Cloning, nucleotide sequence and expression in *Escherichia coli* of a gene (*ompM*) encoding a 25 kDa major outer-membrane protein (MOMP) of *Legionella pneumophila*

ANDREA S. HIGH, STEVEN D. TOROSIAN and FRANK G. RODGERS*

Molecular Pathogenesis and Infectious Disease Group, Department of Microbiology, Spaulding Life Science Building, University of New Hampshire, Durham, New Hampshire 03824–3544, USA

(Received 7 December 1992; revised 25 February 1993; accepted 16 March 1993)

A genomic library derived from a virulent isolate of *Legionella pneumophila* was constructed in *Escherichia coli* JM 83 using the cloning vector pUC19. The clones were screened by filter immunoassay using *L. pneumophila* rabbit polyclonal antisera and in the absence of *in situ* bacterial lysis one such clone, LP 116, expressed *L. pneumophila*-specific antigens on the surface of *E. coli*. Restriction endonuclease digest analysis and agarose gel electrophoresis revealed a fragment measuring approximately 750 bp. Southern hybridization confirmed that the fragment was *L. pneumophila* DNA. Sequencing data showed that the fragment was 810 bp in length with an open reading frame (ORF) of 678 bp. The outer-membrane profiles of the *E. coli* parent, the *L. pneumophila* DNA-contributing strain and clone LP 116 were compared by SDS-PAGE. A protein of 25 kDa was found in outer-membrane preparations of both the clone LP 116 and *L. pneumophila* but not in *E. coli* JM 83. This was in agreement with the molecular mass of the deduced peptide of the mature protein. Immunoblots using *L. pneumophila*-specific polyclonal antiserum confirmed that this 25 kDa outer-membrane protein (OMP) was a *L. pneumophila* polypeptide. Both direct immunofluorescence assay and immunoblots using the commercially produced monoclonal antibody specific for the common antigen of the major outer-membrane protein (MOMP) confirmed that the 25 kDa protein produced by LP 116 was involved with the MOMP complex. The gene encoding this protein has been designated *ompM*. Furthermore, using the fertile chicken egg virulence assay, clone LP 116 producing the 25 kDa MOMP of *L. pneumophila* showed an increase in virulence when compared to the *E. coli* parent strain.

Introduction

Legionella pneumophila, the major etiologic agent responsible for Legionnaires' disease, is a facultative intracellular Gram-negative bacterium that survives and multiplies within human alveolar macrophages and monocytes. This pathogen evades macrophage defences by inhibiting the oxygen-dependent sequelae of phagocytosis and blocking phagosome-lysosome fusion (Horwitz, 1984). As a consequence, the organism

replicates unhindered within the phagosome of infected cells utilizing host cell-derived amino acids as energy sources. Infection of these cells leads to their eventual destruction.

The bacterial virulence determinants and regulatory mechanisms governing the infectious processes of *L. pneumophila* are ill defined. Plasmid-encoded virulence has been reported for other Gram-negative bacteria; however, it has been difficult to link virulence of *Legionella* spp. to factors at the molecular level since pathogenic strains of *Legionella* may or may not possess plasmids. Bacterial cell surface proteins have also been associated with mechanisms of pathogenicity of other Gram-negative micro-organisms. Electron microscopy has shown that *Legionella* organisms attach to mammalian cells in culture prior to engulfment (Oldham & Rodgers, 1985); however, the mechanisms mediating such attachments are unknown. The presence of various structural and non-structural bacterial cell surface and

* Author for correspondence. Tel. (603) 862 2250; fax (603) 862 2359.

Abbreviations: DFA/IFA, direct/indirect immunofluorescence assay; FB/EIA, filter-binding/enzyme immunoassay; LPS, lipopolysaccharide; (M)OMP (major) outer-membrane protein.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L05595.

membrane components (Rodgers & Davey, 1982; Rodgers, 1983) may play important roles in the process of adherence and uptake.

SDS-PAGE of the outer membrane of *L. pneumophila* showed a genus-common 29 kDa protein (Ehret *et al.*, 1984) which was shown to be a component of a large aggregate protein of 95 kDa (Butler *et al.*, 1985). This aggregate composed of 24–29 kDa subunits is stabilized by disulphide bonds and has been reported as structurally similar to the major outer-membrane protein (MOMP) found in chlamydiae; another, albeit obligate, group of intracellular pathogens (Newhall & Jones, 1983). Alternatively, the MOMP complex of *L. pneumophila* has been reported as comprising both 70 and 120 kDa proteins (Hoffman *et al.*, 1992a). Based on its folding properties and ability to form membrane channels, this *Legionella* MOMP behaves as a porin with similar properties to those found on *E. coli* (Gabay *et al.*, 1985). The MOMP shows immunologic similarity to the other serotypes of *L. pneumophila* (Hindahl & Iglewski, 1986) as well as to other *Legionella* species (Butler *et al.*, 1985). However, a role for the *L. pneumophila* MOMP in pathogenicity and immunity has yet to be demonstrated.

