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Original article

Identification and characterization of a type III secretion system in *Chlamydophila psittaci*

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Abstract – *Chlamydiaceae* are obligate intracellular Gram-negative bacteria replicating in vacuoles inside eukaryotic cells. It has been proven that most of them possess a type III secretion system (T3SS) allowing them to transfer effector molecules in the host cell. We examined the existence of a T3SS in *Chlamydophila psittaci* by studying the expression of three essential structural proteins SctW, SctC, and SctN, and one putative effector protein IncA. Immunofluorescence assays showed SctW and IncA to be associated with the bacteria and the inclusion membrane, while SctC and SctN were only localized to the bacteria itself. Immuno electron microscopy could confirm these results for SctW, IncA, and SctC. Unfortunately, SctN was not investigated with this technique. Additionally, we sequenced 14 full-length T3S genes (*scc1*, *sctW*, *sctJ*, *sctL*, *sctR*, *sctS*, *scc2*, *copD1*, *sctN*, *sctQ*, *sctC*, *incA*, *ca037*, and *cadd*) and examined the transcription of 26 *Cp. psittaci* T3S genes namely cluster 1 (*scc1*, *sctW*, *sctV*, *sctU*), cluster 2 (*sctJ*, *sctL*, *sctR*, *sctS*, *sctT*, *scc2*, *copB1*, *copD1*), cluster 3 (*sctD*, *sctN*, *ca037*, *sctQ*, *pkn5*, *sctC*) and non-clustered genes (*incA*, *incC*, *scc3*, *copD2*, *cap1*, *tarp*, *ca530*, *cadd*). The gene expression study indicated the T3S structural protein encoding genes to be transcribed from mid-cycle (12–18 h post infection (p.i.)) on. Genes encoding effector proteins and putative T3S related proteins were expressed early (1.5 h–8 h p.i.) or late (>24 h p.i.) during the developmental cycle. We hereby provided evidence for the existence of a T3SS and possible effectors in avian *Cp. psittaci*.

Type III secretion / *Chlamydia* / *Chlamydophila psittaci*

1. INTRODUCTION

Chlamydophila (Cp.) psittaci belongs to the family *Chlamydiaceae* causing respiratory disease in pet birds and poultry. Pet bird owners, veterinary surgeons, and poultry workers are at risk of becoming infected by this zoonotic agent (reviewed in [1]).

Cp. psittaci is an obligate intracellular Gram-negative bacterium infecting mucosal epithelial cells. Its unique biphasic developmental cycle starts with infectious elementary bodies (EB) preferentially attaching and entrying at the base of cell surface microvilli, where they are often found in association with clathrin-coated pits. Cell entry mechanisms are not fully understood but as in *Chlamydia trachomatis*, type III secretion might be involved [5]. Entered EB can be found within small vesicles, avoiding fusion with lysosomes while trafficking towards the perinuclear region. EB differentiate into metabolically active reticulate bodies (RB), and binary fission within the growing vesicle results in large inclusions. Typically,

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mitochondria are surrounding these large *Cp. psittaci* inclusions, most likely providing these energy parasites with the necessary additional ATP. The mechanism responsible for the recruitment of mitochondria is unknown, but again type III secretion (T3S) effector proteins might play a role. Following binary fission, RB differentiate into intermediate bodies (IB) and finally new infectious EB. Remarkably, newly formed EB of highly pathogenic *Cp. psittaci* strains might escape from the inclusion, spreading diffusely through the cytoplasm of the host cell. From 48 h post infection (p.i.) onwards, cell lysis results in the release of infectious EB. However, localized rupture of the host cell membrane with the underlying inclusion emptying itself into the extracellular surroundings has been observed for highly pathogenic *Cp. psittaci* strains [41]. Additionally, *Chlamydiaceae* are able to persist in host cells as morphologically altered RB, which no longer divide and accumulate chromosomes [18]. Overall, *Cp. psittaci* modulates host cell functions to replicate or persist inside host cells but the mechanisms deployed to subvert host cell functions to benefit the pathogen are poorly understood. Given the current understanding on T3S, one could postulate a role for T3S in some, if not all stages in the *Cp. psittaci* infection.

Genomics of *C. trachomatis*, *C. muridarum*, *Cp. pneumoniae*, and *Cp. caviae* [20, 27, 28, 34] revealed type III secretion system (T3SS) genes for the export of pathogenic proteins analogous to the ones identified in other gram-negative bacteria, with the *Yersinia* ysc-yop weaponry being the best one characterized [6]. *Cp. psittaci* genome sequence data are not available. Meanwhile, evidence for a functional T3SS in *Cp. pneumoniae* [23, 33, 36] and *C. trachomatis* [12] has been presented. Moreover, these investigators showed proteins from *Cp. pneumoniae* and *C. trachomatis*, all of which had in common the presence of a large hydrophobic domain, to be secreted by a *S. flexneri* or *Yersinia enterocolitica* type III secretion machinery [9, 35, 36].

The discovered chlamydial T3S machinery consists of approximately 25 proteins encoded by at least three different conserved gene clusters or pathogenicity islands. Like in other pathogens, the chlamydial T3SS probably spans the inner and outer membrane as well as the plasma membrane (during host cell attachment) or the inclusion membrane (during intracellular replication). As chlamydiae have recently been suggested to possess the most ancient non flagellar T3SS [24, 38], we have chosen to use the unified secretion and cellular translocation (Sct) nomenclature as proposed by Hueck [19] for the conserved structural proteins of the T3SS. Only chaperones and effectors were given species specific names. The exporter, located in the inner membrane of the bacterium, is presumed to consist of SctR, S, T, U, and V. The cytoplasmic membrane associated ATPase SctN, assumed to be the energizer of the system, is in close association with the exporter. The basal body, totally embedded by the exporter, would be composed of SctL, Q, D, and J, where SctJ probably connects the inner and the outer membrane proteins. The bacterial outer membrane is probably penetrated by a secretin, consisting of SctC. Normally, T3SS proteins travel across the bacterial membranes through a hollow conduit defined as the needle of the T3SS. However, needle components like SctI and SctF have not yet been identified in *Chlamydiaceae*. Protein translocation is supposedly mediated by the chlamydia outer proteins (Cop) B1 and D1 and assisted by specific chlamydiaceae chaperones (Scc) 1 and Scc2/Scc3, being homologous to the specific yersinia chaperones (Syc) E and D. Type III sececretion is thought to be regulated by SctW or CopN possibly acting as a cork on a flask preventing or allowing T3S proteins of being translocated across the bacterial membranes. *C. trachomatis* and *Cp. pneumoniae* are supposed to actively modify inclusion membranes with a set of T3S effector proteins, known as inclusion membrane proteins or Incs [10, 30, 36]. Additional chlamydial T3S effector proteins like the translocated actin recruiting phosphoprotein (Tarp) involved in chlamydial entry [4] and the class I accessible protein-1 (Cap1), an inclusion membrane associated protein recognized by protective $CD8^+$ T cells [2, 13], have been described, as well

as Pkn5, a putative serine-threonine kinase [11, 16, 17].

In order to examine the presence of a functional T3SS in *Cp. psittaci* we sequenced 14 full-length T3S genes (*scc1*, *sctW*, *sctJ*, *sctL*, *sctR*, *sctS*, *scc2*, *copD1*, *sctN*, *sctQ*, *sctC*, *incA*, *ca037*, and *cadd*) and focused on the translation and localization of three essential structural proteins (SctW, SctC, and SctN) and one putative effector protein (IncA) using Western blotting and double-label immunofluorescence assays and immuno electron microscopy. Additionally, we examined the transcription of 26 *Cp. psittaci* T3S genes on the RNA level namely cluster 1 genes (*scc1*, *sctW*, *sctV*, *sctU)*, cluster 2 genes (*sctJ*, *sctL*, *sctR*, *sctS*, *sctT*, *scc2*, *copB1*, *copD1*), cluster 3 genes (*sctD*, *sctN*, *ca037*, *sctQ*, *pkn5*, *sctC*), and non-clustered genes (*incA*, *incC*, *scc3*, *copD2*, *cap1*, *tarp*, *ca530*, *cadd*). Results were compared to those of expression studies in *Chlamydia trachomatis* and *Chlamydophila pneumoniae*.

