A Same-Genus Screening Approach Reveals Novel Effectors and New Possibilities for Investigating *Chlamydia* Pathogenesis

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ABSTRACT *Chlamydiae* are obligate intracellular pathogens that rely on secreted effector proteins to establish their intracellular niche. In this issue of the *Journal of Bacteriology*, Yanatori et al. describe a screen for *Chlamydia pneumoniae* effectors, performed in *Chlamydia trachomatis*, which identified several new proteins that are translocated during infection (I. Yanatori, K. Miura, Y. S. Chen, R. H. Valdivia, F. Kishi, J Bacteriol 203:e00511-20, 2021, <https://doi.org/10.1128/JB.00511-20>). More broadly, they demonstrate how new genetic approaches in *C. trachomatis* can be used to characterize the virulence factors of other *Chlamydia* species.

KEYWORDS *Chlamydia pneumoniae*, type III secretion, chlamydia, *Chlamydia psittaci*, *Chlamydia trachomatis*, genetics

Phylogenetically diverse Gram-negative pathogens use type 3 secretion (T3S) systems to deliver unfolded bacterial proteins into eukaryotic cells (1). These secreted “effector” proteins subsequently refold and manipulate the host cell environment to the pathogen’s advantage. T3S systems may play an especially important role in *Chlamydia* spp. because these obligatory intracellular pathogens need to simultaneously counter cell-autonomous defenses and maintain the viability of the host cells they infect to complete their intracellular developmental cycle (2). Studies of, primarily, *Chlamydia trachomatis* T3S effector proteins have revealed important insights into how this pathogen invades host cells, co-opts host cell resources, and evades immunity. However, some of the surrogate systems that have been used to identify putative chlamydial T3S effectors have limitations. Separately, although it has been postulated that some chlamydial T3S effectors could have niche-specific functions (3), less is known about the T3S effectors carried by other *Chlamydia* spp., and testing this hypothesis has been difficult.

The genus *Chlamydia* contains three important human pathogens: *C. trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae* (4). Different serovars of *C. trachomatis* strains preferentially infect the ocular and genitourinary mucosae and are the most common infectious cause of blindness (serovars A to C) and bacterial sexually transmitted infections (serovars D to K), respectively. *C. psittaci* preferentially infects respiratory tract cells and can cause the severe zoonotic pneumonia psittacosis. Humans are incidental hosts of *C. psittaci*, which is primarily a pathogen of birds and rarely transmitted between infected humans (5). *C. pneumoniae* was first identified in 1980 when this pathogen was associated with unexplained, but relatively mild, acute upper respiratory tract infections in college students (6). *C. pneumoniae* was initially believed to be a human-adapted *C. psittaci* variant, but genome sequencing revealed that these pathogens carry distinct repertoires of T3S effectors and other virulence factors, suggesting that these pathogens diverged long ago and have subsequently undergone extensive niche adaptation (7). We now know that more than 50% of adults in many populations have been exposed to *C. pneumoniae* and that this pathogen is an important cause of community-acquired pneumonia (8).

Chlamydiae alternate between distinct physiological forms during their life cycles (9). Extracellular elementary body (EB) forms are transmitted to susceptible host cells

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from infected tissues and organisms, while intracellular reticulate body (RB) forms replicate inside a specialized intracellular vacuole (inclusion). Although EBs exhibit limited metabolic activity, they carry a T3S apparatus that secretes preloaded effectors into target cells to trigger EB uptake into a nascent inclusion formed from the host cell plasma membrane (10). Once these EBs differentiate into RBs, they synthesize and secrete various effector proteins in sequence to coordinate successive steps in chlamydial development (11). Since RBs are physically separated from their host cell by the inclusion membrane, secreted effector proteins mediate cross talk between these pathogens and their hosts. Known functions of chlamydial effector proteins include blocking fusion of the inclusion with degradative lysosomes, promoting fusion of the inclusion with nutrient-bearing exocytotic vesicles, and inhibiting host immune signaling (2).