Investigations of the outer membrane of *L. pneumophila* are crucial to determining pathogenicity at the cellular level but have been hampered by difficulties in separating cell surface components. Using a molecular cloning approach we have developed an *E. coli* clone expressing a surface protein of *L. pneumophila* to facilitate such studies. This gene encodes a 25 kDa protein which, when expressed, shows properties similar to those of the MOMP of *L. pneumophila*. Furthermore, as determined in the fertile chicken egg virulence assay (Tzianabos & Rodgers, 1989), the *E. coli* clone expressing this *L. pneumophila* 25 kDa MOMP shows an increase in virulence when compared with the *E. coli* parent strain.

Methods

Strains, cultural conditions and antisera production. *L. pneumophila* serogroup 1 strain Nottingham N₇, originally isolated from the sputum of a fatal case of Legionnaires' disease, was further passaged twice on L-cysteine-supplemented buffered charcoal yeast extract agar (BBL) was added α -ketoglutarate (BCYE- α) and maintained in 1% (v/v) serum containing 10% (w/v) sorbitol at -70°C . Preparations of *E. coli* strain JM 83 containing the plasmid vector, pUC19 encoding ampicillin resistance, were stored at -70°C in nutrient broth (Difco) containing 0.8% DMSO. Duplicate *E. coli* preparations without the plasmid vector were similarly maintained. *L. pneumophila* N₇ was inoculated onto BCYE- α agar and grown at 37°C for 24 h in a humid atmosphere. *E. coli* JM 83 without the plasmid vector was grown in nutrient broth, while the strain containing pUC19 was grown in nutrient broth with $50\text{ }\mu\text{g}$ ampicillin ml^{-1} . Both *E. coli* JM 83 preparations were grown as overnight cultures at 37°C in a shaking water bath.

Polyclonal antisera was raised to *L. pneumophila* N₇ in New Zealand white rabbits. Multiple subcutaneous inoculations of whole, formalin-

fixed organisms suspended in Freund's incomplete adjuvant were administered at 0, 2 and 4 week intervals.

Preparation of DNA and filter binding immunoassay. Genomic DNA was isolated by a modification of the method described by Marmur (1961). The lysozyme step was omitted from the original procedure as it was found that this did not lyse *L. pneumophila*. The concentration of genomic DNA from this isolation procedure was determined by the diphenylamine (DPA) test (Burton, 1968). The cloning vector, pUC19, was isolated from *E. coli* JM 83 by the alkaline lysis method (Birnboim & Doly, 1979) and was treated with $10\text{ }\mu\text{g}$ RNAase ml^{-1} prior to digestion.

L. pneumophila DNA was partially digested with *Hae*III (IBI), while the plasmid vector was cleaved with *Sma*I (BRL) or *Pst*I (IBI) and subsequently used in the ligation studies with the *L. pneumophila* DNA fragments using T4 ligase (BRL). All treatments were performed according to the manufacturers' instructions. The resultant *L. pneumophila* genomic DNA fragments and pUC19 plasmid constructs were introduced into *E. coli* JM 83 by the transformation procedures of Maniatis *et al.* (1982). The resultant bacterial suspension was inoculated onto lactose-containing violet red bile (VRB) agar supplemented with $50\text{ }\mu\text{g}$ ampicillin ml^{-1} . Colourless colonies were selected from VRB agar and grown in nutrient broth containing $50\text{ }\mu\text{g}$ ampicillin ml^{-1} . Surviving cultures were frozen at -70°C in 0.8% DMSO.

E. coli clones expressing *L. pneumophila* antigens were detected by filter-binding in an enzyme immunoassay (FB/EIA) (Engleberg *et al.*, 1984). Colonies were removed by direct application onto nitrocellulose filters and these were treated by baking in a vacuum oven at 60°C . Organisms were not lysed prior to the baking process. In addition, similar organism samples were formalin-fixed to prevent bacterial lysis and added as drops to nitrocellulose filters which were air-dried. Oven-dried or formalin-fixed air-dried filters were incubated overnight at room temperature in a 1:800 dilution of *L. pneumophila* N₇ polyclonal rabbit antisera. The filters were incubated for 2 h in 1:3000 goat-anti rabbit horseradish peroxidase (HRP) conjugate and immersed in colour developer containing 0.05% (w/v) 4-chloro-1-naphthol and 0.15% (v/v) hydrogen peroxide in a 5:1 solution.