2. MATERIALS AND METHODS

2.1. Sequencing of *Cp. psittaci* T3S genes

As *Cp. abortus* has in an evolutionary context been linked to the *Cp. psittaci* lineage, wherein *Cp. psittaci* strains resemble what might have been recent ancestors of *Cp. abortus* [39], 28 putative T3S genes were identified in the at that time still unannotated *Cp. abortus* genome. Subsequently, alignments (Clustal X, European Bioinformatics Institute, Hinxton, Cambridge, UK) of these loci with *Cp. caviae*, *Cp. pneumoniae*, *C. trachomatis*, and *C. muridarum* orthologues were performed to design consensus primers by Web Primer[1], with the option to amplify exact endpoints of *Cp. psittaci* T3S cluster 1 (s*cc1*, *sctW*, *sctV*, *sctU*), cluster 2 (*sctJ*, *sctL*, *sctR*, *sctS*, *sctT*, *scc2*, *copB1*, *copD1*), cluster 3 (*sctD*, *sctN*, *ca037*, *sctQ*, *pkn5*, *sctC*), and non-clustered genes (*incA*, *incB*, *incC*, *scc3*, *copB2*, *copD2*, *cap1*, *tarp*, *ca530*, *cadd*). Ca037 is the orthologue of the CCA00037 protein in *Cp. caviae*. Ca530, being the orthologue of another *Cp. caviae* protein (CCA00530) was included because its gene expression may be subject to phase variation [37]. Primer sequences are given in Table I.

Cp. psittaci genotype D strain 92/1293 was grown in BGM cells and infections were carried out as described previously [40]. Genomic DNA was extracted as described by Wilson et al. [43] and putative *Cp. psittaci* T3S genes were PCR amplified and ligated into pGEM®-T (Promega, Leiden, The Netherlands) for sequencing.

Obtained gene sequences were compiled and analyzed with Clustal X (European Bioinformatics Institute), while predicted translation products were further analyzed with Simple Modular Architecture Research Tool (SMART 5, European Molecular Biology Laboratory, Heidelberg, Germany) revealing specific domains, repeats and motifs [22] and Conserved Domain Architecture Retrieval Tool (CDART, National Center for Biotechnology Information, Bethesda, MD, USA) examining protein similarities across significant evolutionary distances [15]. IncA and SctW were examined for possible serine, threonine, and tyrosine phosphorylation sites with the NetPhos server (version 2.0, Center for Biological Sequence Analysis, Lyngby, Denmark) [3].

2.2. Expression of SctW, SctC, SctN, and IncA and antibody production

We intended to prove the expression of a T3SS in the highly pathogenic *Cp. psittaci* genotype D strain 92/1293 before going to transcription studies of T3S genes. Therefore, we focused on SctW as presumed T3S regulator, SctC probably penetrating the outer membrane, SctN as putative T3S energizer and finally IncA as a well-known T3S effector protein. In order to construct SctW, SctC, SctN, and IncA gene products possessing histidine-containing (HT) N-terminal leader sequences, purified PCR products were ligated into pET-30 Ek/LIC (Novagen, VWR, Leuven, Belgium) followed by heat-shock transformation of *E. coli* BL21(DE3) cells and induction with 0.1 mM IPTG (Promega). HT-proteins were purified (Talon Spin Columns, BD Biosciences, Erembodegem, Belgium) and identified by mass spectroscopy. Only SctW-HT, SctC-HT, and IncA-HT were successfully expressed. Probability based Mowse scores as determined by the Mascot server [25] were respectively 77, 66, and 60. HT-leaders were removed using the Novagen enterokinase cleavage kit.

[1]Web Primer: DNA and Purpose Entry [on line] (1997–2007) http://seq.yeastgenome.org/cgi-bin/web-primer [consulted 19 December 2007].

Table I. Primer sequences for amplification of full length *Cp. psittaci* TTS genes.

Cluster	Gene	Forward primer sequence[a]	Reverse primer sequence[b]	Size (bp)
Cluster 1	*scc1*	ATGCAAAATCAGTTTGAACAAC	TTATAAATGATACATGCCTAGGACA	441
	sctW	ATGGCTGCATCTGGAGGA	TTAAGACCAGGGATAAGGTTTG	1202
	sctV[c]	ATGAATAAGCTACTCAATTTTGTC	TTAGAAAATCTGAATTCTTCCTAA	2130
	sctU	ATGGGTGAAAAAACAGAAAAGG	TTATAGATTATCGGGTTGGTTAATG	1083
Cluster 2	*sctJ*	ATGTTTCGTAGTTCTATTTCTTG	CTAGACACCCTCATTTTCTTCG	943
	sctL	ATGAAGTTTTTTAGTCTAATTTTTAAACATGAC	TTATTCTTGCTTGTTCTGAGCTTC	657
	sctR	ATGCGTTTCATTTTTCGTACT	CTATTTAAAGCTAATCATGAGACCT	918
	sctS	GTGATAGCACTCGCCGCAA	TTATTTCCATTTATAAAAGTTTTGGAAGA	285
	sctT	ATGGCAATCTCTTTACCAGAGC	CTAAAGGACTTTAGGATTAGATCCG	870
	scc2	ATGAGCAAGCCTACTTCAAATAATTC	TTAATTTTTTTTTCCGGAGTTT	684
	copB1	ATGTCTCTTTCTACCTCAGGTCCA	TTATACTGCTGATGCTAAGCCT	1485
	copD1	ATGACATCAGGAGTTAGTGGAA	TTAGCTAAAGATTGCAGAAGTTG	1323
Cluster 3	*sctD*	/[d]	/	
	sctN	ATGGAWGAGTTMMMRGACAGATTTCG	TCYTTAACAACYTTYTCTGC	1329
	ca037	ATGGAATTAAATAAAACATCCGAG	TTATAAACGTGCTTCTTCAACTT	846
	sctQ	ATGACAGTAGCAGCGGAACCTA	TTAGACTTCTAGAACTCGAATTCCT	1116
	pkn5	/[d]	/	
	sctC	TTAAAAGGGAAACAAATTACTTGTG	TTATTGAGCTTCTATTTCTAGCATT	2730
Non-clustered	*incC*	ATGACCTCTGTAAGAACCGATTT	TTAATTCCCTGTTAATCTCGTTTCG	555
	incB	ATGTCAACAACCACGCCATT	TCAAGCTTCTATTTGAGAATTGT	609
	incA	ATGACATCGACGGTAGAATCTGCT	TTACTGGTCATATATTGGGAA	1143
	scc3	ATGTCACCAAGCACGCTCC	TTATGCATGGTTTTTTATATCCAAG	597
	copB2	ATGTCTTCCTGGCTCGCA	TTACTTTAGAGATTCCAGAAACGT	1515
	copD2[c]	ATGACAGCATCTTGTGCAGTAACAG	CTATTGGTAGAGGCTGCGAACA	1479
	cap1	ATGGCATCTGTAACACCACAAG	TTATGAATGAGCCCATGCG	975
	tarp[c]	ATGTCGAGTCCAATTAATAATCAGC	CTACGAGCGCCGTTGTGG	2613
	ca530[c]	ATGCCAGGAATTATTTTACATTCCC	CTAATTCAGCGTTCTTTCTCCTGTC	1623
	cadd	ATGAAGGAGAGGATTTATACGGTCC	TTAATGACCGCAATTACAACGAC	807

[a] All forward primers carry the GACGACGACAAG adaptor at the 5′ end.
[b] All reverse primers carry the GAGGAGAAGCCCGGT adaptor at the 5′ end.
[c] No amplicon obtained.
[d] No working full length primers were designed.

