RESEARCH LETTER

A Legionella pneumophila collagen-like protein encoded by a gene with a variable number of tandem repeats is involved in the adherence and invasion of host cells

Liesbeth Vandersmissen, Emmy De Buck, Veerle Saels, David A. Coil & Jozef Anné
Laboratory of Bacteriology, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium

Abstract

Legionella pneumophila is a Gram-negative, facultative intracellular pathogen and the causative agent of Legionnaires’ disease, a severe pneumonia in humans. Analysis of the Legionella sequenced genomes revealed a gene with a variable number of tandem repeats (VNTRs), whose number varies between strains. We examined the strain distribution of this gene among a collection of 108 clinical, environmental and hot spring serotype I strains. Twelve variants were identified, but no correlation was observed between the number of repeat units and clinical and environmental strains. The encoded protein contains the C-terminal consensus motif of outer membrane proteins and has a large region of collagen-like repeats that is encoded by the VNTR region. We have therefore annotated this protein Lcl for Legionella collagen-like protein. Lcl was shown to contribute to the adherence and invasion of host cells and it was demonstrated that the number of repeat units present in lcl had an influence on these adhesion characteristics.

Introduction

Legionella pneumophila is a Gram-negative, facultative intracellular pathogen, found worldwide in freshwater systems, where it replicates in various protozoa. Man-made aquatic systems, such as shower heads, whirlpools and air-conditioning systems, are the main sources of human infection. After inhalation of contaminated aerosols, L. pneumophila can replicate in alveolar macrophages and will finally kill and lyse these macrophages and cause severe pneumonia, known as Legionnaires’ disease (Fields, 1996; Fields et al., 2002; Steinert et al., 2007). The outer membrane of Gram-negative bacteria is the site of contact between the bacteria and host cells and outer membrane proteins therefore play an important role in the host–pathogen interaction. In L. pneumophila, several Omps are characterized as important virulence factors, for example Momp or ‘major outer membrane protein’, which plays a role in attachment to host cells (Bellinger-Kawahara & Horwitz, 1990), the heat shock protein Hsp60 (Gardu˜no et al., 1998), important for attachment and invasion of a HeLa cell model, Mip or ‘macrophage infectivity potentiator’, playing a role in intracellular replication (Cianciotto & Fields, 1992), the adhesion molecules LigA (Fettes et al., 2000) and LaiA (Chang et al., 2005), LvgA that would function in resistance mechanisms (Edelstein et al., 2003), and Lpa, the plasminogen activator homologue (Vranckx et al., 2007). Two proteomic maps, showing the outer membrane proteome and proteins present in outer membrane vesicles, also reveal several virulence-related Omps (Galka et al., 2008; Khemiri et al., 2008).

The genus of Legionella comprises approximately 50 species and 15 serogroups (Pourcel et al., 2007). This diversity has led to the development of multiple genotyping methods for epidemiological studies (Cazalet et al., 2004, 2008; Gaia et al., 2005; Pourcel et al., 2007), and variable number of tandem repeats (VNTRs) analysis is one of the methods used for the classification of outbreaks of infectious diseases (van Belkum, 2007). VNTRs represent a single locus showing interindivdual length variability. They are already found in virulence-related genes of L. pneumophila (Newton et al., 2007; D’Auria et al., 2008) and would...
therefore also be an important aspect in host–pathogen interaction.

The VNTR analysis performed at our lab (Coil et al., 2008) identified a gene with a VNTR region that displayed a high homology with eukaryotic collagen. Here, we describe the initial characterization of this L. pneumophila gene, lpg 2644, with a VNTR region, encoding an outer membrane motif and containing a collagen-like repeat region. The gene was therefore annotated lcl (Legionella collagen-like).

Materials and methods

Origin of bacterial strains and growth conditions

The origin of strains and the selection based on sequence-based type (SBT) and repeat pattern are described in detail elsewhere (Coil et al., 2008).

Legionella strains were grown at 37 °C on buffered charcoal yeast extract (BCYE) agar plates or in buffered yeast extract broth supplemented with L-glutamate, l-cysteine and ferric pyrophosphate (Edelstein, 1981), Escherichia coli was grown in Luria–Bertani medium (Miller, 1972), and if necessary, supplemented with ampicillin (50 µg/mL) or chloramphenicol (25 µg mL⁻¹).

Chromosomal DNA isolation

Strains were grown overnight in 5 mL of BCYE. Genomic DNA was isolated from 1 mL of this culture using a Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer’s recommendations. The quality of the DNA was assessed by agarose gel electrophoresis.