Clone LP 116, expressing antigens of *L. pneumophila* created from the DNA manipulations, was screened for plasmid content (Kado & Liu, 1981) and was digested with *Sma*I or *Pst*I (IBI) to release the *L. pneumophila* DNA insert. All samples were compared to pUC19, which had been similarly digested with *Sma*I or *Pst*I, by agarose gel electrophoresis. Gels were transferred to nitrocellulose filters for Southern blot analysis (Southern, 1975). Genomic DNA from *L. pneumophila* N₇ digested with *Hae*III was labelled with [α - ^{32}P]dCTP (NEN) using a nick translation kit (BRL) and was used to probe gels for the presence of *L. pneumophila* DNA.

DNA sequencing and analysis. Both strands of the *L. pneumophila* DNA insert were sequenced directly from the vector employing the dideoxy chain-termination method described by Sanger *et al.* (1977) using a Sequenase sequencing kit (USB). Samples were labelled with [^{35}S]dATP (NEN) and M13 primers (USB) or primer extensions obtained from the novel DNA sequence (National Biosciences). PCR was performed using a GeneAmp kit and thermocycler (Perkins Elmer Cetus) to amplify regions of the fragment. Sequences obtained from these experiments were compared to those registered with GenBank and analysed by the Genetics Computer Group (GCG) Sequence Analysis Software Package (Devereux *et al.*, 1984).

OMP analysis. The outer membranes of *E. coli* JM 83, *L. pneumophila* N₇ and the clone expressing antigens of *L. pneumophila* by FB/EIA were isolated as described by Wenman *et al.* (1985). The protein content for each sample was determined spectrophotometrically by the Lowry method. Protein samples were subjected to SDS-PAGE (Laemmli, 1970) and silver-stained (Oakley *et al.*, 1980). Gels treated

with periodic acid were stained by the method of Tsai & Frasch (1982). Duplicate gels were used in immunoblot analysis (Towbin *et al.*, 1979) using a Tansblot cell (Bio-Rad) and the filters probed with *L. pneumophila* rabbit antisera or MOMP-specific monoclonal antibody followed by HRP-conjugated antiserum as described for colony transfers.

E. coli JM 83, *L. pneumophila* N₇ and the clone LP 116 were tested by indirect immunofluorescence assay (IFA) using *L. pneumophila* polyclonal rabbit antisera and fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum. In addition, surface expression of *Legionella* membrane antigens was further investigated with a direct immunofluorescence assay (DFA) using a commercially prepared monoclonal antibody (Genetic Systems) raised against an OMP of *L. pneumophila* (Gosting *et al.*, 1984). This monoclonal antibody was shown to have specificity for the MOMP of *L. pneumophila* (Nolte & Conlin, 1986). Specimens were mounted in glycerol containing diazobicyclo-2,2,2 octane (DABCO) and examined by UV epifluorescence microscopy.

Virulence assay. Fertile white Leghorn chicken eggs (UNH Poultry Farm) were inoculated into the yolk sac with 10-fold dilutions of *L. pneumophila* N₇, *E. coli* JM 83 or the *E. coli* clone LP 116 to assess virulence (Tzianabos & Rodgers, 1989). The lethal dose₅₀ (LD₅₀) for each of these was calculated by the method of Reed & Meunch (1938).

Results and Discussion

L. pneumophila chromosomal DNA was partially cleaved with *Hae*III, fragments were ligated into pUC19 and transformants isolated in *E. coli*. These were screened by reaction with the rabbit anti-*L. pneumophila* antiserum in FB/EIA dot-blot assay. One positive clone, LP 116, of 129 stable clones derived from 1123 transformants, was studied further. Because these assays did not involve lysis of the bacteria, the *L. pneumophila* antigen detected in the *E. coli* clone, LP 116, appeared to be surface-located. This was confirmed using the anti-*L. pneumophila* polyclonal antiserum in IFA studies.

