, 1990). This finding, combined with the physical characteristics of the protein and the sequence homology, leave no doubt as to its identity as the *L. pneumophila* PAL.

PAL is one of the most potent immunogens of *L. pneumophila*. Its prominence as an antigen probably explains why it was so readily cloned in our early experiments and in experiments by others who also screened an *E. coli* genomic library with *Legionella* immune serum. The reason for its immunogenicity may relate to its lipoproteinaceous nature. The Braun lipoprotein has long been known to be a B-cell mitogen (Melchers *et al.*, 1975), and its mitogenicity can be reproduced by various synthetic lipopeptides that mimic its *N*-terminus (Wiesmuller *et al.*, 1983). The synthetic analogues differ from lipoprotein *N*-termini only in that their lipid components are uniformly composed of palmitic acid. The tripalmitoyl dipeptide *N*-palmitoyl-S-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-(*R*)-cysteine-serine (or Pam_s–Cys–Ser) is as potent a polyclonal B-cell activator as longer lipopeptide analogues (Prass *et al.*, 1987). This is noteworthy since this lipopeptide may also mimic the *N*-terminus of the *L. pneumophila* PAL.

In addition to their mitogenicity, synthetic lipopeptide analogues have also been shown to act as potent immunoadjuvants (Lex *et al.*, 1986). A significant adjuvant effect was recently demonstrated for the lipotripeptide Pam_3–Cys–Ser–Lys–, covalently bound to the hapten, dinitrophenol (Reitermann *et al.*, 1989). Again, this analogue mimics the *L. pneumophila* PAL sequence. Similar synthetic analogues have been shown to activate macrophages and to induce the production of IL-1, IL-6, and TNFα (Hauschildt *et al.*, 1990).

The implications of these lipoprotein properties for bacterial infections are speculative. However, immunogenic surface proteins from a variety of pathogenic bacteria are now known to be lipoproteins. These include the H.8 outer membrane protein of the pathogenic

Neisseria species (Cannon, 1989), the P6 protein (or PAL) of *H. influenzae* (Murphy *et al.*, 1986), the Braun lipoprotein analogue of *Brucella abortus* (Gomez-Miguel *et al.*, 1988), OspA and OspB of *Borrelia burgdorferi* (Bergstrom *et al.*, 1989), and the 34 kDa membrane protein of *Treponema pallidum* (Swancutt *et al.*, 1990). The *H. influenzae* PAL has been shown to be a target for bactericidal antibody, even in type b encapsulated strains (Green *et al.*, 1987). This conserved molecule is therefore a potential vaccine candidate. Likewise, monoclonal antibodies directed against this PAL have been useful in non-culture diagnostic methods for *H. influenzae* (Groeneveld *et al.*, 1989). Whether or not the *L. pneumophila* PAL will prove similarly useful for preventive or diagnostic use against Legionnaires' disease is open to investigation.

The upstream partial open reading frame, which we have temporarily designated as *orfU*, seems to be required for the maintenance of *pal* in multiple copies. We were never able to isolate a transposition into this gene on a plasmid that contains *pal*, and we were never successful in cloning *pal* apart from *orfU*. We succeeded in isolating *orfU* and *pal* to separate replicons in the same strain; however, these constructs were exceedingly unstable. We speculate that *E. coli* may not tolerate multiple, isolated copies of *pal* and that the *orfU* region may exert a necessary moderating effect, e.g. by encoding a negative transcriptional regulator or a protein that effects PAL at the post-translational level. Transcriptional analysis of *pal* will resolve this issue.

It is unknown whether PAL has a role in the pathogenesis of Legionnaires' disease. As a possible mitogen and immunoadjuvant, it may influence disease by modulating the immune response. Alternatively, certain bacterial membrane lipoproteins, e.g. TraT, have been found to interfere with complement deposition, increase bacterial serum resistance, and marginally improve the growth of *Salmonella typhimurium* in a mouse model of infection (Rhen and Sukupolvi, 1988). More recently, genes encoding membrane lipoproteins that confer serum resistance in *Vibrio cholerae* were found to be activated by the global regulator of virulence in that species (ToxR; Parsot *et al.*, 1991). Whether or not PAL participates in serum resistance of *L. pneumophila* or in other aspects of virulence is entirely speculative, but these questions might be addressed by generating isogenic *pal*$^-$ mutants or by manipulating the expression of PAL in *L. pneumophila*. In this study, several transferable plasmids were constructed with insertion mutations in *pal* or *orfD* that can be used for allelic exchange experiments in *L. pneumophila*.

Experimental procedures

Bacterial and plasmids

L. pneumophila AA100 is a clinical isolate that retains a high level of virulence for cultured macrophages and guinea-pigs. *L. pneumophila* AA103 is a nalidixic acid- and streptomycin-resistant derivative of AA100. *L. pneumophila* strains were grown on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYE-α) (Edelstein, 1982).

