

APPENDIX

Sequencing of *Cp. psittaci* T3S genes: discussion on the in silico analysis of the *Cp. psittaci* 92/1293 sequenced T3S genes

The specific chlamydia chaperone 1 or Scc1 is encoded by the *scc1* gene located in T3S gene cluster 1 and has already been detected in *C. trachomatis* purified EB, RB, and infected HeLa cultures 20h p.i. using immunoblotting [9] but not yet in *Cp. pneumoniae* [20]. Immunofluorescence staining of *C. trachomatis* Scc1 suggested that this protein was retained by chlamydiae, resulting in a homogenous fluorescent distribution within the bacterial cytoplasm. Additional evidence for this cytoplasmic distribution was given by Fields et al. [11], showing the absence of Scc1 in chlamydial membrane fractions. The absence of transmembrane domains in *Cp. psittaci* Scc1 suggests that its location will probably be within the bacterial cytoplasm like observed for *C. trachomatis* [9]. Scc1 proteins are homologous to the SycE family of cytoplasmic chaperones to LcrE (SctW) homologues [15], with a helix binding groove [22]. Scc1 was thought to be the chaperone of the putative T3S regulator SctW, but strange enough, no interaction between Scc1 (SycE) and SctW (LcrE) of *Cp. pneumoniae* could be detected [27], despite the fact that chaperones are expected to assist proteins that are encoded in close proximity on the genome [7]. In contrast, interaction between Scc3 (LcrH-2), not encoded by gene cluster 1, and SctW (LcrE) was observed for *Cp. pneumoniae*, *Cp. psittaci*, and *C. trachomatis* [27].

C. trachomatis SctJ was shown to be similar to the periplasmic lipoprotein YscJ of *Yersinia* spp. and was already detected in purified *C. trachomatis* EB, RB, and infected HeLa cell cultures throughout the whole developmental cycle [11]. The protein was only detergent soluble and purely chlamydia-

localized. Thus, it does not reach the inclusion membrane like Incs do. Its function might be bridging the inner and outer bacterial membrane [2] and/or being a basal needle complex component analogous to its homologue PrgK in *Salmonella* [18]. The *Cp. psittaci* SctJ sequence was shown to contain a transmembrane domain, indicating that the protein will probably be bacterial membrane localized.

As proposed by Fields and Hackstadt [9], *C. trachomatis* SctL might interact with SctN, as has been shown for *Yersinia* YscL and YscN [17]. A similar interaction could be present between *Cp. psittaci* SctL and SctN, and the presently found coiled-coil could play an important role in this process.

Cp. psittaci SctR, encoding part of the exporter revealed five transmembrane regions, where only three or four were expected [13, 16]. However, only one amino acid was located in between the fourth and fifth domain, indicating that they are either hard to distinct or they form one single domain.

Cp. psittaci SctS, also encoding part of the exporter, contains two transmembrane domains, confirming the prediction of Hueck [16]. SctS expression in *Cp. pneumoniae* and *C. trachomatis* could be observed from mid-cycle on [11, 28]. Whether the protein is functional in *Cp. psittaci* needs to be investigated since no transcript was detected with RT-qPCR (this study).

Scc2 (SycD or LcrH-1) is a predicted chaperone known to be localized to both the cytoplasmic and the membrane fractions of *C. trachomatis* EB, as equal amounts were detected in the water and detergent soluble fractions following immunoblotting

[11]. As no transmembrane regions were found in *C. trachomatis* Scc2, nor in *Cp. psittaci* Scc2, this membrane localization could only be the consequence of Scc2 association with an integral membrane protein. All known Scc2 and Scc3 chaperones of chlamydiae, including the one from the presently examined species *Cp. psittaci*, have three tetratricopeptide repeats (TPR) and as these domains are very important in protein-protein interactions and formation of multi-protein complexes [19], the interaction with this integral membrane protein could occur through the TPR. However, their binding partners are not yet known, except for Scc3, which interacts with SctW and may function as its chaperone [27]. If the interaction occurs through TPR, it might be worth testing the interaction between Scc2 and SctW, although the Scc2 and Scc3 proteins are clearly different. These two putative chaperones have molecular masses of 25.9 kDa (231 AA) and 20.3 kDa (172 AA) and pIs of 9.35 and 5.83, respectively [27]. Moreover, both have a TPR with different amino acids at positions 8, 20, and 27 of the triple repeat. Fields et al. [8] have shown that both Scc2 and Scc3, after heterologous expression in *Yersinia*, are able to associate with the translocon component YopD. Why exactly *Chlamydiaceae* have two *lcrH* genes remains an open question, as well as why the encoded chaperones operate at different times in the developmental cycle, as the temporal expression data of Slepkin et al. [28] as well as present data seem to suggest.