Identification of the VNTR region

Standard PCRs were carried out using SuperTaq (HT Biotechnology). PCR amplification of the VNTR region of the lpg 2644 gene was accomplished with the primers 5'-TCACATCAGATACG-3' and 5'-TTCCCCAGCTTACGG-3', designed on the chromosome of L. pneumophila Philadelphia-1. Chromosomal DNA from the different Legionella isolates was used as a template.

Sequencing of the VNTR region

The VNTR DNA fragments of lpg 2644 of all 108 strains were cloned into pGEM-T Easy (Promega), introduced into TG1 competent cells and the constructs were purified using the Wizard Plus SV Miniprep DNA purification system (Promega). The size of the insert was checked through electrophoresis, using the initial PCR product as a reference for size. One clone that contained an insert of the exact size was selected for sequencing. Sequencing reactions were performed on this template DNA at the VIB Genetic Service Facility (Antwerp, Belgium).

Eukaryotic cell lines and growth conditions

Acanthamoeba castellanii ATCC30234 was cultured in Acanthamoeba medium (PYG712) at room temperature. The THP-1 or U937 cell line was differentiated into macrophage-like cells by treatment with phorbol 12-myristate 13-acetate for 72 h in RPMI medium, containing 10% heat-inactivated fetal calf serum and 2 mM l-glutamine, at 37 °C and 5% carbon dioxide (CO₂). The A549 cell line, a lung epithelial cell carcinoma, was maintained in DMEM medium, supplemented with 10% heat-inactivated fetal calf serum and 2 mM pyruvate, at 37 °C and 5% CO₂.

Protein purification and refolding

The lcl gene was amplified from L. pneumophila ATCC33152 genomic DNA and provided with a His₆-tag at its 5'-end using the forward primer 5'-TACATGCAACCAGTACCATCACACATACAAAAGCAATCCGGC-3' and the reverse primer 5'-TAGGATCTTCTGAAAAGGTCTCTTACAGC-3'. This DNA fragment was cloned as a BamHI/NdeI restriction fragment in the shuttle vector pMMBN (Promega) according to the manufacturer’s recommendations. The quality of the DNA was assessed by agarose gel electrophoresis.

Overexpression of the Lcl protein in L. pneumophila

The lpg 2644 gene, containing 19 repeat units of 45 nucleotides, was amplified from the L. pneumophila ATCC33152 genomic DNA with the primer pair 5'-TACATGCAACCAGTACCATCACACATACAAAAGCAATCCGGC-3' and 5'-TAGGATCTTCTGAAAAGGTCTCTTACAGC-3'. This fragment was cloned as an NdeI/EcoRI restriction fragment in the shuttle vector pMMBN and electroporated in L. pneumophila Philadelphia-1 [wild
type (WT)/pMMBNlcl]. Expression of the lcl gene was induced by addition of 100 μM IPTG. Cloning procedures led to a spontaneous recombination of the VNTR region, resulting in an lcl gene containing 14 repeat units designated WT/pMMBNlcl(14).

**Cell fractionation**

Fractionation of *L. pneumophila* Philadelphia-1 cultures was performed as described before (Vranckx et al., 2007). Briefly, the supernatant was concentrated by trichloroacetic acid (TCA) precipitation (20% TCA final concentration) and the cells were lysed in a French pressure cell. To extract the inner membrane proteins from the membranes, the sediment was resuspended in 1.5 mL 10 mM Tris (pH 7.5) containing 1.5% sarkosyl and centrifuged. The outer membrane proteins were resuspended in 500 μL 10 mM Tris, pH 7.5, and 10 mM EDTA, containing 1% Triton X-100.

The quality of the cellular fractions was controlled by testing for the presence of DnaK, a cytoplasmic protein, LepB, an inner membrane protein, and Lpa, an outer membrane protein.

**Adhesion–invasion assays**

WT bacteria grown to the stationary phase were added to a monolayer of A549, macrophage-like cells or *A. castellanii* (5 × 10³ cells per well) at a multiplicity of infection (MOI) of 100. Bacteria overexpressing Lcl were added to a monolayer of A549 or macrophage-like cells also at an MOI of 100. For sampling, after 30 and 60 min, the supernatant was removed and the wells were washed three times with medium to remove the extracellular bacteria. Attached bacteria and those that had already entered the eukaryotic cells were collected following lysis of the host cells with ice-cold distilled water and subsequently quantified by plating serial dilutions onto BCYE agar plates. To investigate the role of Lcl in adhesion and invasion, the experiment was repeated with bacteria (5 × 10⁷ bacteria mL⁻¹) preincubated with Lcl-specific antibodies (20 μg mL⁻¹ bacteria culture) at 37°C for 1 h before they were placed in contact with the eukaryotic cells. As a control, experiments were repeated with a xylanase C (XlnC) antibody. XlnC is a *Streptomyces lividans* secreted protein (Faury et al., 2004) and the XlnC antibodies were of the same isotype and produced under the same conditions as the Lcl-specific antibodies.