Restriction analysis of the recombinant plasmid, pLP116, revealed a band which hybridized to ³²P-labelled *L. pneumophila* DNA confirming that this fragment released from pLP116 was derived from *L. pneumophila* DNA. The restriction map for pLP116 is presented in Fig. 1. Nucleotide sequencing data determined that the recombinant plasmid pLP116 contained an *L. pneumophila* DNA insert of 810 bp in length which consisted of an ORF of 678 bp (Fig. 2). The amino acid content deduced from the DNA sequence gave a protein of 226 amino acids, of which the first 20–22 inferred a signal peptide. The deduced molecular mass of the mature protein was approximately 25100 or 25400 Da, depending on the actual cleavage site. Also it was determined that the protein was cysteine-rich, containing nine cysteine residues. This DNA sequence, designated *ompM*, encoding the 25 kDa MOMP showed no homology with other *L. pneumophila* OMP genes, including the *mip* gene producing the 24 kDa OMP (Engleberg *et al.*, 1989), the *ompS* gene responsible for the

28/31 kDa OMP (Hoffman *et al.*, 1992b) or with other prokaryotic DNA currently on file with GenBank.

Other studies have encountered difficulties associated with the cloning and expression of *L. pneumophila* OMPs in *E. coli*. These difficulties have been linked to differences in the –35 and –10 consensus sequences as well as in A-T-rich regions of the promoter sequence for an *L. pneumophila* 28/31 kDa OMP (Hoffman *et al.*, 1992b). This did not appear to be the case for the promoter region of *ompM*. The –35 consensus sequence was typical of an *E. coli* promoter and this may have been responsible for expression of the *L. pneumophila* antigen in the *E. coli* host. As has been reported (Hoffman *et al.*,

Fig. 1. Restriction map of pLP116. The shaded area is the ORF of *ompM* encoding the 25 kDa MOMP of *L. pneumophila*. Solid arrows denote regions sequenced directly from the plasmid. Dashed arrows indicate regions sequenced using PCR-amplified product.

	-35	-10	
1	GTGATCGAGTTGACACGGACTTTAAGTCACACACTAACCCCTACGAAGCAGTTA		53
54	CCCCTACGGGCGAGATCAAGGAGACTCTACCCATGCTTAAGTCTCGTTTACTCT		107
		met leu lys ser arg leu leu	
108	ACGCAACGCTAGCAGCAGTGGCAATCGTAGCACTTCCGAGACGTGTGATCGAG		161
	tyr ala thr leu ala ala val ala ile val ala thr ser ^ glu thr ^cys asp arg		
162	ACTCGAGCCATTGTACGAAATCGATACGATTGATCGCTCAGTACAGTCCTTGAT		216
	asp ser ser his cys thr lys ser ile arg leu ile ala gln tyr ser pro trp met		
217	GGCAGCTCTCAAGCTACTGAATAACGTGACAAATGTCATGCTCTCTAGATCATA		272
	ala val ser lys leu leu asn asn val thr met ser cys leu ser leu asp his		
273	CTGTACCTATTAGATTATGCTACAGTGTGCGAGTGGTTCGTCGTCAGTCAGTGAC		327
	thr val pro ile arg phe met leu gln cys arg val val arg arg gln ser val thr		
328	TATTGAGAGATTGACGTCAGCTGTTGAGTGCCTACTCGATGGCTAGATTCCGA		381
	ile glu arg leu thr ser ala val lys leu leu leu pro phe ser gly val		
382	TGTGTCTCTAGTAGACCTAAACCCACCTTGGGGGACGGGGGGGATACGAGGAGG		434
	val ser cys arg pro lys pro thr leu gly ala ala gly gly tyr glu glu		
435	GGTATTGAGGGGGTTTGCACGTTGTTAAACTACTGCTACCCCTTCAGTGGGGTTC		488
	gly tyr ser gly phe ala arg val lys leu leu leu pro phe ser gly val		
489	GGTATCCGTCAGCAGTCGTACGGGAGTACGCCGATGGTCTACAGATCTTCAGGA		544
	arg tyr pro ser ala val val arg glu tyr ala asp gly ala thr asp leu arg		
543	TCTGTACGAGACTCTAGGTCTCCACGCGACCGCAGACCGTTGACAACATCA		597
	ile cys his glu thr leu gly leu his ala thr ala asp his val asp asn tyr gln		
598	AAGGGACGTAGGGGTGCGATGGGTATGCGGGTATGCGAGTATCGGGGTG		650
	arg asp val gly val ala trp val gly ile gly val cys glu tyr arg gly		
651	TGTTTCTAGGGTCTTGCTTATAGCAAGACTTCGCTCACTGCTATGCGTACTCAAC		706
	val phe leu gly val leu leu ile ala arg leu arg ser leu leu cys val leu asn		
707	GACGCACGAGGCCACCGTTACATTGCTCTAAAGGATGCGTCCAAAAGGGAGAAA		760
	asp ala arg gly his arg tyr ile ala leu lys asp ala ser lys arg glu lys		
761	GGATAAGCATGCGTCAAGGTAGGTGGTGAACATTGTCGGTCACTTAG		810
	gly *		

Fig. 2. Nucleotide sequence of the 810 bp 25 kDa MOMP gene of *L. pneumophila*. The –35 and –10 promoter sequences are overlined. The Shine–Dalgarno sequence is underlined. The 678 bp ORF begins at nucleotide 86 with the initiating methionine (met) of the deduced amino acids. The region of inferred signal peptide is shown overlined with a dashed line and potential cleavage sites are indicated by ^. The termination signal is at *.

