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Phenotype versus genotype of the 19 kD peptido-glycan associated protein of *Legionella* (PplA), among *Legionellae* and other Gram-negative bacteria

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The protein PplA (19 kD) cloned from a genomic library of *Legionella pneumophila*, Philadelphia 1, represents a peptido-glycan associated outer membrane protein in recombinant *E. coli* K-12 and *L. pneumophila*. It exhibits distinct sequence homology to lipoproteins of *Haemophilus influenzae* and *E. coli*. A *pplA* specific DNA probe generated by PCR was used in Southern hybridizations of chromosomal DNA of *Legionella* strains and other Gram-negative pathogens. Under conditions of high stringency, hybridization could only be observed in *L. pneumophila* isolates, but all other *Legionella* strains tested displayed hybridization under lower stringency. No signals appeared after hybridization of chromosomal DNA from a variety of other bacteria. Using anti-PplA monospecific polyclonal antibodies in Western blots, it was demonstrated that PplA related proteins of nearly the same size are found in all *L. pneumophila* isolates and in a variety of, but not all, the *Legionella* species analysed here.

Key words: *Legionellae*; peptido-glycan associated protein; *ppl*; Southern hybridization; stringency; polymerase chain reaction (PCR).

Introduction

Legionellae are the cause of the pulmonary illness known as Legionnaires' disease. The genus *Legionella* comprises of more than 30 species, which can be further subdivided by serotyping.[1] *Legionella pneumophila*, serogroup 1, are the most prevalent agents of legionellosis. The natural habitat of *Legionellae* is the aquatic environment. They can be easily isolated from domestic water systems, often in association with free living amoebae.[1,2] *Legionellae* are able to multiply intracellularly in lung macrophages, leading to severe tissue damage in the course of the infection.[3]

Prolonged passage of *Legionellae* over laboratory media leads to a spontaneous loss of virulence,[4] but presently little is known about the factors determining virulence of *Legionellae*. Genetic approaches revealed that a 24 kD membrane associated

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protein, termed Mip ('macrophage infectivity potentiator') is important for initiation of infection of target cells.[5,6] Furthermore, two proteins exhibiting haemolytic activities have been cloned recently in *E. coli* K-12.[7,8] The major secretory protein (Msp), a metalloprotease of 38 kD, seems to have no influence on virulence.[9] It is still unclear whether or not another haemolytic protein, termed legiolysin (39 kD), contributes to virulence. Also, a 60 kD heat shock protein with immunodominant epitopes and a peptidoglycan associated protein of 19 kD were cloned and analysed recently.[10-12]

In a previous study, we reported on the molecular analysis of the *L. pneumophila* specific 19 kD protein. Subcloning experiments and sequence analysis revealed homology of this protein to lipoproteins of *Haemophilus influenzae* and *Escherichia coli*.[13] The 19 kD protein was found to be associated with the murein layer of recombinant *E. coli* and *L. pneumophila*. Therefore, it was termed peptido-glycan associated protein of *Legionella* (Ppl).[13] In this study we analysed the genetic presence and the expression of *ppl* homologous sequences in *Legionellae* and other Gram-negative bacteria.

Results

Distribution of ppl *sequences in Gram-negative bacteria*

In order to determine the presence of *ppl* sequences among *Legionellae* and other Gram-negative bacteria, chromosomal DNA was isolated from a variety of strains. After digestion with *Cla* I, Southern hybridization was carried out using the 523 bp *pplA* specific fragment.[13] As can be seen from Fig. 1 and Fig. 2 (see Table 1) under high stringency conditions only *L. pneumophila* isolates displayed hybridization to this DNA probe. Interestingly, the size of the resulting *Cla* I fragment did not correspond to that of the cloned fragment. Also, differences could be detected among the various *L. pneumophila* strains according to the *Cla* I hybridization pattern. Using various other restriction enzymes, colinearity between the gene structure in the chromosome of the original strain *L. pneumophila*, Philadelphia 1, and the *ppl* specific plasmids could be confirmed (data not shown). Under conditions of lower stringency, distinct hybridization could be observed in all other *Legionella* strains tested (Fig. 1, Fig. 2 and Table 1). Genomic DNAs isolated from *E. coli, Shigella flexneri, S. sonnei, Serratia marcescens, S. liquefaciens, Salmonella typhimurium, Pseudomonas aeruginosa, Aeromonas sobria*, and *Bordetella pertussis* did not exhibit signals after hybridization to the *ppl*A sequence.