Alternatively, for measuring adhesion to host cells, experiments were performed with immobilized purified, refolded Lcl protein. Lcl and BSA (negative control) were immobilized as films on flat-bottomed microtiter 96-well plates (Nunclon) at a concentration of 5 μg per well overnight at 4°C. Films were blocked with 1% BSA, washed with phosphate-buffered saline (PBS), followed by addition of 100 μL of eukaryotic cell suspension (5 × 10⁵ cells mL⁻¹) to each well and incubation at room temperature for 1 h. Nonadherent cells were removed by two washes with PBS, and those that adhered to the films were stained with crystal violet. Plates were read at A595 nm. Additionally, the immobilized films were preincubated with Lcl-specific antibodies (20, 2, 0.2 μg per well) for 30 min on ice before adding the eukaryotic cells.

**Coimmunoprecipitation assays**

Coimmunoprecipitation experiments were carried out using a host cell lysate in combination with refolded Lcl protein. First, pelleted A549 cells or macrophage-like cells were resuspended in solubilization buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 0.2% Triton X-100) and sonicated. Samples (500 μL) of the lysate (0.5 μg μL⁻¹) were incubated with refolded Lcl protein (10 μg in total) for 1 h at 4°C, rotating end over end. Sepharose A powder (10 mg) was added to the 500 μL mixture and further rotated for 1 h at 4°C, followed by centrifugation (5 min, 1000 g). The supernatant was subsequently incubated with Lcl-specific antibodies or complement component C1q receptor (C1qR)-specific antibodies rotating for 1 h at 4°C. This incubation step was followed by addition of 10 mg sepharose A powder again. After 1 h at 4°C, the immunoprecipitates were isolated by centrifugation (5 min, 1000 g) and washed four times with 150 μL solubilization buffer. After resuspension in 2 × SDS loading dye, the samples were boiled and the immunoprecipitated proteins were visualized by immunodetection with Lcl-specific antibodies. As a control, samples containing only lysate and Lcl protein without antibodies and samples only containing antibodies were also incubated with the protein A sepharose powder.

**Statistics**

Statistical analyses were performed using the standard Student t-test with equal variances.

**Results and discussion**

**Characterization of the encoded Lcl protein**

Bacteria have evolved a spectrum of adaptations and sophisticated mechanisms to manipulate host physiology and cellular functions for the bacteria’s own benefit. This adaptation to host cells is reflected in the genome of *L. pneumophila*, which encodes for an abundance of eukaryotic-like proteins (Cazalet et al., 2004).

Lcl is predicted to encode a 49.6 kDa protein with GXY collagen-like repeats. Enzymatic assays were performed to confirm the collagen-like structure. Lcl and rat tail collagen type I reacted in the same way on collagenase and trypsin...
incubation (data not shown). Furthermore, the GXY repeats were encoded by the VNTR region and a change in the number of repeat units had an influence on the number of GXY repeats and consequently on the collagen-like protein structure. Some of the Legionella eukaryotic-like proteins have already proven their role in virulence and show that these eukaryotic-like proteins are putative candidates to play a role in the L. pneumophila pathogenesis (Cazalet et al., 2004). Therefore, the study of eukaryotic-like proteins, such as Lcl, is important to define the survival strategies of this intracellular parasite.

Virulence factors are also often outer membrane proteins or secreted proteins and previous studies have already identified several outer membrane proteins of L. pneumophila that are involved in the adhesion and invasion of host cells (Mintz et al., 1992; Chang et al., 2005; D’Auria et al., 2008).

Different cellular fractions (the cytoplasm, inner membrane, outer membrane and supernatant) were tested for the presence of Lcl. Separation of the cellular fractions by SDS-PAGE, followed by immunodetection with Lcl-specific antibodies, revealed an immunoreactive band in the outer membrane protein fraction and the extracellular fraction (Fig. 1a). Proteins used as a control were present in the expected fractions (Fig. 1b–d). The results of the cellular fractionation demonstrated that Lcl is an outer membrane protein that can also be found in the extracellular fraction. This could be due to the fragmentation of Lcl, situated at the cell surface, into the extracellular space, or Lcl could have an additional function as a secreted protein. Other work has also yielded conflicting results regarding the localization of Lcl (DebRoy et al., 2006; Galka et al., 2008; Khemiri et al., 2008), which is probably due to the different techniques used. As Lcl contains the characteristic C-terminal consensus AAVRAVRAF, with a hydrophilic amino acid only in position 3, the outer membrane localization is most likely.

