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Recombinant expression of Mimivirus L725 ORFan gene product

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Summary. – Mimivirus was the first discovered amoebal giant virus. The Mimivirus virions are covered by a dense layer of approximately 130 nm-long fibers, the length and shape of which diverge from those of other viruses. Here, we aimed at expressing the L725 protein to further confirm and study its role as a fiber-associated protein. We report *Escherichia coli* expression of the L725 protein, which is encoded by a Mimivirus ORFan, was previously identified by proteomics in purified viral fibers and demonstrated to be a fiber-associated protein by RNA-silencing experiments. The expressed protein was recognized by anti-Mimivirus fiber or anti-Mimivirus L725 polyclonal antibodies. This study is the only expression, to our knowledge, of a product from a Mimiviral ORFan gene.

Keywords: Mimivirus; giant virus; fiber; ORFan; gene expression

Introduction

Mimivirus was the first amoebal giant virus discovered, in 2003, and it founded the family *Mimiviridae,* which currently encompasses dozens of members (La Scola *et al.*, 2003; Raoult *et al.*, 2004; Aherfi *et al.*, 2016). These viruses have giant capsids and genomes. In addition, their genomes harbor considerable proportions of genes encoding putative proteins of unknown function and ORFans (Raoult *et al.*, 2004; Boyer *et al.*, 2010; Kuznetsov *et al.*, 2010). Thus, in Mimivirus, such genes account for 54% of the gene repertoire. Moreover, among the 114 proteins detected in the virion, 57% are hypothetical and 39% are encoded by ORFans (Renesto *et al.*, 2006).

One specific feature of Mimivirus is a dense layer of fibers, the morphology and size of which differ from those in other viruses. These fibers are ~125–140 nm in length and ~1.4 nm in diameter, cover the entire capsid except for one of its vertices (Xiao *et al.*, 2009; Klose *et al.*, 2010; Kuznetsov *et al.*, 2010), and have been described as glycosylated and resisting protease and collagenase treatment (Xiao *et al.*, 2009; Boyer *et al.*, 2011). Culturing of Mimivirus 150 times in germ-free amoebas led to the emergence of a strain devoid of fibers, along with a 16% reduction of the viral genome (Boyer *et al.*, 2011). This Mimivirus strain exhibited reduced pathogenicity for its amoebal host and resistance to infection by virophages, which suggested the role of fibers in both host and virophage infections.

Several Mimivirus proteins have been involved in the formation of these fibers, although their precise structure and composition has not been fully resolved (Boyer *et al.*, 2011; Klose *et al.*, 2015). The 224-amino acid-long product of ORFan gene L725 (YP_003987254) was among the three Mimivirus proteins, with R135 (a putative GMC-type oxidoreductase) and L829 (a hypothetical protein), identified in purified fibers by 2D-gel electrophoresis coupled with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Boyer *et al.*, 2011). We previously silenced the genes encoding these proteins using short interfering RNA, which led to reduction in size

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Abbreviations: FAPs = Mimivirus fiber-associated proteins; MALDI-TOF MS = matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PAGE = polyacrylamide gel electrophoresis; Trx = thioredoxin

or deformation of the Mimivirus fibers (Sobhy *et al.*, 2015). We therefore proposed that these proteins could be annotated as Mimivirus fiber-associated proteins (FAPs). Here, we aimed at expressing the L725 protein to further confirm and study its role as a fiber-associated protein. We report the expression of the L725 protein.

Materials and Methods

For protein expression, the Mimivirus L725 gene was cloned into a Gateway pETG20A expression vector (Invitrogen, USA). This vector (obtained from Renaud Vincentelli, AFMB, Marseille, France) included thioredoxin (Trx), a hexahistidine-tag and *Tobacco etch virus* protease, and was used to transform the *Escherichia coli* Rosetta strain. *E. coli* was then grown at 37°C overnight in Luria-Bertani medium at a 1:20 ratio, moved to ZYP media (with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol), kept at 37°C until an optical density corresponding to 600 nmol/l was reached, then at 17°C overnight. Thereafter, bacteria were centrifuged (3,000 x g, 4°C, 10 min), re-suspended in a lysis buffer (150 mmol/L NaCl, 0.25 mg/ml lysozyme, 0.1 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml DNase I and 20 mmol/l $MgSO_4$) and stored at -80°C for ≥1 h. Frozen cultures were then incubated at 37°C, and bacterial debris were removed by centrifugation (12,000 x g, 30 min, 4°C). Lastly, protein purification was performed on a nickel-affinity column and by size-exclusion chromatography (ÄKTA avant 25 chromatography system (GE Healthcare, USA)). The protein concentration was measured with a NanoDrop instrument (Thermo Scientific, USA).