Fig. 3. OMPs subjected to SDS-PAGE as described in Methods. Lanes: 1, *E. coli* JM 83 with pUC19; 2, *E. coli* LP 116; 3, molecular mass standard (lysozyme, 14300; β -lactoglobulin, 18400; ovalbumin, 43000 and BSA 68000); 4, *L. pneumophila* N₇.

1992b), clones containing *L. pneumophila* genes often show high levels of instability. Likewise, there were problems with the stability of the LP 116 clone. Often it would not grow in the presence of 50 μ g ampicillin ml⁻¹ due to possible toxic effects of the encoded material. Total elimination of plasmid from recovered frozen aliquots of the clone occurred following several freeze-thaw cycles or after long term storage of the LP 116 preparations at -20°C . Because of difficulties maintaining sufficient proportions of pLP116, it was necessary to enhance regions of the recombinant plasmid through the use of PCR. Initial sequencing was performed using standard plasmid preparations (Wang *et al.*, 1988). Approximately 350 bp were sequenced before instability of the LP 116 clone resulted in plasmid preparations of insufficient purity for sequencing. Plasmid preparations of the clone LP 116 were used as templates for the PCR reaction. Consequently, 17mer primers were constructed from known sequences which allowed PCR amplification of the unsequenced portion of the recombinant plasmid. The resulting PCR products were used in subsequent sequence analysis and from this the *L. pneumophila* pLP116 insert was determined to be 810 bp.

Although the immunofluorescence as well as the dot blot and colony transfer immunoassays indicated that the protein expressed on the surface of the *E. coli* clone

Fig. 4. Gels of OMPs electrically transferred to nitrocellulose and treated with *L. pneumophila* polyclonal rabbit antisera (left panel) and MOMP-specific monoclonal antibody (right panel). Lanes: 1, *E. coli* JM 83 with pUC19; 2, clone LP 116; 3, *L. pneumophila* N₇; 4, *E. coli* JM 83 with pUC19; 5, clone LP 116; 6, *L. pneumophila* N₇.

LP 116 was derived from *L. pneumophila* N₇, it remained to be established that the protein encoded by this gene was normally expressed on the outer surface of *L. pneumophila*. Isolation of the OMPs of clone LP 116 and of *L. pneumophila* N₇ showed that the antigenic material in LP 116, as well as in *L. pneumophila*, was expressed in the outer membrane. Examination of the OMP profiles of *E. coli* LP 116 revealed a band of the same size as one found in *L. pneumophila* N₇ that was not found in the parent *E. coli* strain (Fig. 3). The 25 kDa band present in both the clone LP 116 and *L. pneumophila* N₇ showed cross reactivity in immunoblot procedures using *L. pneumophila*-specific polyclonal rabbit antisera (Fig. 4, lanes 2 and 3) as well as with the MOMP-specific monoclonal antibody (Fig. 4, lanes 5 and 6). In other studies examining the behaviour of this protein, electrophoresis of OMP samples of the clone LP 116, the parental *L. pneumophila* strain and the *E. coli* parent strain prepared in non-reducing buffers demonstrated that the 25 kDa MOMP (OmpM) did not enter the gel (data not shown). This was in agreement with other reports indicating that *L. pneumophila* MOMP was a

Fig. 5. OMP profiles after treatment of polyacrylamide gels with periodic acid. LPS in OMP samples is sensitive to periodic acid oxidation and therefore normally stains darkly; however, MOMP does not stain with this technique. The lack of staining of the 25 kDa band in *L. pneumophila* and in the LP 116 clone indicated that this band was the MOMP. Lanes: 1, clone LP 116; 2, *E. coli* JM 83 with pUC 19; 3, *L. pneumophila* N₇.

component of a 95–100 kDa OMP complex (Ehret *et al.*, 1984; Butler *et al.*, 1985; Gabay *et al.*, 1985; Hindahl & Iglewski, 1986). Examination of polyacrylamide gels of outer-membrane preparations which had been produced under reducing conditions and then oxidized with periodic acid prior to silver staining, showed dark lipopolysaccharide (LPS) banding patterns but did not stain the 25 kDa region for *L. pneumophila* and the clone