Female New Zealand White rabbits were subcutaneously immunized with recombinant SctW, SctC, or IncA according to internationally recognized guidelines. Animal experiments were approved by the local ethical committee (reference number: EC 2005/12). Sera were tested in ELISA and Western blotting using recombinant proteins and cell lysate of *Cp. psittaci* 92/1293 (serovar D), GR9 (serovar C), or 84/55 (serovar A) infected and uninfected BGM cells. For all strains a band at the predicted molecular weight could be observed. There was no crossreactivity of the generated polyclonal antibodies against *C. muridarum*, *C. trachomatis*, *Cp. abortus*, *Cp. felis*, or *Cp. pneumoniae* purified EB and RB. For SctN, *Cp. pneumoniae* cross-reacting mouse anti-SctN (kindly obtained from R. Lugert, Germany) was used in further experiments.

2.3. Western blotting

BGM cells at a concentration of 2×10^5 per mL, which were either infected or left uninfected, were solubilized in SDS sample buffer containing 2.5% β-mercaptoethanol and boiled for 5 min. Aliquots of 20 µL, taken at 15 min, 5 h, 22 h, 30 h, and 48 h p.i. were separated by SDS-PAGE as described by Laemmli [21] followed by immunoblotting with rabbit polyclonal anti-SctC, -SctW, and –IncA, and mouse polyclonal anti-SctN [23]. Biotin-labeled anti-rabbit (Molecular Probes,

Invitrogen, Merelbeke, Belgium) or anti-mouse antibodies (Amersham Biosciences, GE Healthcare, Diegem, Belgium) followed by horseradish peroxidase-labeled streptavidin (Zymed, Invitrogen) and 3-amino-9-ethylcarbazole (AEC, Sigma, Bornem, Belgium) were used for visualization.

2.4. Double-label fluorescence microscopy

To determine the translation and localization of SctC, SctN, SctW, and IncA during the developmental cycle, fluorescence double-labeling experiments were conducted from 1 till 50 h p.i. using a 5 h time interval. All dilutions and washing steps (three) were performed with 1% BSA in PBS. BGM cells were grown and infected with *Cp. psittaci* 92/1293 (MOI 13) as described previously [42]. At each time point, infected monolayers were fixed with methanol and blocked with 5% (w/v) FCS in PBS. Cell layers were first incubated with anti-SctC, anti-SctN, anti-SctW or anti-IncA for 1 h at 37 °C, rinsed and then incubated with the FITC-labeled anti-rabbit IgG second antibody (Dako, Heverlee, Belgium) for SctC, SctW, and IncA detection or the Alexa Fluor 546-labeled anti-mouse IgG second antibody (Dako) for SctN detection. Cell layers for SctC, SctW, and IncA detection were rinsed and subsequently bacteria were labeled with a MOMP-specific mouse monoclonal antibody (MAb) 7B6III [14], while bacteria in cell layers for SctN detection were labeled with an anti-92/1293 rabbit polyclonal antibody. Hereafter, monolayers were rinsed and finally, labeling of the bacteria with the MAb 7B6III or with the anti-92/1293 rabbit polyclonal antibody could be visualized by using the Alexa Fluor 546-labeled anti-mouse IgG and the FITC-labeled anti-rabbit IgG, respectively. Fluorescent images were acquired with a 60× apochromat objective of a confocal laser scanning microscope (Radiance 2000, Bio-Rad, Nazareth, Belgium).

2.5. Immuno electron microscopy

Cp. psittaci 92/1293 infected and mock infected (negative control) BGM monolayers seeded on thermanox slides were used. At 15 min, 5, 22, 30, and 46 h p.i. the medium was gently aspired and the monolayers were immersed in 1 mL fixative (2% paraformaldehyde + 0.05% glutaraldehyde in 0.1 M phosphate buffer) (Sigma) for 2 h at 4 °C and subsequently rinsed overnight in phosphate buffer at 4 °C. Next, they were dehydrated in a graded concentration series of ethanol (70, 90, 95%; 15 min each + 3 × 100%; 30 min each) at RT. The blocks were then impregnated with LR White resin (2 × 1 h + overnight at 4 °C) (Electron Microscopy Science, Hatfield, UK). After polymerization at 4 °C, ultrathin sections (∼70 nm) were cut on an Ultracut UCT cryomicrotome (Leica Microsystems, Groot-Bijgaarden, Belgium). Sections were transferred to formvar-coated copper grids (Gilder, Laborimpex, Brussels, Belgium), and pretreated with 0.1% glycine in tris-buffered saline (TBS) for 10 min at RT, followed by a 2 × 5 min rinse in the same buffer solution, and subsequently a 30 min pre-treatment with 10% normal goat serum (Dako) in 0.03 M TBS and 0.1% coldwater fish gelatin (Sigma). Incubation with the primary rabbit antisera (SctW (1/50), SctC (1/50), and IncA (1/200)) lasted for 17 h at 4 °C. The rabbit pre-immunization serum (1/50) was used as negative control. After rinsing, the sections were exposed to gold-conjugated goat anti-rabbit IgG (GAR/G20; British BioCell, Cardiff, UK, diluted 1/20) for 2 h at RT. Finally, after rinsing in TBS (2 × 5 min) and BiDest (10 × 2 min), the sections were postfixed with 2% glutaraldehyde (1 × 5 min) and contrasted with uranyl acetate and lead citrate. Examination was done with a Philips CM10 transmission electron microscope at 80 kV (FEI Systems, Eindhoven, The Netherlands).

2.6. T3S gene expression analysis

Total RNA from 2×10^5 infected BGM cells (MOI 13) was prepared using the Total RNA Isolation Reagent (TRIR, ABgene, Westburg, Leusden, The Netherlands) according to the manufacturer's protocol. RNA from uninfected cells served as a negative control. After RNA extraction, samples were treated with RNase-free amplification grade DNase I (Promega) following the manufacturer's instructions and were confirmed to be DNA-free by performing a PCR for the *Cp. psittaci* 16S rRNA gene. Half a microgram of total RNA was reverse transcribed for 2 h at 47 °C using selective hexamers (5′TTANNN3′/5′CTANNN3′). Reverse transcription was followed by an incubation at 75 °C for 10 min. Each RNA sample was spiked with 5 ng coliphage MS2 control present in the Reverse-IT™ 1st Strand Synthesis Kit (ABgene). A reverse transcriptase minus control was performed

Table II. Oligonucleotide primers used for RT-qPCR analysis.

