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ANALYSES OF *CHLAMYDIA TRACHOMATIS* L2 IN THE BACKGROUND OF THE HOST CELL PROTEOME – INCLUSION MEMBRANE PROTEINS.

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Introduction

The *Chlamydia trachomatis* elementary body (EB) proteome has been characterized with 2D-gel electrophoresis and identification of proteins with tryptic digest and subsequent protein identification by mass-spectrometry (MS)(Shaw et al. 2002). The number of proteins that can be identified has increased after digesting complex protein samples and thereafter separating the peptides by liquid chromatography (LC) coupled MS/MS. The technique is called gel-free proteomics in contrary to 2D-gel based proteomics. Using this technique Skipp et al. (2005) identified 328 proteins in purified EB of the 889 encoded in the genome (Thomson et al. 2008). The technique has also been used to characterize the outer membrane complex of *C. trachomatis* L2 and to find *in vivo* cleavage sites of the proteins, especially between the auto-transporter and passenger domain of the polymorphic outer membrane proteins (Pmp), (Birkelund et al. 2009). Saka et al. (2011) identified 485 proteins in purified EB and reticulate bodies (RB). Due to the high quality of the data it was possible for them to quantify 373 of the proteins. Fifty-nine proteins were only found in RB, and in EB the *in vivo* cleavages of the Pmp proteins could confirm the results of by Birkelund et al. (2009). Saka et al. identified 13 Inclusion membrane proteins (Inc) in purified RBs and EBs. Due to the type III secretion of Inc to the inclusion membrane we expected to find additional Inc proteins by analyzing the entire infected host cell.

By combining ultra high pressure LC (UHPLC) and a mass-spectrometer with a dynamic range of 10^5 and sub-ppm mass-accuracy, it is now possible to identify 7,093 proteins in a HeLa cell sample using MS/MS (Wiśniewski et al. 2009). Therefore, we decided to characterize expression in *C. trachomatis* L2 infected host cells (HeLa) after 43 hrs of infection.

Methods

Semiconfluent monolayers of HeLa cells (75 cm^2) were infected with *C. trachomatis* L2 (434/Bu), for 30 minutes with the infectious dose of 1 IFU/cell and then incubated for 42 hrs and 30 minutes in RPMI-1640 medium containing 10% FCS and gentamicin. Medium was also changed on un-infected HeLa cells. Cells were washed 3 times in PBS, and the cells were solubilized in 5% sodium deoxycholate (SDC), 50 mM triethylammonium

bicarbonate (TEAB) and phosphatase inhibitors. The samples were heated to 90°C for 5 minutes. Filter-aided sample preparation (FASP) and digestion with trypsin was performed according to León et al. (2013) using 10 kDa spinfilters for buffer exchange. The samples were reduced with 12 mM Tris(2-carboxyethyl)phosphine hydrochloride, alkylated with 40 mM iodoacetamide and digested with trypsin (1:50). All reactions were done in 0.5% SDC and 50 mM TEAB. After digestion, formic acid was added to 0.5% and SDC was removed with ethyl acetate extraction.

Samples were analyzed on a Thermo Electron QExactive mass spectrometer.

RESULTS

The trypsin-digested samples were separated with a UPLC system. The system was online coupled to a QExactive mass spectrometer. A 4-hrs UPLC (8-30%) gradient was used. The 12 highest intensity peptides were selected per second to high-energy collision dissociation (HCD) and MS² spectra were obtained. Spectra were extracted from raw files with Thermo Proteome Discoverer and searched with a Mascot server for *C. trachomatis* L2 peptides using all CHLT2 UnProtKB sequences.