For the polyacrylamide gel electrophoresis (PAGE), a mix consisting of 20 µl of sample and 40 µl of loading buffer (Laemmli solution (Bio-Rad, USA) and dithiothreitol 350 mmol/l)) was heated at 95°C for 5 min, and then was loaded on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel in a protein sample buffer (10x Tris-Glycine-SDS (Euromedex, France)). Then, PAGE was performed and gels were stained by Coomassie blue. For western blotting, proteins were transferred onto nitrocellulose membranes and incubated with mouse anti-Mimivirus fiber or anti-L725 polyclonal antibodies obtained as previously described (Sobhy *et al.*, 2015), and diluted in blocking buffer (phosphate-buffered saline (PBS), 0.3% Tween 20 and 5% nonfat dry milk; 1:5,000 and 1:10,000 dilutions, respectively). Membranes were probed with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (Amersham, UK).

MALDI-TOF MS analysis was performed as previously described (Boyer *et al.*, 2011). Briefly, bands were cut and conserved in 20% ethanol, washed for 5 min in H_2O and dissolved in acetonitrile. Then, samples were incubated with 10 mmol/l dithiothreitol for 45 min at 56°C, then with 55 mmol/l iodoacetamide and incubated for 45 min in the dark. The samples were washed by H_2O and acetonitrile, and centrifuged for 30 min to remove excess H_2O. The proteins were digested by trypsin (12 ng/pl; Promega, USA) and incubated for 45 min at 4°C, then for 2–3 h at 37°C. Peptides were re-suspended in alpha-cyano-4-hydroxycinnamic acid matrix solution and spotted on the MALDI-TOF target. Analyses were performed on an Ultraflex spectrometer (Bruker Daltonics, France), and proteins were identified using the Mascot software (http://www.matrixscience.com).

For transmission electron microscopy, samples were fixed by glutaraldehyde and cacodylate buffer and deposited on 400-mesh nickel grids coated with formvar/carbon (Electron Microscopy Sciences, PA, USA). Grids were immersed in NH_4Cl (50 mmol/l), washed in PBS three times for 5 min, then immersed twice for 10 min in blocking buffer (1% normal goat serum, 1% bovine serum albumin (BSA), and 0.2% Tween 20 diluted in PBS). Thereafter, grids were incubated with anti-fiber polyclonal antibody diluted 1:100 in blocking buffer overnight at 4°C. After four 10-min washes, grids were incubated for 90 min in biotin (Beckman Coulter, USA), diluted 1:100 in blocking buffer, then washed with 0.1% BSA-PBS (4x5 min) and 0.01% cold water fish skin (CWFS) gelatin-PBS (3x10 min), incubated for 90 min with streptavidin labeled by 10-nm gold nano-particles (Aurion, The Netherlands) diluted 1:100 in 0.01% CWFS gelatin-PBS, then washed with PBS. After incubating with PBS-2.5% glutaraldehyde for 15 min, grids were washed with PBS (2x10 min) and distilled water (10 min). Finally, they were contrasted by uranyl acetate for 20 min, immersed in water 60 times, and viewed with a FEI Tecnai F20 microscope operating at 200 keV.

Results

The expression of Mimivirus recombinant-L725 protein fused with Trx was achieved in the *Escherichia coli* Rosetta strain, as shown by SDS-PAGE as a faint band compatible with the estimated molecular mass of L725-Trx (42 kDa) (Fig. 1a). By western blotting, after incubation of expressed protein with mouse anti-L725-Trx antibodies, a band was observed at the expected molecular mass of L725-Trx (Fig. 1b). In addition, after incubation with mouse anti-Mimivirus fiber antibodies, which were verified to bind to the Mimivirus virion layer using electron microscopy and immunogold labeling (Fig. 1d), a band was observed at the expected molecular mass of L725-Trx (Fig. 1c). The L725 gene product fused with Trx and hexahistidine-tag (L725-Trx) was found to have a concentration of 0.5 mg/ml as determined with a NanoDrop spectrophotometer (Thermo Scientific). Moreover, L725 protein expression was confirmed by MALDI-TOF MS (Supplementary Fig. S1). Taken together, these results suggest that L725 gene product was obtained and L725 is a Mimivirus fiber-associated protein.

