The Journal of Microbiology, December 2009, p. 768-773
DOI 10.1007/s12275-009-0103-0

Vol. 47, No. 6

Characterization of a Spontaneous Avirulent Mutant of *Legionella pneumophila* Serogroup 6: Evidence of DotA and Flagellin Involvement in the Loss of Virulence

Maria Scaturro[1]*, Stefania Meschini[2], Giuseppe Arancia[2], Fontana Stefano[1], and Maria Luisa Ricci[1]

[1]*Department of Infectious, Parasitic and Immune-Mediated Diseases,*
[2]*Department of Technology and Health, Istituto Superiore di Sanità, 00161-Rome, Italy*

(Received April 1, 2009 / Accepted July 16, 2009)

The pathogenesis of *Legionella pneumophila* mainly resides in its ability to inhibit the phagosome-lysosome fusion, which normally prevents the killing of the host cells. In order to characterize the molecular alterations that occurred in a spontaneous avirulent mutant of *Legionella pneumophila* serogroup 6, named Vir-, we investigated the ability of the mutant to adhere to and multiply in the WI26VA4 alveolar epithelial cell line and in human macrophages, when compared to its parental strain, Vir+. We also determined the co-localization of bacteria with LAMP-1 to gain an insight into the phagosome-lysosome fusion process. Additionally, we determined the flagellin expression and *dot*A nucleotide sequencing. We observed a lack of expression of flagellin and an in-frame mutation in the *dot*A gene. The data obtained strongly suggest the loss of virulence of the mutant could probably be due to the absence of flagellin and the dysfunctional type IV secretion system, resulting from the DotA protein being severely compromised.

***Keywords*:** *Legionella*, pathogenesis, *dot*A, flagellin

Legionella pneumophila, the causative agent of the atypical pneumonia known as Legionnaire's disease, is a Gram-negative facultative intracellular pathogen, widespread in natural aquatic environments, that is able to exploit protozoa as well as macrophages to replicate (Fields *et al.*, 2002; Molmeret *et al*., 2004). *Legionella* after infecting susceptible individuals, through the inhalation of contaminated aerosols, is engulfed by the macrophages within a vacuole that typically does not fuse with lysosomes and maintains near neutral luminal pH (Horwitz, 1983; Horwitz and Maxfield, 1984). Shortly after uptake by coiling phagocytosis, several signals prime a cascade of events, not completely known at molecular level, which alter phagosome trafficking (Roy, 2002). During the 2 h after uptake, the *Legionella*-containing vacuole (LCV) is surrounded by smooth vesicles derived from the endoplasmic reticulum and it becomes a protective replicative niche (Roy *et al*., 1998).

The intracellular life cycle of *L. pneumophila* is typically an alternation of the replicative and transmissive phases. The latter phase is specifically characterized by expression of virulence traits and motility (Dietrich *et al*., 2001). The type IV secretion system encoded by the *dot*/*icm* locus is known to play a fundamental role in creating the permissive organelle for the replication inside phagocytic host cells (Brand *et al*., 1994; Roy, 2002; Bitar *et al*., 2004; Chen *et al*., 2004). Recently, a lot of effector proteins have been identified as substrates translocated by components of the Dot/Icm secretion system and found on the outer surface of the LCV (Murata *et al*., 2006; Ninio and Roy, 2007).

We found a spontaneous avirulent mutant, here after named as Vir-, obtained from a *L. pneumophila* serogroup 6 clinical strain called Vir+, by multiple passages on agar plates. Stable loss of virulence was demonstrated by inoculating the Vir- strain in suckling mice, adult A/J mice and guinea pigs (Castellani Pastoris *et al.,* 1997, where Vir- is referred as "Monza 3p50"). The Vir- strain showed a fatty-acid profile characterized by a lower proportion of the cyclopropane 17 (17cyC) with respect to the Vir+ strain (Castellani Pastoris *et al*., unpublished data), and on the agar plates formed colonies of different morphologies characterized by the absence of satellite micro-colonies. Here we report the results of a study concerning the characterization of both functional and genetic factors of the Vir- mutant and present evidence of the role of flagellin and DotA proteins in the loss of virulence by the *L. pneumophila* mutant.