Cluster	Gene	Forward primer sequence	N[a]	Reverse primer sequence	N	Size (bp)
Cluster 1	*scc1*	ACTTCGGGAAACATTGCTGT	0	TGCCTAGGACATGTAGATCAGG	0	292
	sctW	CATGATTCGGGATTCTCAGG	0	CAATACCTTGAAGGGCAGGA	0	302
	sctV	CAGTAGAACCTTTGGGGGAGA	0	CAAAGCCACCATCAATAGGAA	0	181
	sctU	GGAAAAGTTTGAGGTTAAACAGGA	0	TTTCCTTCATCCCAAAGCTG	0	315
Cluster 2	*sctJ*	TTAAACGCTTGGTTGCCAGT	0	TGGATCGAAGAACCCTTTTG	0	299
	sctL	GGCTTTTTCAGCTCTGCTTG	0	GAAACAATGGCTTCTGGATGA	0	288
	sctR	ACGCAATACGCCAAAAAGTC	0	CAACCATCCACCATCACAAC	0	291
	sctS	ACTCGCCGCAAGCTTTAGAT	0	GGAAGATTTGAGAAGCAAAACG	0	254
	sctT	TCNCCTCATGGGATCTTCTATC	1	TGAAGGCTTTCAANGCAGAA	1	322
	scc2	CCGGATTAACTCTCCAGCAA	0	CTTCGGTTTGGTCGATCTTC	0	303
	copB1	TCTACACCNCAAGCTGTACCC	1	AGAGTGGTCTCNAATGTTTTGTT	1	323
	copD1	GGTCCCAAAGGACTTCAACA	0	CCCGAGATCATGCTTTGTTT	0	299
Cluster 3	*sctD*	GCAGCTCANGAAGAAGAAGAAA	1	TGATGAAAGACAAAGCNTCCA	1	310
	sctN	TGATGGTCACGTTGTCTCGT	0	TTGCGAGGCTTCCTCATAAT	0	301
	ca037	TCCAGTGAGGTTGTTGTGGA	0	AACCAGCAAGCTGAGAAGGA	0	301
	sctQ	TGCGGATTAGACGGAAAAAC	0	TCAACAAAACGACCACCAAA	0	299
	pkn5	CTTCGTAAGTCAATGGGGTCA	0	CCATCACCTATTTCCCATGC	0	291
	sctC	CTTGCAAGTCCCTGATGGTT	0	GGGGTGTTGACATTCAGGAG	0	298
Non-clustered	*incC*	TGACCTCTGTAAGAACCGATTT	0	GCCATAGGACCNCTGCAA	1	320
	incA	AACATGGTGGATGCTGTGAA	0	TCTGCCGTTTCTGTGGTAGA	0	299
	scc3	TTTCCNGATGATTTGGANAGTT	2	GCATANGCATGTAAAGCTTGNG	2	275
	copD2	CCAAGAGCAACAACANCATAAA	1	TTAGCTTGACTTTCNTAACTTTCAA	1	309
	cap1	CCTANATGATGACAGCNAACCA	1	GCCAANCGAACTGAAGCAG	1	283
	tarp	CAACAGCAGAGCAAGCNGTA	1	ACAGANGGGGTGGATAGGAA	1	493
	ca530	GCCGNTGTCATGCTTCNTAT	2	NCGTATNTCTTGAATAGTNAGNTGC	4	315
	cadd	CAATCAAAAACACATGTTAAACCA	0	TTGAGCTGCTGCACTAGGAA	0	301
Normalizing	16*s*	GTCAAGTCAGCATGGCCCTT	0	CCCAGTCATCAGCCTCACCT	0	300
	rsbV2	GAAGGNGCGTTAGATGCTGT	1	CATTCTGTTCTGATTGGCANA	1	333
	MS2	UNKNOWN SEQUENCE	0	UNKNOWN SEQUENCE	0	462

[a] N represents the number of degenerations per primer.

to check for DNA contamination. All the experiments were performed twice.

Following cDNA synthesis, cDNA amplification was performed for 26 T3S genes, the *Cp. psittaci* 16S rRNA normalization gene and the MS2 spike using the Absolute™ QPCR SYBR® Green Mix (ABgene). The DNA polymerase was initially activated for 15 min at 94 °C. Then 55 cycles of amplification were carried out according to the following protocol: cDNA was denatured at 94 °C for 20 s, primers (Tab. II) annealed at 55–60 °C for 30 s and extended at 72 °C for 40 s with a final incubation at 72 °C for 10 min. Program settings included acquisition on the FAM/Sybr channel in the extension step and a gain of six. Quantification was done by using standard graphs of the cycle threshold (Ct) values obtained by testing tenfold serial dilutions (10^6 to 10^3 molecules/µL, except for 16S rRNA: 10^9 to 10^1 molecules/µL for normalizing purposes) of the purified PCR products. All samples and standards were tested in duplicate. Ct-values of the samples were automatically converted into initial template quantities (N_0) by use of the RotorGene Software 6.0 (Westburg) using imported

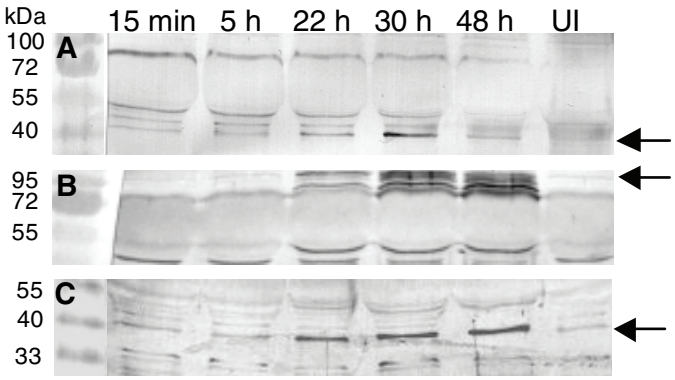

Figure 1. Detection of chlamydial proteins (SctC, SctW, IncA) by immunoblotting. Proteins from whole cell lysates of *Cp. psittaci* 92/1293 infected (different timepoints) or uninfected (UI) BGM-cell monolayers were resolved in 12% (w/v) acrylamide gels. Immunoblotting was performed with anti-sera generated in rabbit. Proteins were visualized by probing with biotin labeled anti-rabbit antibodies, streptavidine-HRP and development with AEC. Arrows indicate the bands at the expected molecular masses: A: SctW (43 kDa), B: SctC (99.5 kDa), C: IncA (41.8 kDa).

standard curves from previous runs. Quantification results of the coliphage MS2 RNA were used to correct for inter-sample variability, while quantification results of the chlamydial 16S rRNA were used to correct for bacterial growth during the developmental cycle. In order to use multiple messages for normalization, we additionally evaluated *rsbV2*, relatively constantly expressed during the *C. trachomatis* developmental cycle from 1 h to 48 h p.i. [8]. Unfortunately, *Cp. psittaci rsbV2* was only expressed from 12 h p.i. onwards and could therefore not be used for normalization. Expression categories were defined as early (1.5–8 h p.i.), middle (12–18 h p.i.) and late (by 24 h p.i.).

To compare the two independent samples (gene expression ratios of 26 genes from two independent infection experiments, each 16S rRNA normalized) at the same timepoint, a Paired Student's T-test was used (SPSS Inc., Chicago, IL, USA). Secondly, an analysis of variance (ANOVA, SPSS Inc.) with post hoc analysis was performed along the time axis to determine significant upregulation time points for each gene.

3. RESULTS

3.1. Sequencing of *Cp. psittaci* T3S genes

The following 14 full-length T3S genes were successfully sequenced: cluster 1 genes *scc1* and *sctW*, cluster 2 genes *sctJ*, *sctL*, *sctR*, *sctS*, *scc2*, and *copD1*, cluster 3 genes *sctN*, *ca037*, *sctQ*, and *sctC*, and finally the non-clustered T3S genes *incA* and *cadd*. An overview of the properties and similarities of these 14 full-length *Cp. psittaci* T3S genes as defined by SMART and CDART is shown in Table III. The NetPhos server predicted 27 possible phosphorylation sites for *Cp. psittaci* IncA and 30 for SctW. We aligned the four *Cp. psittaci* IncA sequences available in GenBank® (National Center for Biotechnology Information) with our own genotype D strain *Cp. psittaci* 92/1293 IncA sequence at both the DNA and protein level. Protein identity varied from 99.0% (with strain NJ1 belonging to the same genotype D) to 96.1% (with VS225, a genotype F strain) and similarity was between 99.2% (again with NJ1) and 96.6% (with both VS225 and CP3, the latter being a genotype B strain).