Taken into account the composition of the CopB1/CopD1 operon and a hydrophobicity profile reminiscent of the *Yersinia* YopB/YopD translocon components [29], these proteins seem to be essential for the chlamydial T3SS. Since no transmembrane domains were found in the *Cp. psittaci* CopD1, the putative protein is suggested to have water-soluble properties and thus to be located in the cytosol. The fact that the *C. trachomatis* CopB1 was found in the aqueous fraction of purified EB lysate may support this hypothesis [11]. Both CopB1 and CopD1 are probably inserted in the inclusion membrane by the T3SS before they can exert their function.

Proteins belonging to the SctQ family are peripheral cytoplasmic proteins with a highly conserved C-terminus and a variable N-terminus. They are homologous to the flagellar proteins FliM and FliN [1, 13]. Functionally, they are supposed to link the conserved structural components to variable species-specific components [16]. Evidence was found by yeast two- and three-hybrid assays with YscQ, YscK, and YscL [17]. As both *Cp. psittaci* SctQ and SctL full length sequences were analyzed in this study, it will be possible in the future to check whether this interaction also occurs in *Chlamydiaceae*.

Cp. psittaci Ca037 is an orthologue of the *Cp. caviae* CCA00037 protein. In *Cp. caviae* CCA00037 is localized in the host cell cytosol [30]. The absence of any transmembrane domains in the *Cp. psittaci* orthologue indicates that it might also be cytosol localized.

We were unable to determine the whole coding sequence for SctC, as the first 710 nucleotides were missed. This was not completely surprising as the first 250 amino acids of chlamydial SctC proteins constitute a mostly hydrophilic domain that is comparatively divergent in primary sequence among chlamydial species: AA 1 to 250 of *C. trachomatis* SctC possess only 50% and 32% sequence identity to those of *Cp. caviae* and *Cp. pneumoniae*, respectively [10]. As in *C. trachomatis* SctC, a RhoGAP domain responsible for GTPase activation of Rho-like GTPases was present in *Cp. psittaci* SctC protein sequence. Rho-like GTPases are activated by guanine nucleotide exchange factors and inactivated by GTPase-activating proteins (GAP). The RhoGAP domain could influence actin polymerization of the host cell. Additionally, like in *C. trachomatis* SctC, a TPR was present which could assist in the formation of multimeric complexes [12, 19].

Cp. psittaci IncA possesses two transmembrane domains probably corresponding to the bilobed hydrophobic domains of 50-80 AA found in all T3S inclusion membrane proteins so far [4, 24]. The t₃SNARE domain recently found in *Cp. caviae* IncA [5] was also present in *Cp. psittaci* IncA. SNARE proteins