(Fig. 5). This resistance to silver staining under these conditions indicated that the 25 kDa surface-expressed protein in the clone was characteristic of MOMP (Tsai & Frasch, 1982; Hindahl & Iglewski, 1986). These findings were confirmed by DFA using a commercially produced monoclonal antibody raised to the common antigen of the MOMP of *L. pneumophila*. The quality of fluorescence of the *L. pneumophila* N₇ DNA-contributing strain, the clone and the *L. pneumophila* positive control supplied with the kit was comparable, whereas the *E. coli* parent strain did not fluoresce (Fig. 6). Similar fluorescent results were obtained using *L. pneumophila* N₇-specific polyclonal antisera. In the immunoblot assays of OMP profiles using goat-antimouse HRP conjugate and MOMP-specific unconjugated monoclonal antibody further established this protein as a component of the MOMP complex of *L. pneumophila* (Fig. 4).

Although the molecular mass of this protein has been reported to range between 24 and 29 kDa, variations in reported molecular mass would depend on both the methods used to isolate the OMP and on how much LPS and peptidoglycan remained attached to the MOMP complex of the *L. pneumophila* outer-membrane sample. Indeed, like the MOMP of *Neisseria gonorrhoeae* (Hitchcock, 1984), the MOMP of *L. pneumophila* is known to be tightly bound to LPS (Gabay *et al.*, 1985; Hindahl & Iglewski, 1986) and resists complete dissociation in SDS at 100 °C. The OMP found in this study was resistant to denaturation and could not migrate through polyacrylamide without the powerful reducing agent 2-mercaptoethanol in the sample buffer. In many respects this was not surprising as the deduced amino acids in this protein contained nine cysteine residues. Furthermore, this *ompM*-derived protein showed antigenic similarity

Fig. 6. Immunofluorescence of strains treated with commercially produced *L. pneumophila* MOMP-specific FITC-labelled monoclonal antibody. (a) *L. pneumophila* N₇; (b) clone LP 116; (c) *E. coli* JM 83 with pUC19. Clone LP 116 showed fluorescence comparable to *L. pneumophila*, whilst the *E. coli* parent strain was negative.

Table 1. LD₅₀ values for *E. coli* JM 83, *L. pneumophila* N₇ and the *E. coli* recombinant inoculated into chicken embryo yolk sacs

Strain and designation	LD ₅₀ endpoint*	Range
Parent <i>E. coli</i> JM 83 with pUC19	1.7 × 10 ⁹	8.8 × 10 ⁸ –3.3 × 10 ⁹
<i>L. pneumophila</i> N ₇	4.6 × 10 ²	3.8 × 10 ² –5.4 × 10 ²
<i>E. coli</i> clone LP 116	2.5 × 10 ⁴	1.9 × 10 ⁴ –3.3 × 10 ⁴

* Ten eggs were inoculated per dilution with 0.1 ml samples and LD₅₀ data were calculated from three separate experiments. Control eggs were injected with PBS and in this group no deaths occurred up to 8 d post-inoculation (300 fertile chicken eggs used in evaluation).

to the *L. pneumophila* outer-membrane antigen which reacted with the MOMP monoclonal antibody. Although it appeared from the present study that the OmpM protein had similar electrophoretic behavioural characteristics to those observed for the MOMP of *L. pneumophila* (Butler & Hoffman, 1990), the sequence data indicated that it was not the same protein as the 28/31 kDa OMP reported by Hoffman *et al.* (1992b). The virulence of the strain in those studies was not recorded; however, it did have an electrophoretic profile similar to an attenuated derivative of the virulent *L. pneumophila* N₇ strain developed in our laboratory (High & Rodgers, 1991). Although the precise role of the MOMP in virulence is unclear, it seems probable that the expression of the OmpM in virulent isolates differs from that in avirulent strains. Whether the MOMP monoclonal antibody reacts with the 28/31 kDa OMP remains to be determined.

The MOMP of *L. pneumophila* was reported to be a porin (Gabay *et al.*, 1985). Bacterial porins have been implicated in the pathogenicity of other Gram-negative bacteria by acting as colicins, by influencing the uptake of molecules which may either benefit the organism or destroy the host cell, as well as by inhibiting phagocytosis (Misra & Benson, 1988; Smarda, 1988; Tufano *et al.*, 1988). Furthermore, porins may be crucial to the survival and growth of some intracellular bacteria within infected cells. The role of porin-like structures in the pathogenicity of *L. pneumophila* remains to be elucidated. At present, there is no evidence that OmpM functions as a porin when cloned and expressed in *E. coli*.