Materials and Methods

Bacterial strains

L. pneumophila strains used in this study were clinical isolates of *L. pneumophila* serogroup 6 (Vir+) and its avirulent spontaneous mutant (Vir-). For all assays, bacteria were grown on buffered activated charcoal and yeast extract (BCYE) agar plates (Oxoid, France) at 37°C with 2.5% CO_2 for 48 h. The single colonies were sub-cultured in (ACES)-buffered yeast extract (AYE) broth until the post-exponential phase corresponding to an optical density (OD)

* To whom correspondence should be addressed.
(Tel) 390649902856; (Fax) 390649387112
(E-mail) maria.scaturro@iss.i

Table 1. Oligonucleotide primers

Primer	Sequence	Source
flaA F	GCACTTTCGGAACTAAAG	This study
flaA R	TGTTTGAGCTGCCCTATTGAC	This study
5'-UTRflaA F	GCCAGCTCATAAATTAGAACCCAG	This study
5'-UTRflaA R	GGGCTGTGAGCGACGCCACATTAG	This study
rt-pcr flaA F	CAGTATCGGCAGCATAAAAG	This study
rt-pcr flaA R	GTCACAGTTAAACCGTCAG	This study
dotA F1	GATCGCATTGGTACTAGCCT	This study
dotA R1	GGTCAACGGTTTTATTCCCG	This study
dotA F2	TTGCCTTGGCCGTATGATGG	This study
dotA R2	GCTTCCTCACCCCATTGCAT	This study
dotA F3	GTGTGGATTTTGAATGCCGG	This study
dotA R3	TAATAAGAAGGGCTCCTGCG	This study
16S F	CGTAGAGATCGGAAGGAACACC	This study
16S R	GCGTGGACTACCAGGGTATCT	This study

Note: F, forward; R, reverse; UTR, Un Translated Region; rt-pcr, reverse transcription-PCR

at 600 nm of >2.8.

Cell cultures

WI26A4 type I pulmonary epithelial cell line and human macrophages were used both in adhesion and intracellular growth experiments. WI26VA4 cells were cultured in F12K medium (Gibco, UK) containing 10% foetal bovine serum (FBS) (Gibco). Human peripheral blood monocytes were isolated by Lympholyte® -H gradient centrifugation, according to manufacturer's instructions (Cedarlane, Canada) and induced to differentiate into macrophages over 6 days of culture in RPMI 1640 (Gibco) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Gibco) antibiotic solution, at 37°C with 5% CO_2.

Genomic DNA extraction and PCR

Genomic DNA from Vir+ and Vir- strains was extracted according to standard protocols, including hexadecyltrimethyl ammonium bromide precipitation (CTAB) (Sambrook and Russell, 2001).

Gene-specific oligonucleotide primers, listed in Table 1, were made by Primm s.r.l. (Italy). Amplicons corresponding to cDNAs were purified with QIAquick PCR Purification kit (QIAGEN, Germany) and the sequences were determined by BigDye terminator reaction kit and analysed with 310 ABI DNA automated sequencer (Applied Biosystems, USA).

Reverse-Transcription (RT)-PCR analysis

Total RNA was extracted from Vir+ and Vir- strains at the late exponential phase (OD 2 at 600 nm) by using the QIAGEN RNA/DNA Mini kit (QIAGEN), as recommended by the manufacturer. Purified RNA was incubated with 1 U/μg of RNase-free DNase (Fermentas Life Science, Germany), at 37°C for 60 min and then re-purified. The RNA concentrations were determined by absorbance at 280 nm. RT-PCRs were performed with SuperScript OneStep RT-PCR with Platinum Taq (Invitrogen, UK), according to the manufacturer's instructions, by using the "rt-pcr flaA F" and "rt-pcr flaA R" primer pairs (Table 1) and 100 ng of total RNA, at 50°C for 30 min. Reverse transcription reactions were followed by 30 cycles, with cycle duration of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. Purified RNAs were analyzed for genomic DNA contamination, performing a PCR reaction with the same primers but adding 0.5 U *Taq* polymerase (Applied BioSystems).

Adherence assay

WI26VA4 epithelial cells and human macrophages were placed on 24-well tissue culture plates at a concentration of 6×10^5 cells/well, in triplicate. Before plating, human macrophages were washed 3 times with 1× phosphate-buffered saline (PBS) to remove the antibiotics. Cell monolayer was treated for 30 min with 1 μg Cytochalasin D (Sigma-Aldrich, USA), and then washed with PBS. Vir+ and Vir- bacteria were then added at MOI of 100. The 24-well plates were centrifuged at 1,000×g for 5 min and then incubated for 60 min at 37°C in 5% CO_2. After incubation, culture medium was removed and the cells were lysed with 1 ml sterile distilled water. Total bacterial CFU was determined for both Vir+ and Vir- strains, by serial dilutions cultured on BCYE-agar plates. Bacterial concentration corresponding to the MOI of 100 was calculated with an optical density of 600 nm and verified by counting CFU on BCYE-agar plates. The degree of adhesion was expressed as previously described (Stone and Abu Kwaik, 1998) and statistical significance was assessed by the Student's T-test.