Strain distribution of lcl

The VNTR region of lcl of all 108 strains was amplified by PCR (see Materials and methods). The resulting PCR fragments of different sizes led to the identification of 12 polymorphisms ranging from 7 to 19 repeats of 45 nt. The repeat distribution of lcl in the 108 strains is bimodal, with a preference for 8 or 13 and 14 repeats (Fig. 2a). For clinical or environmental strains, there was no correlation between the repeat number and the place of origin, because the observed pattern of repeat distribution in clinical and environmental strains was comparable with the overall distribution (Fig. 2b and c). The hot spring isolates, on the other hand, showed almost no variation; only 3 of the 12 occurring polymorphisms were present, with a preference for 14 repeat units (Fig. 2d). It was also observed that strains that had the same seven-gene SBT could still have a different copy number for the lcl VNTR region (data not shown). As addition of the seventh gene neuAc to the previously used six-gene SBT scheme enhanced the discriminatory power (Ratzow et al., 2007), it is possible that additional genes can even further discriminate the presently established SBT types. Therefore, our sequencing data reinforce the observation made by Pourcel et al. (2007) that the VNTR region of lcl (lpms31) is very diverse and could be an additional tool to distinguish among isolates. However, we have to be cautious about its genetic variability. If this genetic variability is extremely large, the chance of two isolates of the same population being different is almost equal to the chance of two isolates of different locations being different. This problematic genetic variability was previously shown for three other L. pneumophila loci: fliC, proA and mompS (Coscolla et al., 2006). This emphasizes that attention has to be paid when selecting a gene for discrimination purposes. More specifically, the genetic variability of the gene has to be high enough to discriminate between different isolates, but small enough to be stable in the period between the outbreak and epidemiological typing.

**Lcl plays a role in L. pneumophila adhesion and invasion of host cells**

Based on the knowledge that mammalian collagen plays a role in cell adhesion (Kadler et al., 2007), the role of Lcl in the adhesion of L. pneumophila Philadelphia to host cells was examined.

For this, we attempted to prepare an Lcl-negative mutant. Notwithstanding many attempts, using (1) homologous recombination with a resistance cassette with flanking regions of the lcl gene of different sizes; (2) point mutagenesis; and (3) different resistance cassettes and shuttle vectors,
Fig. 2. Frequency distribution of the VNTR profiles in (a) the 108 *Legionella pneumophila* isolates, (b) the clinical isolates, (c) the environmental isolates and (d) the hot spring isolates. Above each bar, the number of strains is indicated.

Fig. 3. Functional characterization of Lcl using *Legionella pneumophila* Philadelphia-1 cells (WT) (black bars) and WT cells preincubated with Lcl-specific antibodies (AB) (gray bars) in an adhesion–invasion assay (a) A549, (b) differentiated U937 and (c) Acanthamoeba castellanii cells. (d) WT cells preincubated with XlnC antibodies in an adhesion–invasion assay. The number of adhering cells was determined by crystal violet staining. *P < 0.05 (significantly different vs. the WT control). The results represent the mean of three independent experiments. Statistical analyses were performed using the standard Student t-test with equal variances.
a correct insertion was never obtained, even after screening thousands of colonies. Whether the failure of obtaining a deletion mutant is due to the presence of the VNTR regions present in the lcl gene is not known, but VNTR regions are known to have greater instability.

Because we were not able to obtain an Lcl-negative mutant, the role of Lcl in L. pneumophila adhesion and invasion of host cells was determined indirectly by comparing the Philadelphia WT cells and the same cells preincubated with Lcl-specific antibodies. The preincubation of $5 \times 10^7$ L. pneumophila Philadelphia-1 cells with 20 μg Lcl-specific antibodies for 30 min decreased the adhesion to A549 and macrophage-like cells by 59% ($P = 0.0015$) and 39% ($P = 0.006$), respectively (Fig. 3a and b). In contrast, adhesion and invasion of the amoebae A. castellanii was less affected, because adhesion of WT cells preincubated with Lcl-specific antibodies for 30 min decreased adhesion by only 14% ($P = 0.37$) (Fig. 3c). To ensure that the decreased adhesion and invasion rate was a consequence of the fact that the Lcl antibodies covered Lcl and was not due to possible side effects of the antibodies, experiments were repeated with XlnC antibodies of the same isotype as a control. The results obtained with the latter antibodies showed no difference in the adhesion and invasion of host cells compared with nontreated WT cells (Fig. 3d).