Expression of ppl *sequences in Gram-negative bacteria*

In a further approach to clarify the expression of PplA related proteins we employed anti-PplA monospecific polyclonal antibodies in Western blot analyses of whole cell extracts derived from the strains surveyed above. From Table 1 and Fig. 3 it can be seen that *L. pneumophila* isolates reacted very strongly in Western blots exhibiting proteins of the same size as the cloned PplA protein of 19 kD. Among the other *Legionella* strains, reacting proteins of similar size could be detected in *L. longbeachae* (serogroups 1 and 2), *L. micdadei, L. dumoffii*, and *L. bozemanii* (serogroup 1). *L. hackeliae* (serogroups 1 and 2), *L. oakridgensis, L. feeleii* (serogroups 1 and 2), *L. gormanii, L. jordanis, L. israelensis* and the non-*Legionella* strains did not display any reaction.

Discussion and conclusions

All the *L. pneumophila* isolates tested in this study, including an avirulent derivative of the Philadelphia 1 strain, express proteins with a size of 19 kD reacting with anti-

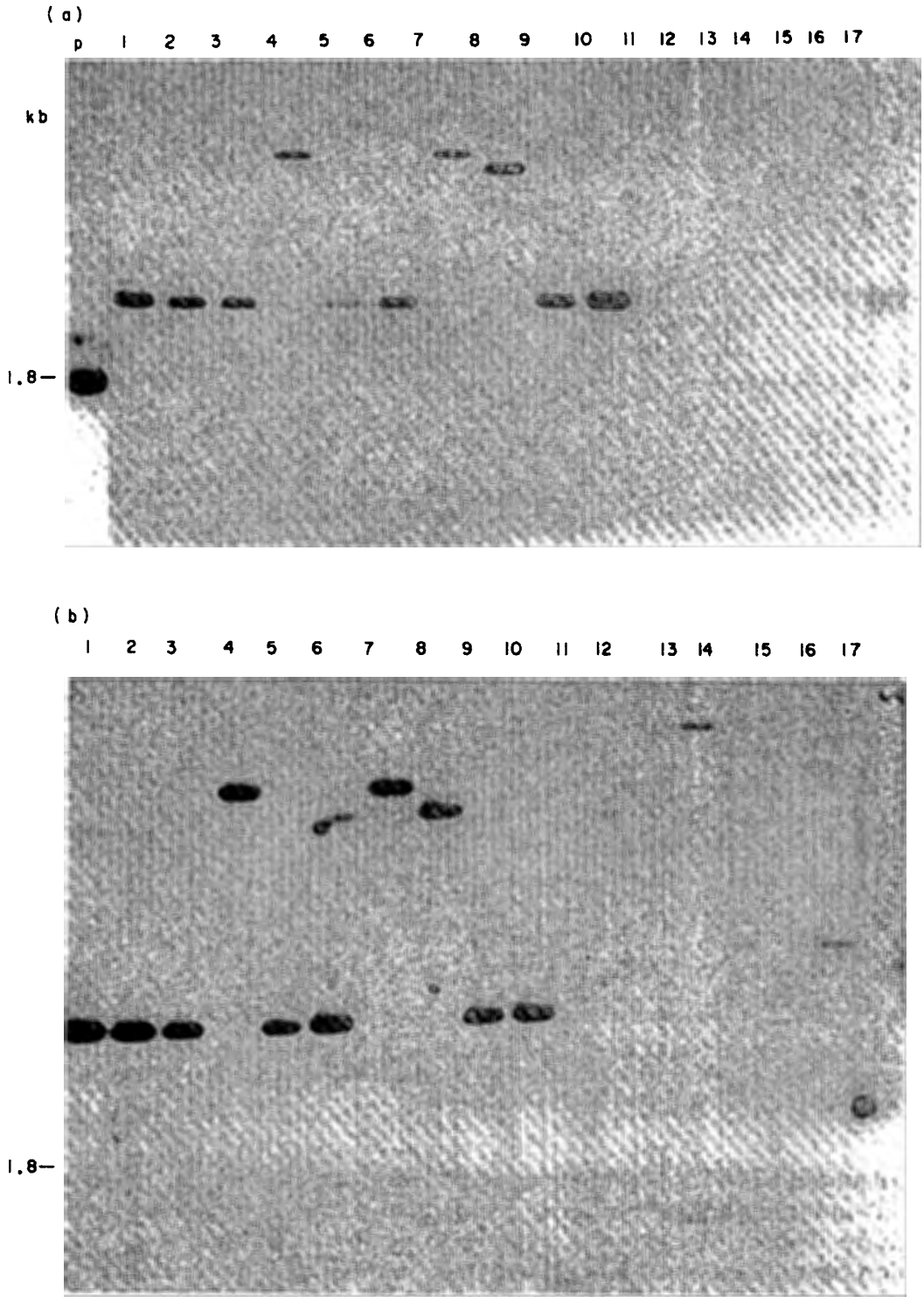

Fig. 1. Southern blot analysis of *Cla* I cleaved genomic DNA isolated from strains 1–17 (Table 1) under conditions of (a) high and (b) low stringency (see Materials and methods). A 523 bp *ppl*A specific fragment generated by PCR was used as the DNA probe. As a control, plasmid DNA of pBLL 30 digested with *Cla* I was separated in lane P.

PplA polyclonal antibodies. A variety of *Legionella* non-*pneumophila* strains reacted as well, but there were others which did not. Among the various Gram-negative strains tested no reaction to the antiserum could be observed. Some of the *Legionella* strains analysed here were previously tested by Hindahl and Iglewski[12] with antiserum specific for a 19 kD protein of *L. pneumophila*. Their data are in complete agreement with the