Discussion

We provide evidence that the L725 gene product, encoded by a Mimivirus ORFan, previously re-annotated as encoding a Mimivirus FAP (Sobhy *et al.*, 2015), was obtained fused to thioredoxin in *E. coli* and recognized by both anti-L725 and

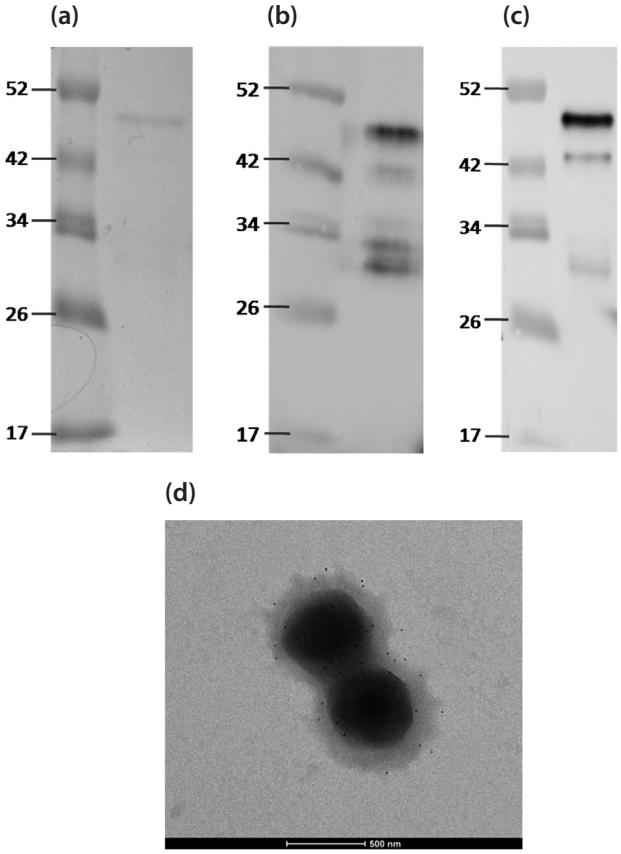

Fig. 1

Polyacrylamide gel electrophoresis (PAGE) and western blot analyses of the expressed L725 gene product

(a) SDS PAGE of the expressed L725 protein fused with thioredoxin (Trx); **(b)** Western blotting of the expressed L725 Mimivirus proteins fused with Trx following incubation with anti-L725 antibodies; **(c)** Western blotting of the expressed L725 protein fused with Trx following incubation with anti-Mimivirus fiber antibodies. **(d)** Transmission electron microscopy with immunogold labeling of the Mimivirus virions using anti-Mimivirus fiber polyclonal antibody. Mouse anti-L725 antibodies were obtained using L725 protein fused with Trx and used at 1:10,000 dilution; anti-Mimivirus fiber antibodies were used at 1:5,000 dilution. Expected molecular masses are as follows: L725 protein: 27 kDa; Trx: 15 kDa; L725 protein fused with Trx: 42 kDa.

anti-Mimivirus fiber antibodies. These results are congruent with previous reports indicating that the Mimivirus L725 gene product, together with three other proteins (R135, L856 and L829), is the component of the Mimivirus fibers (Boyer *et al.*, 2011; Klose *et al.*, 2015; Sobhy *et al.*, 2015). The present study is one of the few studies that expressed a Mimiviral gene (Abergel *et al.*, 2005,2007) and the only one, to our knowledge, that expressed a Mimiviral ORFan. The same method can be used to determine the function of viral structural or nonstructural proteins.

To date, *Acanthamoeba* spp. are the only known hosts for mimiviruses, but macrophages and peripheral blood mononuclear cells were shown to allow Mimivirus replication (Ghigo *et al.*, 2008; Raoult *et al.*, 2007; Pagnier *et al.*, 2013; Silva *et al.*, 2013). The determinants of Mimiviral tropism remain unknown, but the role of the fibers in host infection and virion stability is suspected (Boyer *et al.*, 2011; Boratto *et al.*, 2013; Dornas *et al.*, 2014; Klose *et al.*, 2015). The recent isolation of mimiviruses from humans presenting pneumonia (Saadi *et al.*, 2013a,b) and the detection of sequences best matching mimivirus genomes in the metagenomes generated from various human samples (Colson *et al.*, 2013; Rampelli *et al.*, 2016) are incentive to decipher the mechanisms of entry of these giant viruses into eukaryotic cells. With this in mind, further analyses should specify the structure of the Mimivirus fiber and protein interactions that allow fiber biosynthesis in mimiviruses.

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Supplementary information is available in the online version of the paper.

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