3.2. Western blotting

Western blot studies indicated SctW, SctC, SctN, and IncA to be expressed by *Cp. psittaci* strain 92/1293. Protein bands corresponding to the expected masses of SctW (43 kDa), SctC (99.5 kDa), SctN (48.3 kDa), and IncA (41.8 kDa) were first detected at 15 min, 22 h, 48 h, and 22 h p.i., respectively (Fig. 1). For

Table III. Properties and similarities of the 14 full-length *Cp. psittaci* T3S genes. Properties and Genbank accession numbers of genes sequenced in this study, as defined by SMART and CDART analysis of in silico generated protein sequences (AA stands for amino acids). Homologies of the sequenced T3S genes to those in other *Chlamydiaceae* are presented as the percentage of sequence identity with the corresponding gene in either *Cp. pneumoniae* or *C. trachomatis*.

Cluster	Gene	Protein	Properties and similarities as defined by SMART and CDART	% identity with *Cp. pneumoniae*	% identity with *C. trachomatis*	Accession number
Cluster 1	*scc1*	Specific *chlamydia* chaperone 1	146 AA. Similarity with enteropathogenic *E. coli* (EPEC) CesT, as a chaperone for the T3SS translocated intimin receptor (Tir). Absence of transmembrane domains.	69.1	66.2	DQ299384
	sctW	Secretion and cellular translocation protein W	398 AA, low complexity regions at positions 2 to 35 and 116 to 133. Classified in the InvE family of *Salmonella* spp. invasion proteins by CDART.	65.8	55.2	DQ299385
Cluster 2	*sctJ*	Secretion and cellular translocation protein J	330 AA, low complexity regions at positions 57 to 67, 237 to 251, and 289 to 301, transmembrane domain at position 238–260. Similarity with the Yersinia secretion protein J (YscJ) and the flagellar biosynthesis protein FliF.	74.3	71.2	DQ299387
	sctL	Secretion and cellular translocation protein L	229 AA, coiled-coil at position 35 to 93. Similarity with the flagellar biosynthesis protein FliH.	70.9	67.2	DQ299388
	sctR	Secretion and cellular translocation protein R	305 AA, transmembrane regions at positions 4 to 26, 92 to 114, 134 to 156, 246 to 268, and 278 to 300. Similar to the flagellar biosynthesis proteins FliP and FliO.	72.2	69.6	DQ299389
	sctS	Secretion and cellular translocation protein S	94 AA, transmembrane domains at positions 21 to 43 and 53 to 75, low complexity region at position 19 to 36 within the first transmembrane domain. Similarity with *E. coli* secretion protein S (EscS).	77.4	75.4	DQ299390
	scc2	Specific *chlamydia* chaperone 2	227 AA, low complexity regions at positions 2 to 15, 28 to 35, and 203 to 221 and two tetratricopeptide repeat (TPR) regions below the threshold at positions 78 to 111 and 112 to 145. Presence of the TPR after finding TPR containing homologous chaperones by CDART.	69.7	74.0	DQ299391
	copD1	*Chlamydia* outer protein D1	440 AA, nine regions of low complexity at the following positions: 14–35, 62–102, 134–148, 158–169, 183–197, 202–217, 228–309, 335–345, and 396–419. No similarities found by CDART.	64.8	63.7	DQ299392

Table III. Continued

Cluster	Gene	Protein	Properties and similarities as defined by SMART and CDART	% identity with *Cp. pneumoniae*	% identity with *C. trachomatis*	Accession number
Cluster 3	*sctN*	Secretion and cellular translocation protein N	442 AA, small low complexity region at position 99 to 111, large ATPase associated activity (AAA) domain at position 163 to 345. Identity and similarity higher than 90% for all species within the *Chlamydiaceae*.	77.4	76.8	DQ299393
	ca037	Hypothetical protein	282 AA, low complexity regions at positions 70 to 87, 106 to 125, and 252 to 274.	65.0	62.7	DQ299396
	sctQ	Secretion and cellular translocation protein Q	371 AA, low complexity region at position 288 to 304. Similarity with the flagellar biosynthesis protein FliM.	65.1	63.0	DQ299394
	sctC	Secretion and cellular translocation protein C	First 710 bp of the *sctC* gene were missed. 672 AA, low complexity regions at positions 256 to 265 and 283 to 290. In the same region, a RhoGAP (GTPase activator protein for Rho-like GTPases) domain was found (position 131–297). SMART found a TPR domain below the threshold at position 429 to 462, which was confirmed using CDART. As for SctN, very high conservation of SctC among *Chlamydiaceae*.	76.8	76.1	DQ299395
Non clustered	*incA*	Inclusion membrane protein A	380 AA, transmembrane regions at positions 69 to 91 and 96 to 115 and a coiled- coil at position 224 to 296. A t_SNARE domain, related with intracellular vesicle fusion, endocytosis and exocytosis, was found at position 254 to 318.	46.3	38.5	DQ299386
	cadd	*Chlamydia* protein associated with death domains	271 AA, stop codon at position 14, which was absent in the *Cp. caviae* and *Cp. abortus* orthologues. Transmembrane domain at position 5 to 27, low complexity region at position 244 to 265. Death domain at position 86 to 178, less significant than the threshold, and RasGEFN (Guanine nucleotide exchange factor for Ras-like GTPases) domain (position 15 to 140).	52.7	57.4	DQ299397

SctW, an additional protein band of approximately 80 kDa was observed from 15 min p.i. onwards, as well as two additional bands at 45–50 kDa. For SctC, the 99.5 kDa signal was accompanied by clear signal at 50 kDa and a clear band at ∼80 kDa. For SctN, only a weak signal at 48.3 kDa could be observed at 48 h p.i. (data not shown). A protein band of approximately 53 kDa accompanied the IncA signal of 41.8 kDa.

3.3. Intracellular localization of SctW, SctC, SctN, and IncA

To determine the localization of the chosen T3S proteins, both immunofluorescence and immuno electron microscopy were carried out. SctW was detected from 25 to 50 h p.i. and the signal was most intense from 30 to 35 h p.i. SctW staining was associated with the inclusion with a rim like pattern of more intense fluorescence at the periphery of the inclusion (Fig. 2A). Immuno electron microscopy showed labeling of both the reticulate body and the inclusion membrane (Fig. 3G). Non-specific background labeling was not observed (Fig. 3A). SctC was detected from 15 to 30 h p.i. and the fluoresent signal for *Cp. psittaci* and SctC colocalized inside the host cell (Fig. 2B), which was confirmed by immuno electron microscopy (Fig. 3C, 3D). The fluorescent signal for SctC was, in contradiction to the one for SctW, more evenly distributed throughout the inclusion. SctN was detected from 15 h p.i. until 50 h p.i., with a maximum signal at 30 to 35 h p.i. Like for SctW, SctN staining was more intense at the periphery of the inclusion (Fig. 2C). IncA was expressed from 22 h till 45 h p.i. and was associated with both *Cp. psittaci* and the inclusion membrane (Fig. 3F).

3.4. T3S gene expression analysis

After proving the existence of a *Cp. psittaci* T3SS, RT-PCR assays were carried out on *Cp. psittaci* 92/1293 RNA to investigate the transcription of the 14 currently full-length sequenced *Cp. psittaci* T3S genes and of 14 T3S genes for which full-length sequences could not be obtained but for which consensus RT-PCR primers could be designed (*sctV*, *sctU*, *sctT*, *copB1*, *sctD*, *pkn5*, *incB*, *incC*, *scc3*, *copB2*, *copD2*, *cap1*, *tarp*, *ca530*). RT-PCR primers were first checked by standard PCR and qPCR on *Cp. psittaci* 92/1293 genomic DNA. All primer couples, except the ones for *incB* and *copB2* yielded a specific PCR product of the expected size and melting temperature. *IncB* and *copB2* were therefore excluded from the expression studies. The obtained Ct values were used as a measure for the initial (c)DNA and RNA template quantity and quantification was performed for a total of 28 reactions (26 chlamydial T3S genes, 16S rRNA and the internal MS2 spike).