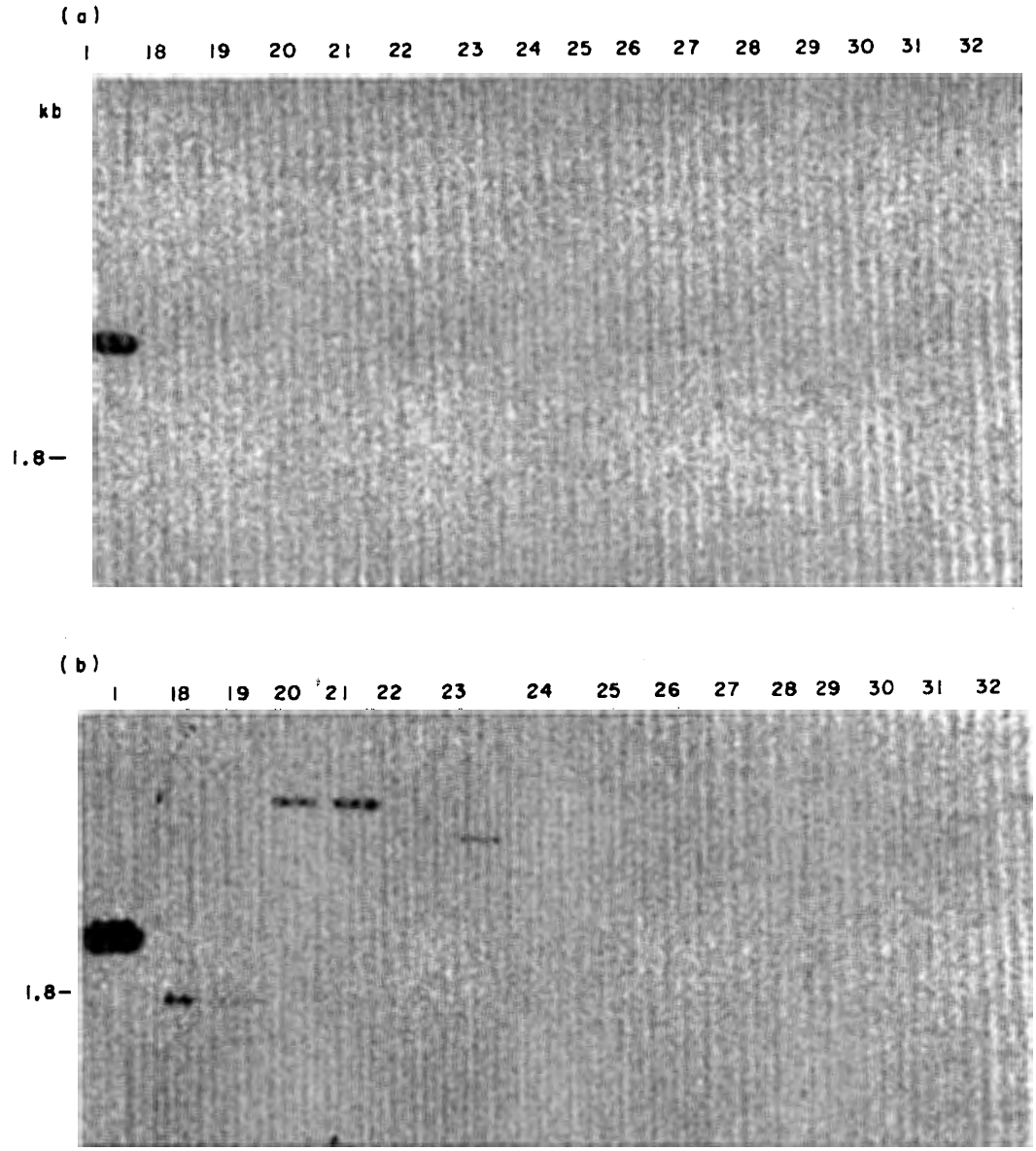

Fig. 2. Southern blot analysis of *Cla* I cleaved genomic DNA isolated from strains 1, 18–32 (Table 1) under conditions of (a) high and (b) low stringency (see Materials and methods). A 523 bp *ppl*A-specific fragment, generated by PCR, was used as the DNA probe.

results presented here, while Engleberg *et al.*[14] found related proteins reacting with an anti-19 kD antibody preparation in *L. gormanii* and *L. jordanis*, which were negative in our study. These discrepancies might be due to the different mode of antibody preparation, so that these authors yielded a higher titer, by which detection of proteins was possible, which share only some common epitopes.

A further approach was attempted by hybridization studies with a *ppl*A specific probe using low and high stringency conditions. Only the *L. pneumophila* isolates reacted with the *ppl*A DNA probe under high stringency conditions, while all other *Legionella* strains not belonging to the species *L. pneumophila*, displayed hybridization signals only under lower stringency conditions irrespective of expression of the PplA related proteins. All the other Gram-negative bacteria tested did not exhibit hybridization signals under the latter conditions. As the PplA protein exhibits only 60% similarity and 37% identity to the Pal protein of *E. coli* on amino acid level, positive hybridization under the conditions used here could not be detected. DNA

Table 1 Distribution and expression of *ppl* sequences among Legionella and other strains

