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

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(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 the 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-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

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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 N₄, 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 (BBY) and incubated 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) and 1% (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 *SmaI* or *PstI* (IBI) to release the *L. pneumophila* DNA insert. All samples were compared to pUC19, which had been similarly digested with *SmaI* or *PstI*, by agarose gel electrophoresis. Gels were transferred to nitrocellulose filters for Southern blot analysis (Southern, 1975). Genomic DNA from *L. pneumophila* N₄ digested with *HaeIII* was labelled with [α-32P]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 [35S]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 (GGG) Sequence Analysis Software Package (Devereux et al., 1984).

OMP analysis. The outer membranes of *E. coli* JM 83, *L. pneumophila* N₄, 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 Tantobloc 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 Nt, 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 (Note & 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 Nt, E. coli JM 83 or the E. coli clone LP 116 to assess virulence (Tzianabos & Rodgers, 1989). The lethal dose (LD50) for each of these was calculated by the method of Reed & Meutch (1938).

**Results and Discussion**

L. pneumophila chromosomal DNA was partially cleaved with HaeIII, 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.

Restoration 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 promotor sequence for an *L. pneumophila* 28/31 kDa OMP (Hoffman et al., 1992b). This did not appear to be the case for the promotor 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., 1992b), the promoter region 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.

**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.
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 µg 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 immunoblot assays indicated that the protein expressed on the surface of the *E. coli* clone

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, 14 300; β-lactoglobulin, 18 400; ovalbumin, 43 000 and BSA 68 000); 4, *L. pneumophila* N₉.

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* N₉; 4, *E. coli* JM 83 with pUC19; 5, clone LP 116; 6, *L. pneumophila* N₉.

LP 116 was derived from *L. pneumophila* N₉, 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* N₉ 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* N₉, 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* N₉ 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 N.

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 N, 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 N,-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 N; (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. $LD_{90}$ values for E. coli JM 83, L. pneumophila N$_r$, and the E. coli recombinant inoculated into chicken embryo yolk sacs

<table>
<thead>
<tr>
<th>Strain and designation</th>
<th>$LD_{90}$ endpoint*</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>Parent E. coli JM 83 with pUC19</td>
<td>$1 \times 10^9$</td>
<td>$8 \times 10^8 -3 \times 10^9$</td>
</tr>
<tr>
<td>L. pneumophila N$_r$</td>
<td>$2 \times 10^9$</td>
<td>$3 \times 10^8 -5 \times 10^9$</td>
</tr>
<tr>
<td>E. coli clone LP 116</td>
<td>$2 \times 10^9$</td>
<td>$1 \times 10^8 -3 \times 10^9$</td>
</tr>
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* Ten eggs were inoculated per dilution with 0.1 ml samples and $LD_{90}$ 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 N$_r$, 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.

$LD_{90}$ 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 $LD_{90}$ 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. Zsigay 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.

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