Intracellular multiplication assay

WI26VA4 cells and human macrophages were also used to determine the Vir+ and Vir- intracellular multiplication. In this case, bacteria were added at a MOI of approximately 0.1. The plates were centrifuged at 1,000×g for 5 min and then incubated at 37°C for 60 min in 5% CO_2. The monolayer was then washed with PBS, incubated with 50 μg/ml gentamicin solution for 60 min and then rewashed to remove the antibiotic. The cells were lysed immediately (time=0) and after 1, 2 or 3 days of infection to determine the CFU.

Phagosome-lysosome fusion assay

Human macrophage suspensions, at a density of 8×10^4 were placed on coverslips in 24-well tissue culture plates and infected with Vir+ and Vir- strains at MOI of 30. After 30 min of incubation at 37°C in 5% CO_2, bacteria outside the cells were removed with PBS washes, and fresh tissue culture medium was added. The plates were kept in the incubator at 37°C for 2 h, and then fixed in 1% paraformaldehyde (P-PFA) for 30 min. The cells were permeated by immersing coverslips in ice-cold methanol for 10 sec and then treated with PBS 2% bovine serum albumin (BSA) (Sigma) for 1 h. Lysosomes were immunostained with human LAMP-specific monoclonal antibody CD107 (Sigma) (1:500) followed by TRITC-labeled goat anti-mouse secondary antibody (1:500) (Sigma-Aldrich). Bacteria were stained with anti-*L. pneumophila* serogroup 6 rabbit polyclonal antibody (1:4,000), followed by FITC-labeled anti-rabbit secondary antibody (1:80) (Sigma-Aldrich). All antibody dilutions were made in PBS buffer. Coverslips were dried and inverted onto 2 μl of mounting media before viewing by confocal microscopy. Slides were washed several times in PBS and

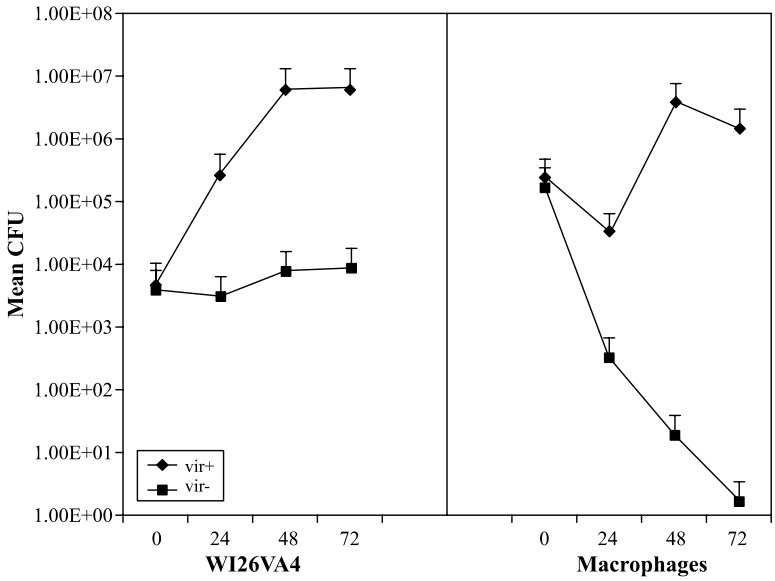

Fig. 1. Growth of Vir+ and Vir- strains in WI26VA4 cells (A) and in human macrophages (B), are shown. The mean CFU±SD was determined in triplicate wells, at 0, 24, 48, and 72 h after infection. The results are representative of 3 independent experiments.

mounted in buffered PBS-glycerine (1:1) solution and observations were performed using a Leica TCS SP2 spectral confocal microscope equipped with argon-helium neon (Ar-HeNe) lasers. The excitation wavelengths were 488 nm for fluorescein staining and 555 nm to evaluate rhodamine; emission lines were collected after passage through a DD488/543 filter in a spectral window ranging from 515~700 nm. Signals from different fluorescent probes were taken in sequential scan mode, which permits the elimination of channel cross-talk and co-localization, were detected in an overlay model. Acquisition parameters were as follow: 63.0/1.4 NA objective; image size, 1,024×1,024; pinhole size, 1 Airy; step size, 0.5 μm. Images were processed by using LCS (Leica Microsystems, Heidelberg GmbH, Germany) and Photoshop (Adobe System Inc., USA) software. The percentage of co-localization with LAMP-1 was determined by scoring at least 100 bacteria of each strain.