LD₅₀ data derived from the chicken embryo lethality assay showed that the *E. coli* JM 83 parent strain was of low virulence, whereas that of the derived clone was much enhanced (Table 1). Due to the complex nature of the infectious process in whole animals, data from the fertile chicken egg virulence assay must be interpreted with caution. Nonetheless, these results suggested that the presence of the OmpM of *L. pneumophila* may be involved in expression of pathogenicity. LPS of *L.*

pneumophila as well as other Gram-negative bacteria has long been associated with antigenic activity and has been considered a virulence factor *per se*. With the problems encountered during the separation of cell surface material from *Legionella*, it would be difficult to define a single factor involved with expression of pathogenicity of the organism. Because the OmpM appeared to be responsible for an increase in virulence of the LP 116 clone as shown by a decrease in LD₅₀ values for the chick embryo, it would appear that this pathogenic character was not LPS-related. As shown in the OMP immunoblots, protein preparations of all samples contained contaminating amounts of LPS. It was also clear from the immunoblot analysis with polyclonal rabbit antisera that there was no antigenic relationship between the LPS of *E. coli* and that of *L. pneumophila*.

No doubt, several factors are involved in the expression of virulence of this and other pathogenic bacteria. Attachment of this facultative intracellular pathogen to host cells followed by penetration of cell membranes with subsequent intracellular survival and replication may be linked to cell surface composition. However, it is not clear precisely what role this protein plays in infectivity and immunity. The *E. coli* clone LP 116, containing this sequenced *ompM* gene from *L. pneumophila* responsible for the expression of the 25 kDa MOMP, will serve as a useful tool for future studies on the cellular and molecular aspects of the pathogenicity of *L. pneumophila*.

We are grateful to Dr Robert M. Zsigray for helpful discussion, Dr Larry Gosting, Genetics Systems, Seattle, WA, USA, for the unconjugated monoclonal antibody and Christine Rodgers for help in preparing the manuscript. This work, which was supported by a Public Health Service grant AI-27929 from the National Institutes of Health (F.G.R.) and by CURF grants from the University of New Hampshire Office of Sponsored Research (A.S.H.), was presented in part at the 91st General Meeting of the American Society for Microbiology, Dallas, TX, USA, Abstract B-71.

References

- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- BURTON, K. (1968). Determination of DNA concentration with diphenylamine. *Methods in Enzymology* **12B**, 163–165.
- BUTLER, C. A. & HOFFMAN, P. S. (1990). Characterization of a major 31-kilodalton peptidoglycan-bound protein of *Legionella pneumophila*. *Journal of Bacteriology* **172**, 2401–2407.
- BUTLER, C. A., STREET, E. D., HATCH, T. P. & HOFFMAN, P. S. (1985). Disulfide-bonded outer membrane proteins in the genus *Legionella*. *Infection and Immunity* **48**, 14–18.
- DEVEREUX, J., HAEGERLI, P. & SMITHIES, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Research* **12**, 387–395.
- EHRET, W., ANDING, G. & RUCKDESCHL, G. (1984). Characterization of membrane proteins from various strains and serogroups of *Legionella pneumophila* and other *Legionella* species. In *Proceedings of the Second International Symposium on Legionella*, pp. 265–268. Edited by C. Thornsberry, A. Balows, J. C. Feeley & W. Jakubowski. Washington, DC: American Society for Microbiology.