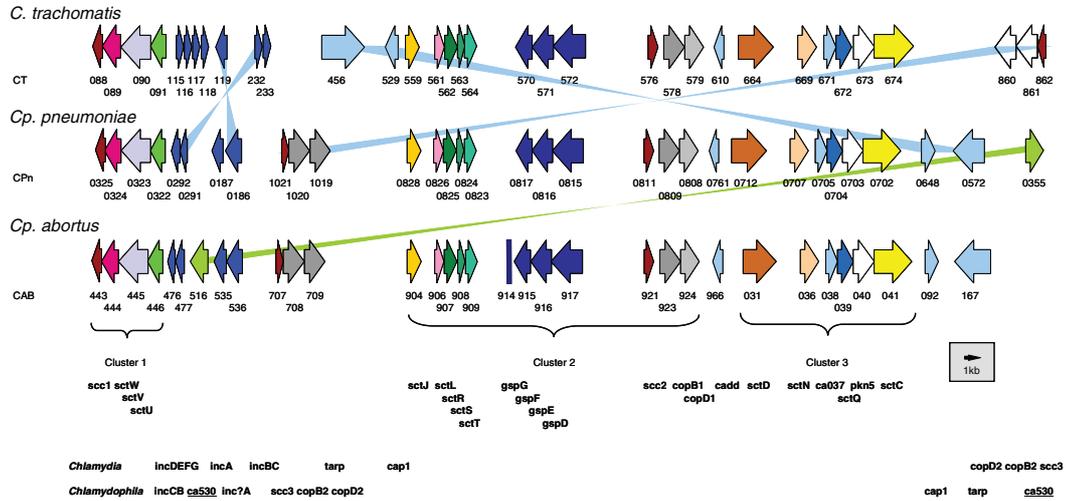


Figure A. Comparison of the genetic organization of the T3S genes of three different species of the *Chlamydiaceae*. Below each species, genes are numbered with the locus tag of the GenBank genome files (respectively NC_000117, NC_000922, and NC_004552). Genetic organization in *Cp. psittaci* is presumed to be highly similar to that of *Cp. abortus* as *Cp. psittaci* strains resemble what might have been recent ancestors of *Cp. abortus* [31]. Positions of corresponding homologues are indicated by color codes. Note that structural proteins (unified nomenclature after Hueck [16]) appear in the same order in both genera, while (putative) effector proteins do not. Distance within the clusters is in scale, between the clusters not.

share a seven amino acids repeat which forms a coiled-coil in the SNARE complex [6]. Coiled-coils are frequently observed protein motifs participating in oligomerization. Thus, the observed coiled-coil in the *Cp. psittaci* IncA (AA 224-296) could be part of the predicted t-SNARE domain (AA 254-318). The *C. trachomatis* IncA is known to facilitate homotypic vesicle fusion [14] and to interact with itself to form multimers [5, 14]. Interaction between IncA and host-proteins has been reported for *Cp. caviae*. The *Cp. caviae* IncA, localized at the host cytoplasmic face of the inclusion membrane, was shown to be phosphorylated by host kinases [23] and oligomerization with other IncA was responsible for interaction between facing membranes [5].

In silico translation of *Cp. psittaci* CADD revealed one stop site. In the same frame, a second start site was found, so the first stop might not be of biological importance. Regarding this, *Cp. psittaci* CADD would not have a transmembrane domain. The overall sequence similarity and "PqqC-like" annotation for

CADD proteins are reflected by the same fold, but the active sites are not conserved and the two proteins are therefore functionally and most likely also evolutionarily unrelated [21, 25]. CADD is consequently an orphan unique to *Chlamydiaceae* species, which further emphasizes its role as a highly specific redox enzyme modulating host cell apoptosis. Additionally, a RasGEFN (guanine nucleotide exchange factor for Ras-like GTPases) domain was found, in overlap with the death domain, which might influence the host cell actin polymerization process.

Unlike in other gram-negative pathogens, who have generally only one cluster, the structural components of the chlamydial TTSS are found on three different conserved clusters (pathogenicity islands) on the genome (Fig. A). The first structural cluster of T3SS genes (*sctU*, *sctV*, *sctW*, and *sctI*) was discovered in *Cp. caviae* [15] (Fig. A, Cluster 1). The *incDEFG* effector cluster [26] is presumably specific for *Chlamydia* since psiblast searches [3] did only reveal homologues

in *C. muridarum* and not in *Chlamydophilae*. *IncA* is immediately adjacent to this cluster in *Chlamydia*. The *incBC* cluster which is found 125 kb after *incA* in *C. trachomatis*, is flipped to *incCB* and lies in front of it in *Chlamydophila*. The second structural cluster contains genes coding for *sctJLRST* as well as *scc2* and *yopB/yopD* homologues (putative translocator components *copB/copD*). A third structural cluster contains *sctD*, *sctN*, *sctQ*, and *sctC* as well as the T3S effector genes *ca037* [30] and *pkn5*. The *tarp* gene is found before the second and third cluster in *Chlamydia* and after it on the complementary strand in *Chlamydophila*. For *scc3*, *copB2*, and *copD2* it is vice versa.

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