Western blot analysis

To analyze flagellin expression, total protein extracts were prepared from Vir+ and Vir- strains at post-exponential growth phase. Equal amounts of protein extract (10 μg) were separated on 12% SDS-PAGE mini-gel and then transferred to nitrocellulose membrane for immunoblot analysis, by using an anti-flagellum serum adsorbed with Vir- strain (Ricci *et al.*, 2005). The serum was diluted 1:500 in PBS 2% BSA. To visualize DotA in Vir+ and Vir- strains, proteins from whole cells and supernatant fraction were prepared as described by Nagai and Roy (2001). For immunoblot analysis, proteins were probed with the monoclonal antibody mAb 2.29 (kindly supplied by C. Roy), diluted 1:500 in PBS.

Results and Discussion

Although it is known that *L. pneumophila* serogroup 6 is able to cause illness in susceptible individuals, currently there is no experimental evidence that describes intracellular behavior for the serogroup 6. Therefore, to determine the molecular mechanisms that caused the loss of virulence of the *L. pneumophila* serogroup 6 Vir- mutant, we investigated 3 important phases of the bacterial infection process in the WI26VA4 alveolar epithelial cell line and human macrophages, such as the adhesion, intracellular multiplication, and phagosome trafficking.

Adherence and intracellular multiplication of Vir+ and Vir- strains

We determined the percentage of adherence for Vir+ and Vir- strains for the WI26VA4 cell line and human macrophages, as an average from 3 different experiments. The percentage adherence of Vir+ bacteria was 3.42% for the WI26VA4 cells and 1.34% for the macrophages; conversely, only 0.25% and 0.3% of Vir- bacteria adhered to the mentioned host cells, respectively. These data highlighted a significant decrease in the ability of the Vir- mutant to adhere to host cells when compared to Vir+ strain.

The WI26VA4 cell line and human macrophages were also used for intracellular multiplication assays. Fig. 1A and B show the CFU obtained from Vir+ and Vir- bacteria, during the 3-day infection period. As shown in Fig. 1A, it was apparent that the Vir- strain survived, but poorly replicated within the WI26VA4; however, was killed by the human macrophages, shown in Fig. 1B. The Vir+ strain however, was able to grow well in the WI26VA4 and in human macrophage host cells.

Flagellin expression and determination of *fla*A gene sequence

We have previously observed by electron microscopy (Ricci *et al.*, 2005), the absence of flagellum in the Vir- strain. In

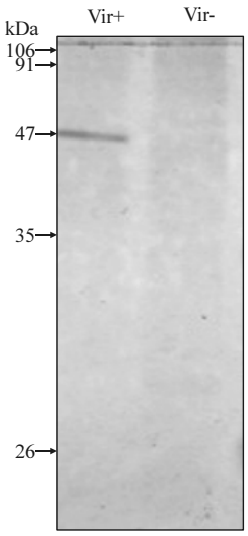

Fig. 2. Flagellin expression was determined on the Vir+ and Vir- total protein extracts by electrophoresis on SDS-PAGE followed by immunoblot analysis. The flagellin was probed with adsorbed anti-flagellum polyclonal serum and a 47 kDa band was visualized. Molecular weights are shown on the left of the Fig.