No.	Strain[a]	Reference	Hybridization with *ppl* (stringency) High	Low	Reaction with anti-PplA antibodies[b]
1.	*L. pneumophila* (S1) Philadelphia 1	ATCC 33152	+	+	+
2.	*L. pneumophila* (S1) XXV, avirulent Philadephia 1	(29)	+	+	+
3.	*L. pneumophila* U1S1 environmental isolate	(29)	+	+	+
4.	*L. pneumophila* U21S6 environmental isolate	(29)	+	+	+
5.	*L. pneumophila* U22S3 environmental isolate	(29)	+	+	+
6.	*L. pneumophila* MSP19S1 environmental isolate	(29)	+	+	+
7.	*L. pneumophila* 685S1 patient isolate	(8)	+	+	+
8.	*L. pneumophila* 667S4 patient isolate	this study	+	+	+
9.	*L. pneumophila* 640S5 patient isolate	this study	+	+	+
10.	*L. pneumophila* 664S6 patient isolate	this study	+	+	+
11.	*L. longbeachae* S1	ATCC 33462	—	+	+
12.	*L. longbeachae* S2	ATCC 33484	—	+	+
13.	*L. dumoffii*	ATCC 33279	—	+	+
14.	*L. bozemanii* S1	ATCC 33217	—	+	+
15.	*L. micdadei*	ATCC 33218	—	+	+
16.	*L. gormanii*	ATCC 33297	—	+	—
17.	*L. jordanis*	ATCC 33623	—	+	—
18.	*L. feeleii* S1	ATCC 35072	—	+	—
19.	*L. feeleii* S2	ATCC 35849	—	+	—
20.	*L. hackeliae* S1	ATCC 33250	—	+	—
21.	*L. hackeliae* S2	ATCC 35999	—	+	—
22.	*L. israelensis*	ATCC 43119	—	+	—
23.	*L. oakridgensis*	ATCC 33761	—	+	—
24.	*Serratia marcescens* W225	Braun unpublished	—	—	—
25.	*Serrati liquefaciens* DSM 30064	Braun unpublished	—	—	—
26.	*Aermonas sobria* AB3	(30)	—	—	—
27.	*Salmonella typhimurium* 05-1/4, 12:i:1,2	Hof unpublished	—	—	—
28.	*Bordetella pertussis* 6564/85	Heesemann unpublished	—	—	—
29.	*Pseudomonas aeruginosa* SS 712	Behringer unpublished	—	—	—
30.	*Shigella sonnei* 5542/89	Heesemann unpublished	—	—	—
31.	*Shigella flexneri* 1265/89	Heesemann unpublished	—	—	—
32.	*E. coli* 536	(20)	—	—	—

[a] S designates the serogroup.
[b] Determined in Western blots using whole cell extracts.

analysis with the *ppl*A-specific DNA probe can be used for discriminating *L. pneumophila* from the *Legionellae* while such a differentiation could not be achieved by immunological analysis using anti-PplA antiserum. Such findings may be useful for the evaluation of diagnostic DNA probes. It is of interest that the *Cla* I hybridization pattern of the strains exhibited heterogeneity to some degree and that the cloned 1.8 kb *Cla* I fragment was not present in this size in the original strain *L. pneumophila*, Philadelphia 1. Differences in DNA modification[15] of the genomes and/or the location of *ppl*A at different positions on the chromosomes of the various isolates, might explain these findings.

It is important to note that PplA related proteins of similar size could also be detected in *Legionella* strains displaying only limited DNA homology. This was reflected by their resultant hybridization under conditions of lower stringency. Similar results have been obtained for Mip-like proteins[6] which can also be found in all *Legionella* isolates irrespective of species although only limited overall sequence homology to the cloned *mip*-gene of *L. pneumophila* exists. In contrast to PplA, Mip-like proteins produced

