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Published in:

Proceedings of the Thirteenth International Symposium on Human Chlamydial Infections, 22-27 June 2014, Pacific Grove, CA, USA

Publication date:

2014

Document Version

Publisher's PDF, also known as Version of record

[Link to publication from Aalborg University](#)

Citation for published version (APA):

Christiansen, G., Sennels, L., Stensballe, A., & Birkelund, S. (2014). LC-MS/MS proteomic determination of DUF582 proteins of chlamydia trachomatis L2. In J. Schachter, G. I. Byrne, M. A. Chernesky, I. N. Clarke, T. Darville, E. W. Hook, III, D. C. Mabey, J. Paavonen, M. Starnbach, R. S. Stephens, P. Timms, ... P. Wyrick (Eds.), Proceedings of the Thirteenth International Symposium on Human Chlamydial Infections, 22-27 June 2014, Pacific Grove, CA, USA (pp. 47-50)

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International Chlamydia Symposium
San Francisco, CA 94110
USA

ISBN 0-9664383-4-5

LC-MS/MS PROTEOMIC DETERMINATION OF DUF582 PROTEINS OF CHLAMYDIA TRACHOMATIS L2

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INTRODUCTION

The coding capacity of the chlamydial genome was revealed by genome sequencing of strain D/UW-Cx (Stephens et al. 1998). Of the 894 likely protein-coding genes 255 (28%) were not similar to any known proteins indicating the uniqueness of the genus *Chlamydia*. Since then multiple chlamydial and parachlamydial genomes have been sequenced (Stephens et al. 2009) but still, many aspects of molecular pathogenesis of chlamydial diseases remain unknown. The first attempt to reveal the protein synthesis in chlamydiae was done using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of *in vivo* S³⁵ labelled proteins. Hereby we showed that among the first proteins to be synthesized were the S1 ribosomal protein, the GroEl-like (HSP60) protein and the DnaK-like protein identified by immunoblotting (Lundemose et al. 1990). Later, when the chlamydial genome became available mass spectrometry (MS) was used to identify the protein content within individual protein spots separated by 2D-PAGE (Shaw et al. 2002; Vandahl et al. 2001). With the development of more sensitive and sophisticated mass spectrometers in combination with advanced separation technology it became possible to make quantitative proteomics (Saka et al. 2011) of purified reticulate bodies (RB) and elementary bodies (EB). Using 2D liquid chromatography – tandem mass spectrometry (LC/LC-MS/MS) Saka et al. (2011) succeeded in identifying 485 non-redundant chlamydial proteins (approximately 54% of the predicted proteins in the *C. trachomatis* L2 proteome). When the proteome of purified EB and RB is analyzed, secreted proteins may be missed. In the present study we performed LC-MS/MS on complete lysates of trypsin digested *C. trachomatis* L2 infected HeLa cells to determine whether members of the *Chlamydia*-specific, leucine-rich *Domain of Unknown Function* (DUF)582 protein family could be found among the identified proteins.

METHODS

Cultivation: *C. trachomatis* L2, strain 434/Bu was cultivated in HeLa cells (ATCC, Rockville, MD, USA) with 1 IFU/cell in RPMI medium as described (Hobolt-Pedersen et al. 2009).

Extraction of proteins: At 43 hpi the *C. trachomatis* L2 infected monolayers of HeLa cells were harvested, washed three times in PBS, and the cells were lysed in 5% sodium deoxycholate (SDC) and 50 mM triethylammonium bicarbonate (TEAB) containing phosphatase inhibitors. After heating to 90°C for 5 min. digestion of the samples with trypsin was performed using filter aided sample preparation using 10 kDa cutoff spin filters. The resulting protein solution was digested with sequencing grade modified trypsin (Promega) at 37°C for 24 hours. After trypsin digestion the samples were acidified with trifluoroacetic acid, extracted with ethyl acetate, dried down and resuspended in 2% acetonitrile and 1% formic acid.