order to investigate the reason for this absence, we first determined by Western blot analyses the expression of flagellin, the major subunit of *L. pneumophila* flagellum. In Fig. 2, the 47 kDa band corresponding to *Legionella* flagellin in the Vir+ protein extracts can be seen, while there is an absence of the Vir-. The *fla*A gene encoding flagellin was later amplified from genomic DNA extracted from both strains and an amplicon of similar length was obtained for each DNA. The nucleotide sequences of the Vir+ and Vir- amplicons were determined on both strands. The amplicons were then compared with each other, and then to those of database *fla*A sequences. Vir- *fla*A sequences were not any different from those obtained for Vir+ *fla*A gene. Both Vir- and Vir+ *fla*A sequences showed just some conservative differences compared to *fla*A sequences present on the database (nucleotide sequence accession number: AJ496382). The *fla*A gene inter-spacer regions, including *fla*A promoter and ribosome binding site (RBS) regions, were also amplified and the amplicons analyzed by sequencing. These sequences showed 100% identity also when compared with the promoter and RBS regions from gene bank. The data (data not shown) suggest that, although flagellin is not expressed in Vir- strain, the sequence of the gene is conserved and therefore the absence of protein seems to be due to a dysregulation of the gene. To investigate the flagellin expression at transcriptional level, RT-PCR analysis on total RNA from both Vir+ and Vir- strains was performed. Although equal amounts of RNA were used, as shown in Fig. 3B and C, no *fla*A transcript was detectable in Vir- strain. In the Vir+ strain, an amplification product of the expected size (about 300 bp) was detected, shown in Fig. 3A. These data indicate that the Vir- strain could have a dysregulation of *fla*A gene at transcriptional level, in the absence of any significant mutation or deletion of the gene.

Adhesion and intracellular multiplication assays confirm that the Vir- strain is similar to a type II C mutant, according to the Horwitz' s designation (Horwitz, 1987). Although it is known that attachment of *Legionella* to epithelial cells and human macrophages is predominantly due to the *pilin* protein, encoded by the *pil*E gene (Stone and Abu Kwaik, 1998), we attribute the decreased capability of Vir- strain to adhere to the host cells due to the absence of flagellum and, consequently, of the motility. Partially to support our hypothesis, by sequencing the *pil*E gene from both the Vir+ and Vir- genomic DNA, no mutation of the Vir- *pil*E gene had been observed (data not shown). Even though the mechanisms are not completely understood (Dietrich *et al.*, 2001; Heuner and Steinert, 2003), it is known that the flagellum positively affects the early phase of *Legionella* infection, allowing the invasion of host cells. On the other hand, during the replicative phase bacteria are non-motile, while they become motile in the later stages of infection (transmissive phase), when they are already inside the host cells and before the cellular lysis, suggesting a strict association between flagellum expression and infection process (Pruckler *et al.*, 1995).

Identification of a mutation in the *dot*A gene and investigation of phagosome trafficking

Data from intracellular multiplication assays suggested severe damage to the Vir- phagosomal maturation pathway, because the ability to alter the host cell defenses, escaping the phagosome-lysosome fusion (Shin *et al.*, 2008), is crucial for *Legionella* pathogenesis. It has been demonstrated that immediately after the interaction with target host cells, *Legionella* Dot/Icm secretion system is required for the efficient formation of the replicative vacuole (Roy *et al.*, 1998;

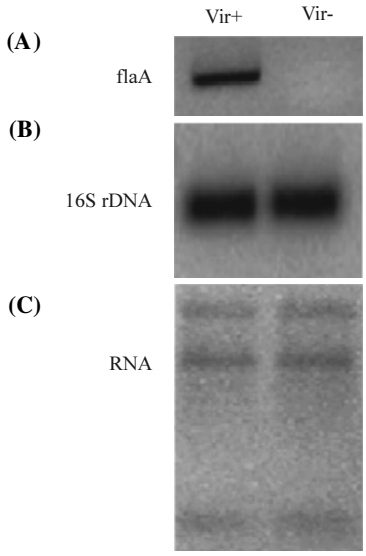

Fig. 3. Vir+ and Vir- total RNA was reverse-transcribed and a region of 300 bp of *fla*A cDNA amplified with specific oligonucleotide primers (A). 16S rDNA was also assayed as control constitutively expressed (B). The relative amount of total RNA in each sample was demonstrated by ethidium bromide staining (C).

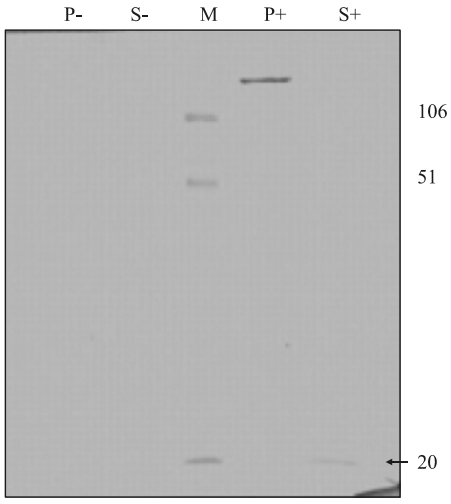

Fig. 4. Immunoblotting analysis was performed to detect DotA. Proteins from whole cells and supernatant fractions of Vir+ and Vir- strains were separated by electrophoresis on 12% acrylamide gel. P- and S- represent proteins from the cellular pellets and from the supernatant fraction of Vir- strain, respectively. P+ and S+ represent proteins from the cellular pellets and from the supernatant fraction of Vir+ strain, respectively. The DotA protein was probed with the monoclonal antibody mAb2.29. The small peptide derived from DotA is indicated by an arrow. Molecular weights of the marker (M) are indicated on the right of the Fig.