To further exclude that masking other adhesion factors caused by steric hindrance of bound antibodies might be the basis of the abovementioned results, Lcl-adhesion assays were performed with immobilized recombinant Lcl protein. Adhesion of the A549, macrophage-like cells and A. castellanii to the immobilized Lcl protein was influenced by preincubation of the protein film with Lcl-specific antibodies. The use of different antibody concentrations demonstrated that the adhesion was specifically hindered by Lcl-specific antibodies, in an antibody concentration-dependent manner. The A549 cells showed an adhesion of 21% ($P < 0.001$), 80% and 95% using 20, 2 and 0.2 μg Lcl-specific antibodies, respectively (Fig. 4a). The influence on the macrophage-like cell line was less pronounced, with a decrease of only 25% ($P = 0.06$) using 20 μg of Lcl-specific antibodies (Fig. 4b). In contrast, no effect of antibody treatment was seen for the adhesion of A. castellanii to the immobilized film of Lcl, as similar results were obtained for the negative control (coated BSA) (Fig. 4c). In conclusion, the results of these incubation assays with Lcl-specific antibodies suggest that Lcl plays a role in the adhesion process of L. pneumophila.

Identification of Lcl interaction partners

Coimmunoprecipitation experiments were performed to investigate the presence of possible partners on the host cells that interact with Lcl.
The eukaryotic C1qR was suggested to be a possible interaction partner, because it is involved in the phagocytosis of microorganisms. This receptor interacts, for example, with the complement factor C1q and lung surfactant A through binding of the collagen-like region of these proteins, resulting in phagocytosis (Hoppe & Reid, 1994; Grubor et al., 2006). Moreover, the C1qR is present on both cell lines that were shown to interact with Lcl. Coimmunoprecipitation experiments using anti-C1qR antibodies and Lcl antibodies indicated an interaction between the Lcl protein of L. pneumophila Philadelphia and the C1qR of the A549 and the U937 cell line (Fig. 5).

**Number of repeat units has an influence on the adhesion characteristics of Lcl**

The previously described adhesion–infection assays were repeated with lung epithelial cells A549 and macrophage cell line U937 using the IPTG-inducible WT/pMMBNlcl, with 19 repeat units, and the WT/pMMBNlcl(14) strain, with 14 repeat units. The WT/pMMBNlcl(14) strain adhered to and invaded the lung epithelial cells significantly better ($P = 0.02$) than WT/pMMBNlcl after 60 min. Moreover, the two overexpression strains adhered better to and showed higher invasion numbers of the lung epithelial cells compared with the WT strain, with a significant difference ($P = 0.004$) between WT/pMMBNlcl(14) and the WT strain (Fig. 6a). For the macrophage-like cell line, the WT/pMMBNlcl adhered and invaded the macrophage cells significantly better than the WT cells after 60 min ($P = 0.04$). The adhesion and invasion of the WT/pMMBNlcl(14) strain, on the other hand, did not differ significantly from that of the WT strain ($P = 0.26$). Additionally, there was a significantly better adhesion and invasion of WT/pMMBNlcl compared with WT/pMMBNlcl(14) ($P = 0.002$) (Fig. 6b).
Firstly, these observations demonstrate that overexpression of Lcl enhanced the adhesion of *L. pneumophila* to host cells. Secondly, the number of repeat units seemed to be an additional factor for adhesion, and finally, it was observed that the effect of variation in repeat number on adhesion is dependent on the host cell used.

**Conclusion**

Here, we described the characterization of a collagen-like protein encoded by a gene with a VNTR region annotated as Lcl. It was demonstrated that Lcl is involved in *L. pneumophila* host cell adhesion and invasion and interacts with the C1qR. Furthermore, it was observed that the number of repeat units likely influences the adhesion characteristics of the encoded collagen-like protein. However, no correlation was found between clinical strains and number of repeat units and further work is required to elucidate the importance of this collagen-like protein in the virulence of *L. pneumophila*.

**Acknowledgements**

This research was financially supported by Onderzoeksfonds K.U. Leuven (OT/05/62) and Research Foundation – Flanders (FWO) (G.0289.06). DnaK, LepB and Lpa antibodies were kind gifts from Dr P. Mazodier and Dr G. von Heijne and L. Vranckx, respectively. We would like to thank Dr J. Peduzzi for the kind gift of THP-1 cells and Dr R. Quarc for the A549 cells. The Research Group of Dr S. Jarraud, Centre National de Référence des légionelles, Lyon, France, is also acknowledged for performing the sequence-based typing.

**References**