Mass spectrometry (MS): MS was done according to Bennike et al. (2013). The protein solution was analyzed on an automated LC-ESI MS/MS system using an Ultimate 3000 UPLC system with a nanopump module. The system was coupled with an emitter for nanospray ionization to a QExactive mass spectrometer (Thermo Scientific, Waltham, USA). Ten μ g of the sample was

loaded onto the C18 reversed phase column (Dionex) and eluted with a linear gradient of 96% solvent A and 4% of solvent B (Bennike et al. 2013) increasing solvent B to 35% on a 240 min ramp gradient. The MS was used in a data dependent mode, selecting the 12 precursor-ions with the highest intensity for fragmentation. Resulting raw files were analyzed using Thermo Proteome Discoverer, and identified by Mascot (Matrix Science) against a *C. trachomatis* L2 Uniprot database. A Homo sapiens database was used to screen the identified chlamydial peptides for homology to human proteins.

Production of polyclonal antibodies: Recombinant CT619 was generated by cloning a PCR product encompassing the *ct619* gene and expressing the protein and immunization of rabbits were done as described by Hobolt-Pedersen et al. (2009).

Immunofluorescence microscopy: HeLa cells, cultivated on cover slips, were infected with *C. trachomatis* L2 (0.5 IFU/cell) and fixed after 43 hours of infection with paraformaldehyde/glutaraldehyde. The infected cells were incubated with the primary antibody specific for CT619 followed by incubation with FITC-conjugated goat –anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, USA). Images were obtained using a Leitz DMBRE fluorescence microscope (Leica, Germany) as described (Hobolt-Pedersen et al. 2009).

RESULTS

Trypsin digestion will cleave proteins at the peptide bond at the carboxyl side of the amino acids lysine (K) and arginine (R) in the primary sequence, thereby cleaving the proteins into smaller fragments. The mass of such peptides reflects the amino acid composition of the peptide. By MS/MS, fragmentation patterns are generated by collision-induced dissociation resulting preferentially in ion dissociation at amide bonds along the peptide backbone, thus generating b- and y-type fragment ions (Roepstorff & Fohlman, 1984) and the amino acid sequence can be determined. By comparison of the obtained results with *in silico* generated tryptic digests of a translated genome the origin of the peptide is determined. Using the translated *C. trachomatis* L2 genome (Stephens et al. 1998) we searched for peptides belonging to the DUF582 protein family (CT619; CT620; CT621; CT711 and CT712 (*C. trachomatis* D genome numbers)). Results are shown in Table 1. Three peptides were identified from CT621, a DUF582 protein previously shown to be secreted by the type III secretion system (T3SS) (Hobolt-Pedersen et al. 2009; Gong et al. 2011; Muschiol et al. 2011).

We found no peptides from the DUF582 family members CT620, CT711 and CT712 but from CT619 we found four peptides that uniquely identified the protein. None of the identified peptides had homology to human proteins.

The MS/MS spectrum of one of the peptides from CT621 is shown in Fig 1. It is seen that there is a clear y-ion spectrum of peaks indicating the high quality of the amino acid sequence of this peptide.

An even better y-ion spectrum of MS/MS peaks is seen in Fig. 2, clearly demonstrating the presence of CT619 in the solution of peptides.

Table 1.

Accession number	expectancy	Peptide sequence
B0B8J6_CHLT2	9.8e-005	R.SLLSLSNDVMR.V
CTL0885/CT621	5.6e-005	R.TQGS�TDYNSTLEIAK.K
	0.00014	R.TQGS�TDYNSTLEIAK.K
B0B8J5_CHLT2		
CTL0884/CT620		
B0B8J4_CHLT2	7.4e-005	K.ASFNTSTGSTPSLR.A
CTL0883/CT619	7.3e-008	R.IIVEQLAVLDSLLR.S
	0.012	R.IIVEQLAVLDSLLR.S
	4e-006	R.IIVEQLAVLDSLLR.S
B0B8T7_CHLT2		
CTL0080/CT711		
B0B8T8_CHLT2CTL008		
1/CT712		

Immunofluorescence microscopy of *C. trachomatis* L2 infected HeLa cells using an antibody specific for CT619 is seen in Fig. 3A, and a Nomarsky image of the same cell in Fig. 3B. The antibody is seen binding to formed structures within the chlamydial inclusion and only a trace of fluorescence is seen in the cytoplasm of the infected cell at 43 hpi, indicating that most of the protein is localized within EB/RB at this stage of infection. We therefore cannot determine conclusively whether CT619 is secreted.