Isberg *et al.*, 2009). As *L. pneumophila* requires DotA to regulate initial phagosome trafficking decisions (Roy *et al.*, 1998), we analyzed the nucleotide sequence of *dot*A gene. Amplicons of about 1,750 bp, corresponding to the 5' region, and the other two different amplicons, each of about 400 bp, separated by a small gap, corresponding to the 3' region, were obtained for both Vir+ and Vir- strains and sequenced both on forward and reverse strands. The *dot*A consensus sequences were aligned and compared with each other and with *dot*A sequences present on the database. Comparison between Vir- and Vir+ *dot*A sequences reveals a C→T nucleotide transition at the position 1006. This in frame transition introduces a stop codon in protein translation, at the amino acidic position 301. Moreover, the expression of DotA protein was determined by Western blot analysis. Protein extracts from whole cells and supernatant fractions of Vir+ and Vir- cultures were probed for DotA using the monoclonal antibody mAb2.29 (Matthews and Roy, 2000), produced against the C-terminus end of DotA, and a band size corresponding to the DotA protein (113 kDa) was evidenced only in the Vir+ protein extracts (Fig. 4). The small protein derived from DotA previously observed by Nagai and Roy (2001), was also evidenced in the Vir+ supernatant fraction (Fig. 4, arrow). The lower intensity of the band corresponding to this small protein was likely due to a slightly lower protein concentration.

The rapid accumulation of the lysosomal glycoprotein LAMP-1 is a marker for phagosomes containing *dot*A mutants (Roy *et al*., 1998). Therefore, we investigated the phagosome trafficking in Vir- and Vir+ by co-localization of bacteria (stained with FITC-conjugated secondary antibody)

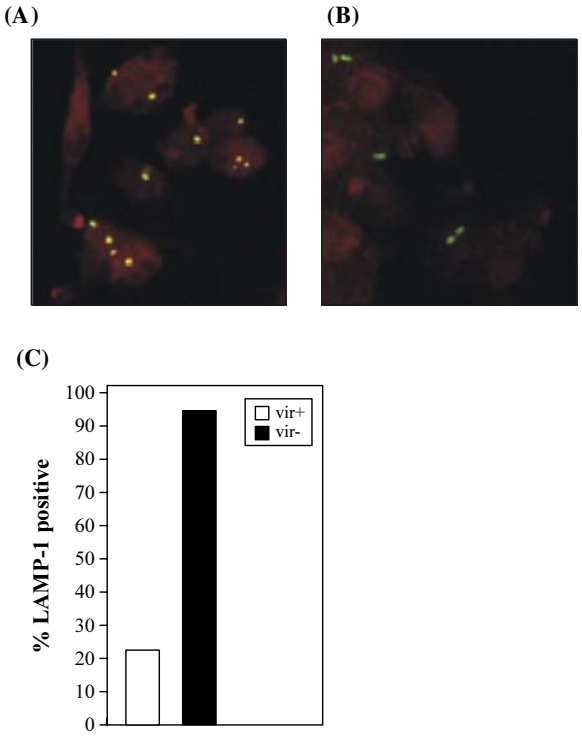

Fig. 5. Confocal immunofluorescent images showed the co-localization of LAMP-1 with phagosomes containing Vir- (A) and Vir+ strain (B). The co-localization of LAMP-1 with phagosomes containing Vir- bacteria is characterized by the orange staining. The percentage of positive phagosomes for LAMP-1 staining (C) is also reported for each strain.

with lysosomes (stained with TRITC-conjugated secondary antibody) visualized by confocal microscopy. In Fig. 5A, the co-localization of Vir- strain with LAMP-1 positive lysosomes results in a typical orange color as shown. On the contrary, phagosomes harboring Vir+ strain did not show appreciable LAMP-1 staining, resulting in a green color, as shown in Fig. 5B. Scoring at least 100 phagosomes for each strain, we calculated that about 95% of the Vir- bacteria were found in the lysosomes, shown in Fig. 5C. The evident localization of Vir- bacteria in the lysosomes implicates the inability of this strain to avoid the lysosomal pathway.