- ENGLEBERG, N. C., DRUTZ, D. J. & EISENSTEIN, B. I. (1984). Cloning and expression of *Legionella pneumophila* antigens in *Escherichia coli*. *Infection and Immunity* **44**, 222–227.
- ENGLEBERG, N. C., CATER, C., WEBER, D. R., CIANCOTTO, N. P. & EISENSTEIN, B. I. (1989). DNA sequence of *mip*, a *Legionella pneumophila* gene associated with macrophage activity. *Infection and Immunity* **57**, 1263–1270.
- GABAY, J. E., BLAKE, E. M., NILES, W. D. & HOROWITZ, M. A. (1985). Purification of *Legionella pneumophila* major outer membrane protein and demonstration that it is a porin. *Journal of Bacteriology* **162**, 85–91.
- GOSTING, L. H., CABRIAN, K., STURGE, J. C. & GOLDSTEIN, L. C. (1984). Identification of a species-specific antigen in *Legionella pneumophila* by a monoclonal antibody. *Journal of Clinical Microbiology* **20**, 1031–1035.
- HIGH, A. S. & RODGERS, F. G. (1991). Electroporation of the major outer membrane protein-encoding genes from virulent to avirulent *Legionella pneumophila*. Abstracts of the General Meeting of the American Society for Microbiology, Dallas, TX, USA, abstract H-9.
- HINDAHL, M. S. & IGLEWSKI, B. H. (1986). Outer membrane proteins from *Legionella pneumophila* serogroups and other *Legionella* species. *Infection and Immunity* **51**, 94–101.
- HITCHCOCK, P. J. (1984). Analyses of gonococcal lipopolysaccharide in whole-cell lysates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis: stable association of lipopolysaccharide with the major outer membrane protein (Protein 1) of *Neisseria gonorrhoeae*. *Infection and Immunity* **46**, 202–212.
- HOFFMAN, P. S., SEYER, J. H. & BUTLER, C. A. (1992a). Molecular characterization of the 28 and 31 kilodalton subunits of the *Legionella pneumophila* major outer membrane protein. *Journal of Bacteriology* **174**, 908–913.
- HOFFMAN, P. S., RIPLEY, M. & WEERTNA, R. (1992b). Cloning and nucleotide sequence of a gene (*ompS*) encoding for the major outer membrane protein of *Legionella pneumophila*. *Journal of Bacteriology* **174**, 914–920.
- HORWITZ, M. A. (1984). Interactions between *Legionella pneumophila* and human mononuclear phagocytes. In *Proceedings of the Second International Symposium on Legionella*, pp. 159–166. Edited by C. Thornsberry, A. Balows, J. C. Feeley & W. Jakubowski. Washington, DC: American Society for Microbiology.
- KADO, C. I. & LIU, S.-T. (1981). Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* **145**, 1365–1373.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**, 208–218.
- MISRA, R. & BENSON, S. A. (1988). Isolation and characterization of *ompC* porin mutants with altered pore size. *Journal of Bacteriology* **170**, 528–533.
- NEWHALL, W. J. & JONES, R. B. (1983). Disulfide-linked oligomers of the major outer membrane protein of chlamydiae. *Journal of Bacteriology* **154**, 998–1001.
- NOLTE, F. S. & CONLIN, C. A. (1986). Major outer membrane protein of *Legionella pneumophila* carries a species-specific epitope. *Journal of Clinical Microbiology* **23**, 643–646.
- OAKLEY, B. R., KIRSH, D. R. & MORRIS, N. R. (1980). A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Analytical Biochemistry* **105**, 361–363.
- OLDHAM, L. J. & RODGERS, F. G. (1985). Adhesion, penetration and intracellular replication of *Legionella pneumophila*: an *in vitro* model of pathogenesis. *Journal of General Microbiology* **131**, 697–706.
- REED, L. J. & MEUNCH, H. (1938). A simple method for determining 50 percent endpoints. *American Journal of Hygiene* **27**, 493–497.
- RODGERS, F. G. (1983). The role of structure and invasiveness on the pathogenicity of *Legionella pneumophila*. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene A* **255**, 138–144.
- RODGERS, F. G. & DAVEY, M. R. (1982). Ultrastructure of the cell envelope layers and surface details of *Legionella pneumophila*. *Journal of General Microbiology* **128**, 1547–1557.
- SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.
- ŠMARDÁ, J. (1988). Colicin K decreases the density of intramembrane particles (IMP) in the cell membrane of *Escherichia coli*. *Journal of General Microbiology* **134**, 1283–1288.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- TSAL, C.-M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- TUFANO, M. A., SOMMESE, L., CAPASSO, C., SCAFFA, F. & GALDIERO, E. (1988). Porin content modifies resistance of *Salmonella typhimurium* to phagocytosis. *European Journal of Epidemiology* **4**, 110–114.
- TZIANABOS, A. O. & RODGERS, F. G. (1989). Pathogenesis and chemotherapy of experimental *Legionella pneumophila* infection in the chick embryo. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene A* **271**, 293–303.
- WANG, L.-M., WEBER, D. K., JOHNSON, T. & SAKAGUCHI, A. Y. (1988). Supercoil sequencing using unpurified templates produced by rapid boiling. *Biotechniques* **6**, 839–843.
- WENMAN, W. M., CHAI, J., LOUIE, T. L., GOUDREAU, C., LIOR, H., NEWELL, D. G., PEARSON, A. D. & TAYLOR, D. E. (1985). Antigenic analysis of *Campylobacter* flagellar protein and other proteins. *Journal of Clinical Microbiology* **21**, 108–112.