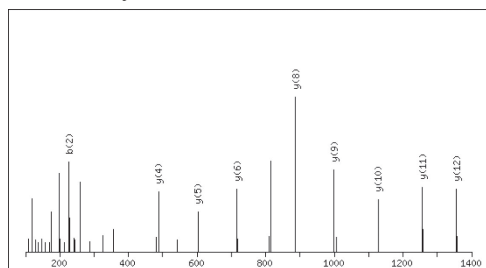


Fig. 1) CT621/CTL0885
R.SLLSLSNDVMR.V

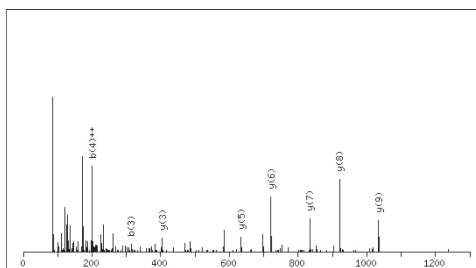


Fig. 2) CT619/CTL0883
R.IIVEQLAVLDSLLR.S

DISCUSSION

LC-MS/MS is a powerful method to identify not only the mass but also the amino acid sequence of a peptide. After digestion with trypsin, the complex peptide mixture is separated by online LC prior to injection into the MS, for accurate mass measurement. There, selected peptides are fragmented in a second MS-event, acquiring accurate fragment masses, thus enabling confident identification of the peptide sequence (Figs. 1 and 2). Using whole cell extract of *C. trachomatis* L2 infected HeLa cells, no sample fractionation was performed prior to MS-analysis, and therefore no proteins were lost. This is in contrast to gel-based proteomics where proteins with extreme pIs frequently are lost, and to proteomics of purified RB and EB from which secreted proteins may be lost.

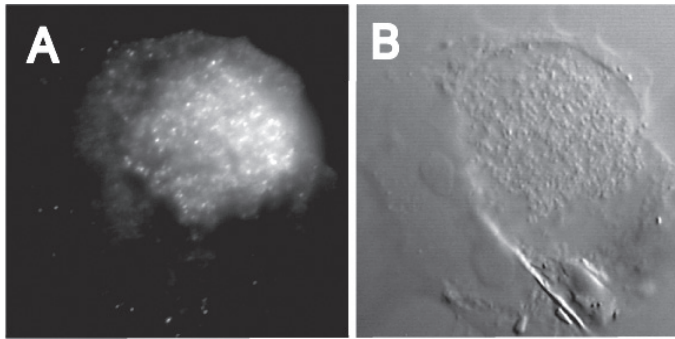


Fig. 3)
 A: IMF anti
 CT619
 B: Nomarsky

As is the case for CT621, CT619 is a member of the DUF582 family of proteins specific for chlamydiae. The size of CT619 is 97 kDa and the protein has a DUF582 domain in its C-terminal end. This domain is characterized by being leucine-rich but in contrast to CT621, CT619 does not contain a leucine zipper, and thus CT619 may serve a function different from CT621. In the proteomics study by Saka et al. (2011) CT619 was identified in the EB fraction in agreement with the immunofluorescence microscopy image in Fig. 3A. Also CT620 and CT711 are expressed at the middle and late phase of the developmental cycle (Muschiol et al. 2011). The reason why we in the present study did not identify peptides from these proteins may be caused by their lower abundance and rapid degradation as demonstrated by Muschiol et al. (2011).

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