Expression was detected for 24 of 26 examined T3S genes as *sctT* and *ca530* were apparently not expressed. Statistical analysis confirmed the absence of significant differences between mRNA expression ratios from two independent infection experiments (Student's T-test). The ANOVA test revealed significant upregulation of gene expression along the time axis. An overview of the upregulation time points before and after 16S rRNA normalization is given in Table IV.

For gene cluster 1, comprising *scc1*, *sctW*, *sctV*, and *sctU*, expression results revealed *sctW* and its putative chaperone *scc1* as well as *sctV* to follow the same transcription pattern with peak expressions at 12 h p.i. during the middle phase of the developmental cycle, and less prominent peak expressions at 36 h p.i. For *sctV*, an additional minor expression peak was present at 4 h p.i. The *sctU* gene transcription seemed to follow the same pattern, with the exception of a first peak at 16 h p.i. instead of 12 h p.i.

For gene cluster 2, represented by *sctJ*, *sctL*, *sctR*, *sctS*, *sctT*, *scc2*, *copB1*, and *copD1*, results were as follows. *SctJ*, encoding the FliF homologue of the flagellar rotor (MS ring), was constitutively highly expressed throughout the developmental cycle, reaching a peak expression at 36 h p.i. *SctL* and *sctR* principally showed the same gene expression pattern, with a primary mRNA peak at 12 h p.i. during the mid-cycle period, and a second one at 36 h p.i. during the late stage of

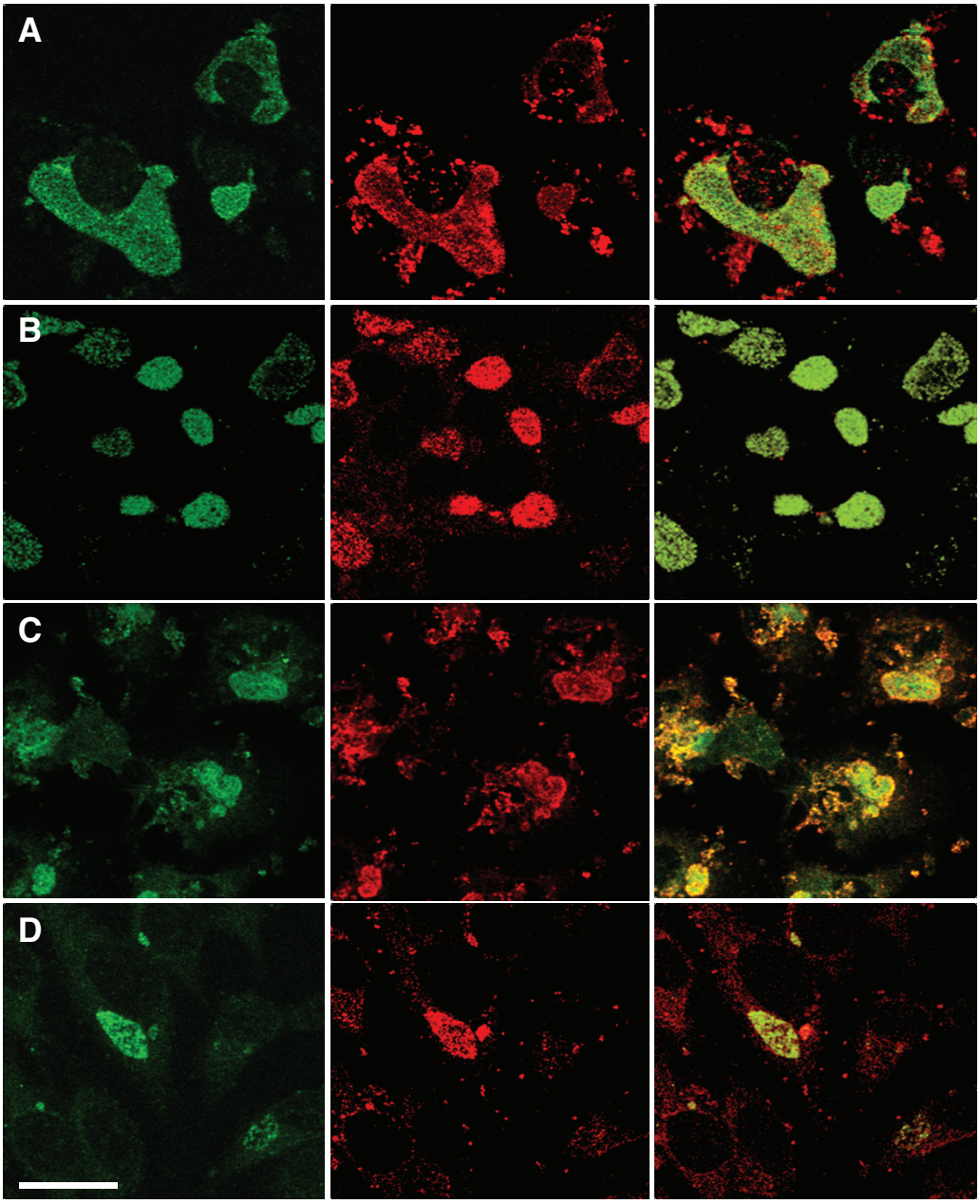

Figure 2. Cellular localization of SctW, SctC, SctN, and IncA (×600) at 28 h p.i. of BGM-cells. Staining was performed as described in experimental procedures and representative confocal images are shown. From left to right: *Cp. psittaci* inclusion staining, T3S protein staining and merged images. A: SctW staining was predominantly found in the inclusion with more intense staining at the periphery. B: SctC colocalized with the bacteria inside the host cell. C: for SctN a more intense staining of the periphery of the inclusion could be observed. D: IncA staining was associated with both *Cp. psittaci* and the outer boundaries of the inclusion. The bar represents 25 µm (a color version of this figure is available online at www.vetres.org).