DotA is a large inner membrane protein of 113 kDa, with 2 periplasmic and 8 hydrophobic transmembrane domains, highly conserved among *dot*A alleles encoded by other *L. pneumophila* serogroups, suggesting an important role for the folding of this secreted protein (Nagai and Roy, 2001). The very precocious transition occurred in the *dot*A gene sequence of the Vir- strain, which probably severely damaged the correct folding of the DotA protein, making it dysfunctional and possibly determined its degradation.

Roy and Isberg (1997) have demonstrated that *dot*A internal deletions damage normal membrane topology and, in particular, deletions of a large C-terminus region compromise the DotA function. On the other hand, the intracellular growth defect of *dot*A-induced mutants may have varying severity depending on the altered nucleotide position, and 1006 position of alteration was a key in preventing fusion,

as previously demonstrated by Berger *et al.* (1994).

It is well documented that *L. pneumophila*'s survival and replication inside macrophages are strongly linked to its ability to manipulate host cell processes, in order to create an intracellular replicative niche. *Legionella* modulates the expression of host cell apoptotic and anti-apoptotic genes to prevent the premature termination of its intracellular replication. In particular, the presence of flagellin and a competent Dot/Icm secretion system are critical for *Legionella* to regulate the apoptotic process (Amer *et al.*, 2006; Molofsky *et al.*, 2006; Abu-Zant *et al.*, 2007; Santic *et al*., 2007; Shin and Roy, 2008). In this context, the loss of virulence of Vir- strain should give an explanation on how severely damaged the type IV secretion system was, and the absence of flagellin signal could determine the de-regulation of the apoptotic process.

In conclusion, this work highlights the intracellular behavior of *L. pneumophila* serogroup 6. We showed that it is substantially similar to that known for the serogroup 1. Furthermore, although the avirulence of the Vir- mutant is most probably also due to other molecular alterations, we believe the absence of the flagellin protein and a severely damaged type IV secretion system, because of the absence of the DotA protein, strongly suggest a loss of virulence.

References

Abu-Zant, A., S. Jones, R. Asare, J. Suttles, C. Price, J. Graham, and Y.A. Kwaik. 2007. Anti-apoptotic signalling by the Dot/Icm secretion system of *L. pneumophila*. *Cell. Microbiol.* 9, 246-264.

Amer, A., L. Franchi, T.D. Kanneganti, M. Body-Malapel, N. Ozoren, G. Brady, S. Meshinchi, R. Jagirdar, A. Gewirtz, S. Akira, and G. Nunez. 2006. Regulation of *Legionella* phagosome maturation and infection through flagellin and host IPAF. *J. Biol. Chem.* 46, 35217-35223.

Berger, K.H., J.J. Merriam, and R.R. Isberg. 1994. Altered intracellular targeting properties associated with mutations in the *Legionella pneumophila dot*A gene. *Mol. Microbiol.* 14, 809-822.

Bitar, D.M., M. Molmeret, and Y. Abu Kwaik. 2004. Molecular and cell biology of *Legionella pneumophila*. *Int. J. Med. Microbiol.* 293, 519-527.

Brand, B.C., A.B. Sadosky, and H.A. Shuman. 1994. The *Legionella pneumophila* icm locus: a set of genes required for intracellular multiplication in human macrophages. *Mol. Microbiol.* 14, 797-808.

Castellani Pastoris, M., E. Proietti, C. Mauri, P. Chiani, and A. Cassone. 1997. Suckling CD1 mice as an animal model for studies of *Legionella pneumophila* virulence. *J. Med. Microbiol.* 46, 647-655.

Chen, J., K.S. de Felipe, M. Clarke, H. Lu, O.R. Anderson, G. Segal, and H.A. Shuman. 2004. *Legionella* effectors that promote nonlytic release from protozoa. *Science* 303, 1358-1361.

Dietrich, C., K. Heuner, B.C. Brand, R.J. Hacke, and M. Steinert. 2001. Flagellum of *Legionella pneumophila* positively affects the early phase of infection of eukaryotic host cells. *Infect. Immun.* 69, 2116-2122.

Fields, B.S., R.F. Benson, and R.E Besser. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin. Microbiol. Rev.* 15, 506-526.