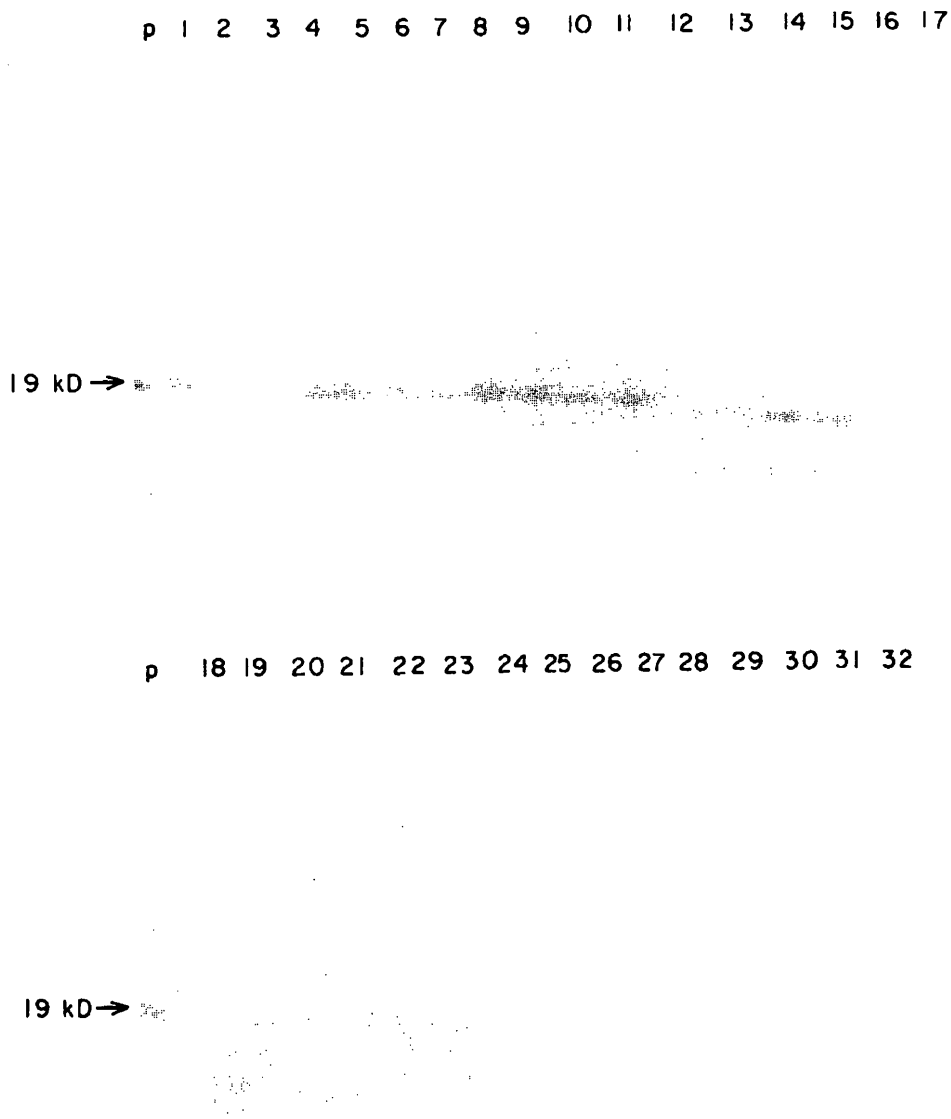

Fig. 3. Western blot analysis of whole cell extracts using anti-PplA antibodies. Strains are designated according to the numbers in Table 1. As a control, whole cell extracts of the recombinant strain *E. coli* DH5α; pBLL 30 were applied (lane P).

by different *Legionella* species strains differ more drastically in size. Furthermore, proteins that are related immunologically and with a similar size can be detected in all *Legionella* species and in other bacteria by using polyclonal and monoclonal antibodies raised against the 60 kD heat shock protein (HtpB) cloned from *L. pneumophila* (Chirinos, unpublished data).[10,16] Using a *L. pneumophila htp*-specific DNA probe under conditions of high stringency in hybridization experiments, signals appeared only in the case of *L. pneumophila* isolates but not in a variety of other *Legionella* species tested (Bender, unpublished data). The presence of immunodominant conserved epitopes on these proteins could explain such findings. Conversely, studies with the haemolytic proteins cloned from *L. pneumophila*, the legiolysin[8] (Bender *et al.*, unpublished data) and the major secretory protein,[17] revealed that expression of immunologically related proteins is detected exclusively in members of the species *L. pneumophila*. These data are of use in evaluating immunological assays used in detection and identification of *Legionellae*.

Materials and methods

Bacterial strains and plasmid. Bacterial strains used in this study are listed in Table 1. The recombinant *ppl* specific plasmid pBLL 30 was described recently.[13] The plasmid was maintained in *E. coli* DH5α under antibiotic pressure (ampicillin, 100 μg/ml).