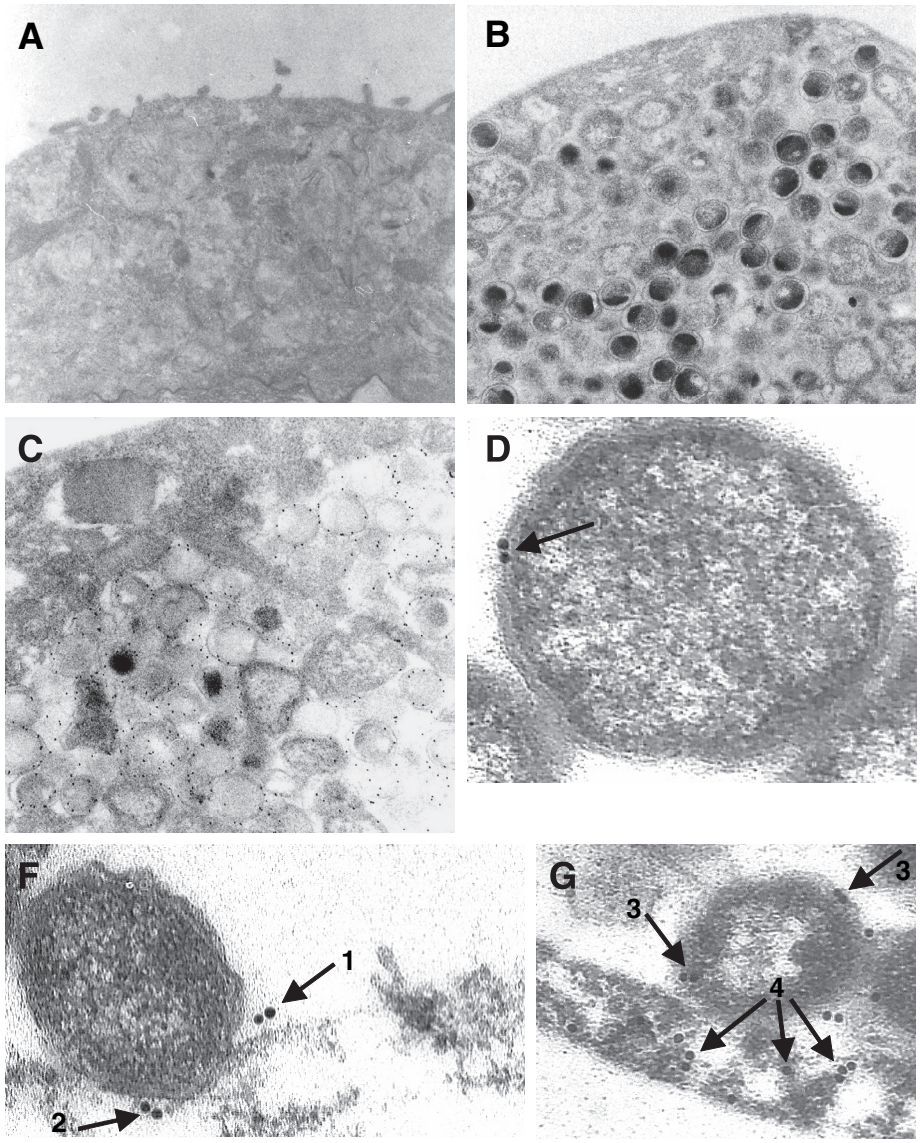

Figure 3. Ultrastructural localization of SctW, SctC, SctN, and IncA at different time points p.i. of BGM-cells. Immuno electron microscopy was performed as described in the materials and methods section. Representative images are shown. A: uninfected BGM monolayer stained with the antibodies generated in rabbits (×3200). B: infected BGM monolayer stained with rabbit pre-immunization serum (×3200). C: overall view of the SctC staining (×3200) at 30 h p.i. of BGM-cells. D: BGM culture, 30 h p.i. with *Cp. psittaci.* Gold labelling (arrow) of SctC, part of the secretin in the outer membrane of a reticulate body (×8900). F: BGM culture, 22 h p.i. with *Cp. psittaci.* Gold labelling of secreted IncA (arrow 1) and localization of the effector protein in the inclusion membrane (arrow 2), (×11500). G: BGM culture, 48 h p.i. with *Cp. psittaci.* SctW gold labelling. Notice the presence of SctW in both the reticulate body (arrow 3) and the inclusion membrane (arrow 4, which at the end of the developmental cycle apparently dissolves [4] near the host cell membrane (×8900).

the developmental cycle. *Scc2* was expressed at 6 h p.i., and only slightly upregulated at 12 h p.i. Its close neighbour *copD1* on the other hand was upregulated at 6 h, 12 h, and especially 36 h p.i.

SctD, *sctQ* and *sctC*, all belonging to the T3S gene cluster 3, were first upregulated at 12 h p.i. and subsequently at 36 h p.i., although the latter signal was rather faint compared to the one observed during the middle stage of the developmental cycle. *SctN* does not seem to be strongly upregulated and its largest relative expression was already observed at 1 h p.i. Expression of *ca037* peaked at 36 h p.i.

A pool of *incA* mRNA seems to exist as maximal relative *incA* expression was observed at 5 min p.i., with a second strong signal at 10 h p.i. This was not the case for *incC*, reaching its maximal expression level at 16 h p.i., which was however still very low in comparison to *incA* expression levels. The *cap1* and *scc3* genes showed their highest relative expression level at 1 h p.i. Expression of *tarp* peaked at 36 h p.i.

4. DISCUSSION

4.1. Sequencing of *Cp. psittaci* T3S genes

A detailed discussion of the results of the in silico analysis of sequenced *Cp. psittaci* T3S genes can be found in the Appendix (available online only at www.vetres.org).

4.2. Sequencing, expression, and intracellular localization of SctW, SctN, SctC, and IncA

SctW of *C. trachomatis* shows similarity to *Yersinia* YopN and would – like in *Yersinia* spp. – be an important regulator of T3S in the absence of contact with a susceptible host cell. In immunoblotting *Cp. psittaci* SctW (∼40 kDa) could be detected from 15 min on, with expression peaking at 30 h p.i. (Fig. 1). This is in agreement with *C. trachomatis* SctW expressed at 20 h p.i. [9]. On the contrary, Lugert et al. [23] could not detect *Cp. pneumoniae* SctW until 48 h p.i. Possible explanations for the two additional bands at 45–50 kDa are phosphorylation as 30 possible phosphorylation sites were predicted by the NetPhos server, and association with a chaperone protein [32]. The ∼80 kDa band could represent a SctW dimer, although, unlike for *C. trachomatis* SctW [9], no coiled-coil domain could be predicted. Previous immunofluorescence experiments in *C. trachomatis* and *Cp. pneumoniae* and present studies in *Cp. psittaci* showed inclusion membrane and inclusion body associated SctW [9,16,23]. This supports the hypothesis of SctW being a soluble protein blocking the transmembrane channel [9, 33].

SctC proteins are the only T3SS proteins that are clearly localized in the outer membrane and they have no flagellar homologue. In *Yersinia*, the SctC homologue YscC is absolutely required for a functional T3SS [26]. Membrane localization was confirmed for *C. trachomatis* [12]. As such, membrane localization was also expected for *Cp. psittaci* SctC, considering the high homology between the chlamydial SctC proteins and the presence of two transmembrane domains. For *C. trachomatis*, polymorphic SctC bands have been observed in immunoblotting, but unfortunately their molecular weights were not mentioned [12]. Similar findings were obtained for *Cp. psittaci* SctC. A 100 kDa protein was expected and detected from 22 h p.i. on as well as proteins of ∼50 kDa and ∼80 kDa (Fig. 1). The physical relevance of the observed polymorphisms remains to be elucidated. It is however possible that the full length protein is processed in fragments of less than 100 kDa. In both immunofluorescence and immuno electron microscopy, SctC colocalized between 15 h and 30 h p.i. with the bacteria in the inclusion, supporting its structural function in the T3SS.

SctN probably delivers the energy for the chlamydial T3SS as *C. trachomatis* SctN contains two nucleotide binding domains [9] and shows high homology with the *Yersinia* ATPase YscN [44] and other bacterial ATPases. As SctN is highly conserved in *Chlamydiaceae*, it was no surprise to find a large ATPase associated activity domain in *Cp. psittaci* SctN. Lugert et al. [23] found *Cp. pneumoniae* SctN in Hep-2 cell lysate at 48 h p.i. using immunoblotting. SctN was very difficult to detect in *Cp. psittaci*, as only a weak

Table IV. T3S gene expression patterns for *Cp. psittaci* in BGM cells. Total RNA from 2×10^5 BGM cells inoculated with *Cp. psittaci* 92/1293 at an MOI of 13 was isolated at 0, 5, 15, 30, and 60 min p.i. and subsequently every two hours until 24 h p.i., and finally at 36, 48, and 60 h p.i. The experiments were performed twice and all samples were tested in duplicate. For each gene the timepoint at which a specific PCR product was first detected is provided (timepoint at which the absolute copy number without 16S rRNA normalization was higher than 100). In order to describe the gene expression profile for each of the genes under study, the timepoint where maximal significant upregulation of the genes was observed is given in the fourth column, as determined by an ANOVA with post hoc analysis along the time axis (both Tukey HSD and Tukey-b test, $P < 0.01$). The last column describes further significant upregulation time points after 16S rRNA normalization, in order of their importance.