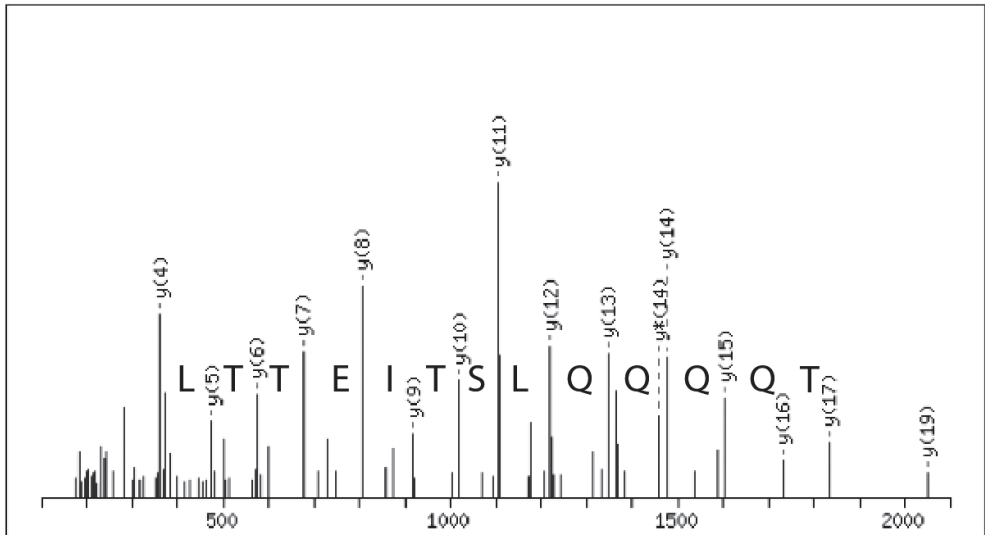


Fig. 1) MS² spectrum of a peptide ion form CTL0882

In figure 1 the MS² spectrum of a fragmented peptide ion of 2350.20 Da is seen. The mass difference between the peaks is analyzed for amino acid residues masses. Since the peptide is ionized in the C-terminal part, the sequence of the peptide is **TTVSETQQQLSTIETTLGSAK**. The peptide is from the Inc protein CTL0882 (Ct618 homolog in D).

The samples were run in triplicates resulting in approximately 900.000 MS² spectra for infected HeLa cells as well as for uninfected cells. 250 C.

trachomatis proteins were identified with more than 2 peptides with an expectancy score of $<e^{-4}$ and a mowse score of >200 in the mascot search program. Accession numbers for predicted Inc proteins according to Lutter et al. (2012) were searched in the output. Seven predicted Inc proteins were identified with at least 2 different unique peptides (Table 1).

Table 1

Accession Number	D homolog	Peptide sequence	Saka	Inc
CTL0371 INCE_CHLT2	CT116	LDNLTPVVR, SSPANEPVQFF	0	E
CTL0373 B0B9M6_CHLT2	CT118	VLSSVQSMPVIDK, SIGDSVSGAFDDINK, SIGDSVSGAFDDINKDNSR	RB	G
CTL0402 B0B9Q5_CHLT2	CT147	SSDATVGLQHQIR, DLIDVVEESIETAK, DLIDTILQMGSAAAGVTPDVDGVHK	RB/NQ	
CTL0466 B0B9W4_CHLT2	CT214	FAAPFLSLIEK, GASIPGISGQADDSLPNR	EB	
CTL0476 B0B9X4_CHLT2	CT223	AHLDVCLGEK, EEISQLEQQQAK, VEANNGINDLSPAPEAK	RB/NQ	
CTL0480 B0B7K4_CHLT2	CT288	QYDILNAAFNK, QLSQIDLTIQAPEK YDSINHSIEEISDR	RB	
CTL0882 B0B8J3_CHLT2	CT618	LLSSATATTLR, TNPDGSFHLDLVSQQR, LSSVNDLFDLISSSCTLAR, TTVSETQQQQLSTIETTLLGSAK	RB	

Six of the proteins were also identified in purified RB/EB by *Saka et al.* (2011). IncE was not found in that study. To control the specificity of our *C. trachomatis* identifications, against the human protein background, we performed a database search of the MS data on the un-infected HeLa in a Mascot search against the L2 database. Only chlamydia Glyceraldehyde-3-phosphate dehydrogenase and PolyA polymerase were unambiguously identified in the L2 database with high scores, due to the conserved peptides between the human and chlamydia proteins.

DISCUSSION

On unfractionated infected HeLa was it possible to identify 250 *C. trachomatis* L2 proteins with high confidence with the FASP method in combination with UHPLC and on-line MS. Saka et al. (2011) identified 426 proteins in purified EB and 269 in RB. However, the current method also gives the possibility to look at the changes in the host cell proteins and

the chlamydial proteins secreted to the host. Saka et al. (2011) found, however, many Inc proteins in their proteomics study of RB and EB even though the proteins should be secreted from the chlamydiae. This is likely due to presence of the Inc family proteins in chlamydiae before secretion. We found IncE (CT116) in addition to the Inc protein found by Saka et al.

The expression of IncE is in good agreement with Lutter et al. (2012) and Almeida et al. (2012) who showed that the *incE* gene in the LGV serovars had been under a positive selection pressure with many nonsynonymous nucleotide substitutions. This was also the case for CT118 and CT147 that we also found expressed. By quantitative PCR CT116, CT118 and CT147 mRNAs were found to be expressed early in the *C. trachomatis* L2 developmental cycle (Lutter et al. 2012). This indicates that the proteins are stable in the host cells. Therefore, the present proteomic study supports the results by Lutter et al. (2012) and Almeida et al. (2012) indicating that polymorphisms in the *Inc* genes are of importance for disease and tissue tropism.

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