Heuner, K. and M. Steinert. 2003. The flagellum of *Legionella pneumophila* and its link to the expression of the virulent phenotype. *Int. J. Med. Microbiol.* 293, 133-143.

Horwitz, M.A. 1983. The Legionnaires' disease bacterium (*Legionella pneumophila*) inhibits phagosome-lysosome fusion in human monocytes. *J. Exp. Med.* 158, 2108-2126.

Horwitz, M.A. 1987. Characterization of avirulent mutant *Legionella pneumophila* that survive but do not multiply within human monocytes. *J. Exp. Med.* 166, 1310-1328.

Horwitz, M.A. and F.R. Maxfield. 1984. *Legionella pneumophila* inhibits acidification of its phagosome in human monocytes. *J. Cell. Biol.* 99, 1936-1943.

Isberg, R.R., T.J. O'Connor, and M. Heidtman. 2009. The *Legionella pneumophila* replication vacuole: making a cosy niche inside host cells. *Nat. Rev. Microbiol.* 7, 13-24.

Matthews, M. and C.R. Roy. 2000. Identification and subcellular localization of the *Legionella pneumophila* IcmX protein: a factor essential for establishment of a replicative organelle in eukaryotic host cells. *Infect. Immun*. 68, 3971-3982.

Molmeret, M., D.M. Bitar, L. Han, and Y.A. Kwaik. 2004. Cell biology of the intracellular infection by *Legionella pneumophila*. *Microbes Infect.* 6, 129-139.

Molofsky, A.B., B.G. Byrne, N.N. Whitfield, C.A. Madigan, E.T. Fuse, K. Tateda, and M.S. Swanson. 2006. Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J. Exp. Med.* 203, 1093-1104.

Murata, T., A. Delprato, A. Ingmundson, D.K. Toomre, D.G. Lambright, and C.R. Roy. 2006. The *Legionella pneumophila* effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. *Nat. Cell Biol.* 8, 971-977.

Nagai, H. and C.R. Roy. 2001. The DotA protein from *Legionella pneumophila* is secreted by a novel process that requires the Dot/Icm transporter. *EMBO J*. 20, 5962-5970.

Ninio, S. and C.R. Roy. 2007. Effector proteins translocated by *Legionella pneumophila*: strength in numbers. *Trends Microbiol.* 15, 372-380.

Pruckler, J.M., R.F. Benson, M. Moyenuddin, W.T. Martin, and B.S. Fields. 1995. Association of flagellum expression and intracellular growth of *Legionella pneumophila*. *Infect. Immun.* 63, 4928-4932.

Ricci, M.L., A. Torosantucci, M. Scaturro, P. Chiani, L. Baldassarri, and M. Castellani-Pastoris. 2005. Induction of protective immunity by *Legionella pneumophila* flagellum in an A/J mouse model. *Vaccine* 23, 4811-4820.

Roy, C.R. 2002. The Dot/Icm transporter of *Legionella pneumophila*: a bacterial conductor of vesicle trafficking that orchestrates the establishment of a replicative organelle in eukaryotic hosts. *Int. J. Med. Microbiol.* 291, 463-467.

Roy, C.R., K.H. Berger, and R.R. Isberg. 1998. *Legionella pneumophila* DotA protein is required for early phagosome trafficking decisions that occur within minutes of bacterial uptake. *Mol. Microbiol.* 28, 663-674.

Roy, C.R. and R.R. Isberg. 1997. Topology of *Legionella pneumophila* DotA: an inner membrane protein required for replication in macrophages. *Infect. Immun.* 65, 571-578.

Sambrook, J. and D.W. Russell. 2001. Molecular cloning: a laboratory manual, 3th ed. Cold Spring Laboratory Press, Cold Spring Harbor, New York, N.Y., USA.

Santic, M., R. Asare, M. Doric, and Y. Abu Kwaik. 2007. Host-dependent trigger of caspases and apoptosis by *Legionella pneumophila*. *Infect. Immun.* 75, 2903-2913.

Shin, S. and C.R. Roy. 2008. Host cell processes that influence the intracellular survival of *Legionella pneumophila*. *Cell. Microbiol.* 10, 1209-1220.

Stone, B.J. and Y. Abu Kwaik. 1998. Expression of multiple pili by *Legionella pneumophila*: identification and characterization of a type IV pilin gene and its role in adherence to mammalian and protozoan cells. *Infect. Immun.* 66, 1768-1775.