Cluster	Gene	First detection	Major upregulation timepoints	Secondary timepoints of upregulation
Cluster 1	*scc1*	12 h	12 h	36 h
	sctW	12 h	12 h	60 h and 36 h
	sctV	12 h	36 h	12 h and 4 h
	sctU	16 h	16 h	36 h
Cluster 2	*sctJ*	5 min	36 h	
	sctL	12 h	12 h	
	sctR	12 h	12 h	36 h
	sctS	/[a]	/	/
	sctT	/	/	/
	scc2	6 h	6 h	12 h
	copB1	36 h	36 h[b]	
	copD1	1 h	36 h	6 h and 12 h
Cluster 3	*sctD*	12 h	12 h	36 h
	sctN	1 h	1 h[b]	
	ca037	10 h	36 h	60 h and 12 h
	sctQ	12 h	12 h	36 h
	pkn5	24 h	*	*
	sctC	12 h	12 h	36 h
Non-clustered	*incC*	16 h	16 h	
	incB	NT	NT	NT
	incA	5 min	5 min	10 h
	scc3	1 h	1 h	12 h
	copB2	NT	NT	NT
	copD2	/	/	/
	cap1	1 h	1 h	
	tarp	12 h	36 h	
	ca530	/	/	/
	cadd	10 h	12 h	60 h
Normalizing	*16S rRNA*	5 min	–	–

[a]No signal observed.
[b]Faint signal.
*No upregulation following 16S rRNA normalization.
NT: not tested.

signal at the expected molecular mass was noticed at 48 h p.i. in immunoblotting. This could be due to the fact that a cross-reacting *Cp. pneumoniae* antibody was used and that cross-reactive epitopes were destroyed during SDS-PAGE. A clear staining of the chlamydial inclusion was noticed in immunofluorescence using the same antibody (Fig. 2), confirming that indeed SctN was present. In *Cp. pneumoniae*, SctN was shown to be associated with the cytoplasmic side of the inner bacterial membrane [23]. This is in accordance with the present immunofluorescence results demonstrating SctN inside *Cp. psittaci* inclusions.

Three different phosphorylated forms of *Cp. caviae* IncA could be visualised in immunoblotting, each with a different molecular weight [7, 29]. Although the NetPhos server predicted 27 possible phosphorylation sites for *Cp. psittaci* IncA (data not shown), only one band of 41.8 kDa could be detected. The protein band observed at 53 kDa might therefore reflect the association of IncA with a host protein located at the place of its insertion in the inclusion membrane, as interaction between IncA and host-proteins has been reported for *Cp. caviae* [29]. Immuno electron microscopy confirmed the predicted membrane associated topology.

4.3. T3S gene expression analysis

The expression of 26 T3S genes was studied during the *Cp. psittaci* developmental cycle in BGM cells and results were compared to T3S gene expression studies in *C. trachomatis* and *Cp. pneumoniae*. Generally accepted temporal expression categories, based on when specific chlamydial mRNA are first detected, were defined as early (1.5 to 8 h), middle (12 to 18 h), and late (by 24 h) [33]. The early-cycle expression category contains predominantly macromolecular synthesis genes and genes encoding products that modify the inclusion membrane, whereas intermediary metabolism and structural components are major representatives of mid-cycle genes. Typical late-cycle genes include those involved in terminal differentiation of RB to EB [31].

Cp. psittaci scc1, *sctW*, and *sctV* gene expression was detected from 12 h onwards, while *sctU* expression was observed from 16 h p.i. onwards. The general trend for all studies in *Chlamydiaceae* [12, 23] is the finding of coding mRNA from mid-cycle (12 h p.i.) onwards, which implicates that T3S cluster 1 proteins are not de novo synthesized during the early stages of the developmental cycle.

Cp. psittaci sctJ expression already occurred 5 min p.i. and *sctJ* was constitutively expressed throughout the developmental cycle. This is in contradiction with results obtained for *C. trachomatis* and *Cp. pneumoniae*, where SctJ mRNA was first detected from mid-cycle onwards (12 h and 8 h p.i., respectively) [12, 31, 33]. However, SctJ is constitutively present in *C. trachomatis* infected HeLa cell lysate collected from 1 h to 18 h p.i. [12], supporting the presence of a functional T3SS apparatus prior to de novo synthesis of its different components. For *sctS* and *sctT* no expression could be observed in *Cp. psittaci*, which is in contrast with the results for *C. trachomatis* and *Cp. pneumoniae* [12,23,33]. *Cp. psittaci copB1* and *copD1* transcripts appeared at 36 h and 1 h p.i. respectively. In *C. trachomatis* however, expression of both genes was first noticed at 20 h p.i. [12], while in *Cp. pneumoniae*, *copB1* was expressed at 48 h p.i. [23]. Because no time points were examined between 24 h and 48 h p.i. in the study of Lugert et al. [23], it cannot be excluded that *Cp. pneumoniae* expresses *copB1* earlier, like in the present study. However, middle to late cycle expression of putative translocator components is strange, since one would expect that they are necessary to guide effector proteins through the inclusion membrane. On the other hand, CopB1 and CopD1 proteins are detected in lysed purified *C. trachomatis* EB, again illustrating that a fully functional T3SS is present at the early stages of the chlamydial developmental cycle [12].

Cp. psittaci sctN was relatively highly expressed at 1 h p.i. and subsequently at rather small quantities during the whole developmental cycle. In *C. trachomatis* and *Cp. pneumoniae*, *sctN* expression was detected at 12 h and

8 h p.i., respectively, and the expression level seemed to be higher than for *Cp. psittaci* SctN [12, 23, 31, 33]. However, as SctN is supposed to be the T3SS energizer, it is unlikely that *Cp. psittaci* SctN would be produced in smaller quantities compared to *C. trachomatis* or *Cp. pneumoniae* SctN. Moreover, immunofluorescence clearly showed the presence of SctN in chlamydial inclusions (this study). Maybe *Cp. psittaci sctN* mRNA is more rapidly degraded, resulting in lower detection. Further examination is needed.

When we turn to the expression results of (putative) effector proteins, *incC* expression was first detected at 16 h p.i. This is in contrast with results obtained for *C. trachomatis* and *Cp. pneumoniae*, where *incC* transcript was already detected at 2 h p.i. [12, 23]. The *Cp. psittaci incA* gene was first expressed at 5 min p.i., followed by a transcription upregulation at 10 h p.i. A comparable result was obtained for *Cp. pneumoniae*, where *incA* expression was also found from the first investigated time point (2 h p.i.) on. *Scc3* expression was already noticed 1 h after infection with *Cp. psittaci*, analogous to what was found for *Cp. pneumoniae* [33]. This corresponds to the protein's putative chaperone function in SctW secretion [32]. Class I accessible protein-1 (Cap1) is inserted into the inclusion membrane of *C. trachomatis* possibly after T3S and is able to stimulate MHC class I $CD8^+$ T-cells [13]. The coding gene is expressed during the early stages (4 h p.i.) of the *C. trachomatis* developmental cycle [2]. Current results show that the *cap1* gene is expressed in *Cp. psittaci*, reaching a maximal relative expression level at 1 h p.i. Transcription of the Tarp encoding gene was found to occur late during the *Cp. psittaci* developmental cycle (Tab. IV). This implicates that Tarp might have the same function as in *C. trachomatis* where it plays an important role in chlamydial attachment and that Tarp is stored in the EB until attachment to a new host cell.

The T3S protein translation study revealed the expression of SctW, SctC, SctN and IncA present in the lumen of the inclusion (SctC and SctN) or associated with the inclusion membrane (SctW and IncA). The present gene expression study clearly demonstrates the transcription of structural components of the *Cp. psittaci* 92/1293 T3SS from 8 h to 12 h p.i. indicating that the T3SS is possibly newly assembled from mid-cycle on. Genes encoding effectors and putative T3S related proteins were expressed early or late during the developmental cycle. In this way, we are the first to provide evidence for the presence of a *Cp. psittaci* T3SS most likely assisting in the establishment of an optimal intracellular environment for bacterial growth. Further research is required to examine the role of T3S in the context of infection biology possibly unique to *Cp. psittaci.* Currently, experiments addressing this issue are in progress.

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