Media, chemicals and enzymes. Legionella strains were cultivated on BCYE agar plates (Oxoid, Wesel, Germany) at 37°C in 5% CO_2–atmosphere for 2–3 d.[18] *E. coli* strains and all other Gram-negative bacteria were grown overnight in Luria Bertani (LB) medium at 37°C, except *Bordetella pertussis*, which was cultivated on Bordet-Gengou (BG, Difco, Detroit, Michigan, USA) containing 15% sheep blood for 5 d at 37°C. Radiochemicals were purchased from NEN Research (Dreilind, Germany). All other chemicals were a gift of Sigma (Deisenhofen, Germany).

DNA techniques. Plasmid and chromosomal DNA were isolated as described earlier.[19,20] For restriction enzyme analysis, DNA was treated with appropriate enzymes and the resulting fragments were separated on 1% agarose gels.[21] *Hind* III cleaved lambda DNA was used as size marker.

PCR amplification. A 523 bp fragment encoding solely PplA[13] was amplified by polymerase chain reaction (PCR) using the bio-med Thermocycler 60 (Braun, Göttingen, Germany). Primers were selected according the sequence published by Ludwig *et al.*:[13] 5'GCCGGATCGTTTTA-TAAACTGGG 3' (position 116–139) and 5'CTTGTTGCCTCATAAATAAACTCTC 3' (reverse position 639–615). Oligonucleotide synthesis was performed by the automated phosphor-amididite coupling method[22] (TIB MOLBIOL, Berlin, Germany). The PCR solution contained 100 mM KCl, 20 mM Tris–Cl (pH 8.3), 0.02% gelatine, 2 mM $MgCl_2$, 200 μM each of the deoxynucleosidetriphosphates, 0.5 μM each of the primers and 2.5 units of *Taq* DNA polymerase (Boehringer, Germany). The total volume for PCR reaction was 100 μl. Approximately 0.5 μg of template DNA (plasmid pBLL 30)[13] was initially denatured at 95°C for 3 min. Then a total of 30 cycles were run, using a three temperature PCR cycle with denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 2 min.

Radioactive labelling. The *ppl*A specific fragment generated by PCR (see above) was isolated from agarose gels[23] and labelled by the method of Feinberg and Vogelstein[24] with the random priming kit purchased from Boehringer, Mannheim, Germany, using P-dCTP.[32]

Southern hybridization. Transfer of DNA fragment to Biodyne nylon membranes was performed according to the method of Southern[25] modified as described by the manufacturer (Pall Bio Support, New York, USA). High stringency hybridization was performed in 50% formamide at 42°C in 5×SSC. The filters were washed three times with 0.1×SSC/0.1% SDS at 56°C. For low stringency conditions, the formamide contents of the hybridization solution were reduced to 25%, and hybridization was carried out at 37°C with 6×SSC. The filters were washed in 2×SSX/0.1% SDS at 56°C three times. These conditions allow approximately 10% or 25% mismatches estimated according to Davis *et al.*[26]

Preparation of anti-PplA antibodies. Polyclonal monospecific anti-PplA antibodies were prepared in rabbits using the *E. coli* K-12 strain expressing the 19 kD protein. For Western blot analysis the antiserum was adsorbed with the *E. coli* strain carrying only the vector (see Ludwig *et al.*[13]).

SDS–PAGE and Western blot analysis. SDS–PAGE was performed according to Laemmli[27] using 10% polyacrylamide gels. Whole cell extracts for Western blot analysis were prepared; after growth of the bacteria at 37°C they were harvested in distilled water. OD_{600} was adjusted to 0.8 and 1 ml of the suspension was centrifuged. After removal of the supernatant, the bacteria were resuspended in 100 μl Laemmli buffer and boiled for 10 min. For SDS–PAGE 10 μl were applied. Western blots were performed as described by Towbin *et al.*[28] using peroxidase-conjugated swine-anti rabbit IgG antibodies (DAKO, Hamburg, Germany).

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