T3S has been characterized more extensively in facultative intracellular bacteria, including *Yersinia*, *Salmonella*, and *Shigella* spp., compared to *Chlamydia* spp., but the "injectisomes" that drive secretion of T3S effectors by these organisms are highly conserved (12). In contrast, T3S effectors are less conserved, and the genes that encode them in *Chlamydia* spp. are not located in pathogenicity islands, unlike other organisms (13). Since few tools for manipulating chlamydial T3S were available until recently, these effectors have often been identified using algorithms that were trained using the secretion signals from T3S effectors of other bacteria (14). Separately, predictions generated by these algorithms have been tested by fusing suspected secretion signals from putative chlamydial effectors to reporter proteins and then testing if the resulting fusion proteins can be secreted by the T3S systems of more tractable surrogate organisms (15–17). This approach has identified several secreted chlamydial proteins that have been verified using complementary approaches. Nonetheless, these surrogate T3S screens have been plagued by false negatives and false positives. Possible explanations for this lack of fidelity include that some chlamydial T3S effectors have diverged sufficiently so that they are not recognized by the T3S injectisomes of other organisms or that some of these chlamydial effectors are actually secreted by T3S-independent pathways.

In this issue of the *Journal of Bacteriology*, Yanatori and colleagues systematically evaluated approximately one-third of the genes in the entire genome of *C. pneumoniae* to identify encoded proteins that were secreted via the T3S system in a *C. trachomatis*-based surrogate secretion assay (18). In addition to the identification and localization of almost 50 novel secreted *C. pneumoniae* proteins in host cells, results of this expansive study raise important questions about the limitations of studying the chlamydial T3S system in surrogate systems.

Yanatori et al. developed a Gateway-compatible *C. trachomatis* expression vector to generate 382 recombinant plasmids encoding different *C. pneumoniae* open reading frames (ORFs) fused to an identical short reporter peptide derived from glycogen synthase kinase 3 β (GSK3 β) and then transformed the resulting constructs into *C. trachomatis*. GSK3 β is phosphorylated by cytosolic kinases in mammalian cells (19). Thus, the investigators were able to differentiate the expression of the fusion proteins in *C. trachomatis* and the subsequent translocation of these proteins into host cells by Western blotting using different antibodies that recognize native and phosphorylated forms of GSK3 β . Strong native and phospho-GSK3 β signals were detected when six known *C. pneumoniae* effectors were expressed in this system, but only native GSK3 β was detected when *C. trachomatis* was transformed with vectors encoding nonsecreted *C. pneumoniae* outer membrane proteins.

In total, 75 secreted proteins were identified in this assay, of which 49 had not been previously reported to be secreted. Ectopic expression of these proteins as epitope tagged-fusions in mammalian cells was then used in colocalization experiments to define the localizations of 40 of these proteins in HeLa cells compared with cellular markers with known localization patterns. Many of the secreted *C. pneumoniae* proteins localized to regions targeted by previously characterized chlamydial effectors, including, but not limited to, the Golgi apparatus, endoplasmic reticulum, nucleus, and plasma membrane. Additional investigation of one *C. pneumoniae* effector (CPj0678) that localized to the plasma membrane using reciprocal immunoprecipitation experiments identified an interaction with the host

protein PACSIN2. Importantly, this PACSIN2-CPj0678 interaction was confirmed in *C. pneumoniae*-infected cells. Separately, a recombinant *C. trachomatis* strain that expressed CPj0678-mCherry redirected PACSIN2-GFP from the cytoplasm to the plasma membrane, demonstrating that gain-of-function experiments can be performed in *C. trachomatis* using foreign T3S proteins.

Beyond the extensive discovery aspects of the Yanatori et al. study, their observations reinforce the idea that caution is warranted when evaluating if chlamydial proteins are secreted by T3S. Intriguingly, only 23 of the 75 proteins that were determined to be secreted in this analysis actually contained predicted T3S sequences. Conversely, Yanatori et al. failed to confirm secretion of 24 effectors that were proposed in prior studies. This information suggests that T3S-independent pathways play a more important role in the delivery of chlamydial effectors than is currently appreciated. However, methods to stably transform *C. pneumoniae* and *C. psittaci* were recently described (20, 21). Thus, it might already be possible to test these questions by comparing the abilities of *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* to secrete both their own and cross-species effectors using approaches described in the Yanatori et al. study (18).

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