E. coli LE392 (*supE*, *supF*) was used to propagate bacteriophage λ 431 (*b*221, *c*I857, P_{am}) carrying either Tn*phoA* (i.e. λTn*phoA*-1) or Tn*phoA*–*oriT*. RecA$^-$ *E. coli* strains HB101 and CC118 were used for most cloning experiments. CC118 has deletion *phoA*Δ20 (Manoil and Beckwith, 1985). *dam*$^-$ *E. coli* strain K3216 (obtained from D. Friedman, University of Michigan) was used to propagate plasmids prior to performing *Bcl*I digestions.

pSMJ11 is a derivative of pBR322 that has a 7.5 kb fragment from *L. pneumophila* AA100 cloned in the *Bam*HI site. pJA1 is a derivative of pBR322 that carries a unique multiple cloning site and which was designed and constructed in this laboratory. pTLP5 was also constructed in this laboratory (by J.A.) for the purpose of gene transfer to *L. pneumophila*. This plasmid carries the same multiple cloning site as pJA1. Further details of its construction will be reported elsewhere; however, for the purposes of this study, it is necessary to know that pTLP5 carries the chloramphenicol acetyltransferase gene of pACYC184, which is selectable in *L. pneumophila*, and the *E. coli rpsL* allele, which confers streptomycin sensitivity in *L. pneumophila* strain AA103.

Tn*phoA*–*oriT* was generated by insertion of an origin of transfer from the IncP group of conjugative plasmids into the *Bam*HI site of Tn*phoA* (Manoil and Beckwith, 1985); this insertion inactivates the cryptic Sm^R gene of Tn*phoA*. Details of this construction will be presented elsewhere (J. Arroyo, in preparation). PhoA fusions were screened by growing colonies on LB media with 40 μg ml^{-1} of 5-bromo-4-choro-3-indolyl phosphate (XP).

Antibodies and sera

Rabbit antisera were prepared by subcutaneous injection with heat-killed *L. pneumophila* or *L. micdadei* at biweekly intervals for 8 weeks. Anti-alkaline phosphatase serum was prepared by injection of *E. coli* alkaline phosphatase (Sigma Chemical Co.) in complete Freund's adjuvant initially and in incomplete Freund's adjuvant for booster immunizations.

Monoclonal antibody 1E9 is a mouse monoclonal antibody generated by immunization with crude outer membrane fractions of *L. pneumophila*. To avoid production of antibodies to lipopolysaccharide, fractions for booster immunizations were isolated from *L. pneumophila* of diverse serogroups. Reactivity of mAb-1E9 with the native and cloned PAL antigen from AA100 was confirmed by immunoblotting; mAb1E9 also reacts with a 19 kDa antigen in other *L. pneumophila* strains representing serogroups 1–8, but has no reactivity with any proteins of *L. micdadei* or *E. coli*, by immunoblot analysis or dot immunoassay of whole bacteria.

Gel electrophoresis and immunoblotting

Bacterial pellets were lysed and proteins were solubilized by boiling in 2% SDS, 5% 2-mercaptoethanol, 50 mM Tris-HCl (pH 6.8) for 2–5 min in different experiments. Insoluble material was removed by centrifugation at 12 000 × *g* for 5 min before loading of samples. In experiments requiring re-extraction of the cell-wall

material, the insoluble pellet was washed once in water, then re-extracted as described.

SDS lysates were separated by polyacrylamide gel electrophoresis (Laemmli, 1970). Proteins were electrotransferred to nitrocellulose membranes over 3 h at 40 volts in Tris-glycine buffer with 20% methanol (pH 8.3). Enzyme immunoassays were performed on blots in 50 mM Tris-buffered saline (pH 7.5) with 0.05% Tween-20 (T-TBS). T-TBS was made with 500 mM NaCl for assays with immune serum and at 150 mM NaCl for assay with mAb1E9. After one hour of blocking in T-TBS, diluted immune sera or neat mAb1E9 hybridoma supernatants were incubated with blots overnight. After three washes in T-TBS, blots were then incubated for one hour in horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulins (Cappel Laboratories). After three final washes, peroxidase activity was detected using 0.06% diaminobenzidine (Sigma Chemical Co.) in 0.003% hydrogen peroxide.

Subcloning

Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979). Restriction fragments for subcloning were isolated from agarose gels by electrophoresis onto DEAE membrane (Schleicher & Schuell) and eluted into high-salt buffer as described (Lizardi *et al.*, 1984). Ligation and transformation of $CaCl_2$-treated *E. coli* were performed using standard methods (Sambrook *et al.*, 1989).

Transposon mutagenesis

E. coli strains carrying plasmids with target sequences were transduced to kanamycin resistance with λTn*phoA*-1 or λ::Tn*phoA–oriT*. Plasmids were then isolated and introduced into CC118 by transformation or were transferred conjugally (in the case of Tn*phoA–oriT*) using a helper strain with conjugative plasmid pRK212.1. If transpositions mapped to the plasmid, the plasmid was isolated by the alkaline lysis technique, and the position of the transposon was mapped with restriction endonucleases.

DNA sequencing

Sequencing of M13 templates was performed using the dideoxy chain termination method (Sanger *et al.*, 1977). M13-based primers were used initially, but for subsequent reactions, oligonucleotide primers were synthesized. Primer extension was catalysed by Sequenase (US Biochemical). DNA and protein sequences were analysed using Microgenie and MacVector software. Searches of the National Library of Medicine Protein Data Bank were supported by both of these software packages.

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