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Short Communication

SHORT COMMUNICATION

Conservation of extrusion as an exit mechanism for *Chlamydia*

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One sentence summary: The authors report that the extrusion mechanism of *Chlamydia* exit from host cells is broadly conserved across divergent *Chlamydia* species and clinical strains.
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ABSTRACT

Chlamydiae exit via membrane-encased extrusion or through lysis of the host cell. Extrusions are novel, pathogen-containing structures that confer infectious advantages to *Chlamydia*, and are hypothesized to promote cell-to-cell spread, dissemination to distant tissues and facilitate immune evasion. The extrusion phenomenon has been characterized for several *Chlamydia trachomatis* serovars, but a thorough investigation of extrusion for additional clinically relevant *C. trachomatis* strains and *Chlamydia* species has yet to be performed. The key parameters investigated in this study were: (i) the conservation of extrusion across the *Chlamydia* genus, (ii) the functional requirement for candidate *Chlamydia* genes in extrusion formation i.e. *IncA* and *CT228* and (iii) extrusion-mediated uptake, and consequent survival of *Chlamydia* inside macrophages. Inclusion morphology was characterized by live fluorescence microscopy, using an inverted GFP strategy, at early and mid-stages of infection. Enriched extrusions were used to infect bone marrow-derived macrophages, and bacterial viability was measured following macrophage engulfment. Our results demonstrate that extrusion is highly conserved across chlamydiae, including ocular, STD and LGV biovars and divergent *Chlamydia* species. Consequently, this exit mechanism for *Chlamydia* may fulfill common advantages important for pathogenesis.

Keywords: *Chlamydia*; extrusion; dissemination; exit; macrophage

Chlamydiae are gram-negative obligate intracellular bacteria that inflict a tremendous public health burden globally. *Chlamydia trachomatis* is the causative agent of trachoma, the leading cause of infectious blindness (Burton and Mabey 2009), as well as the sexually transmitted disease (Gerbase, Rowley and Mertens 1998). Chlamydiae have evolved to infect a wide variety of host species including humans (*Chlamydiae trachomatis, Chlamydiae pneumoniae*), birds (*Chlamydiae psittaci*), rodents (*Chlamydiae muridarum*), guinea pigs (*Chlamydiae caviae*), horses and reptiles (*C. pneumoniae*) (Sachse *et al.* 2015), and more. Despite this diverse host range, *Chlamydia* species are genetically closely related. Members of the family *Chlamydiaceae* share >91% similarity in 16S rRNA sequences, and >88% gene identity with *C. trachomatis* (Sachse *et al.* 2014, 2015), with conservation of

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specific individual gene products limited in some cases. The extent to which virulence mechanisms of *Chlamydia* species are conserved is an active area of investigation.

The mechanisms by which Chlamydia spp. disseminate within a host, or transmit to new hosts, are not well understood. *C. trachomatis* replicates within an inclusion in host epithelial cells, then exits through two distinct mechanisms— extrusion and lysis (Hybiske and Stephens 2007). Extrusion occurs by pinching of the chlamydial inclusion and the plasma membrane, generating a detached, membrane-bound compartment containing infectious *Chlamydia* (Hybiske and Stephens 2007; Chin *et al.* 2012). The benefits of extrusion for *Chlamydia* were recently explored, demonstrating that containment within an extrusion enhances extracellular survival of *C. trachomatis* (Zuck *et al.* 2016). These novel pathogenic structures are also potentially involved in dissemination, immune evasion and transmission. Furthermore, extrusion is known to occur in *C. trachomatis* serovars L2 (CT L2) and D, *C. caviae* (Hybiske and Stephens 2007) and *Chlamydia pecorum* (Doughri, Storz and KP 1972) but had not been more widely explored. In this study, we investigated how broadly the extrusion exit mechanism is conserved among Chlamydia spp.

Inclusion morphology varies greatly between species of *Chlamydia*, ranging in phenotypes from: circular, amorphous and multivacuolar. We hypothesized that variation in inclusion number and morphology within host cells may affect whether these *Chlamydia* can extrude, or dictate extrusion size and downstream outcomes.

To compare inclusion morphological differences between the species in this study, green fluorescent protein (GFP)-expressing HeLa cells were infected with *Chlamydia* and imaged using an inverted GFP microscopy strategy, whereby fluorescent host cells reveal the nonfluorescent inclusion against the GFP labeled host cytoplasm (Zuck, Feng and Hybiske 2015). The following *Chlamydia* species were used in this study: C. trachomatis LGV L2/434, *C. trachomatis* serovar D, *C. psittaci* 6BC, *C. muridarum* Nigg and *C. pneumoniae* TW183. Two additional *C. trachomatis* strains were tested for functional requirement of candidate *Chlamydia* genes in extrusion; CT Ds/2923 (Suchland *et al.* 2000) and CT B/Jali20/OT (validated IncA and CT228 truncation mutants, respectively, Seth-Smith *et al.* 2009; Lutter *et al.* 2013). CT B/Jali20/OT was included in this study based on published data involving CT228 in regulation of extrusion by interaction with host cell protein Myosin phosphatase target subunit 1 (MYPT1) (Lutter *et al.* 2013). Inclusion morphology and numbers varied as expected; CT L2 displayed a single, round inclusion phenotype at 24 h post-infection (hpi), representative of the majority of strains tested (Fig. 1A, left). CT D^{incA-} and *C. pneumoniae*

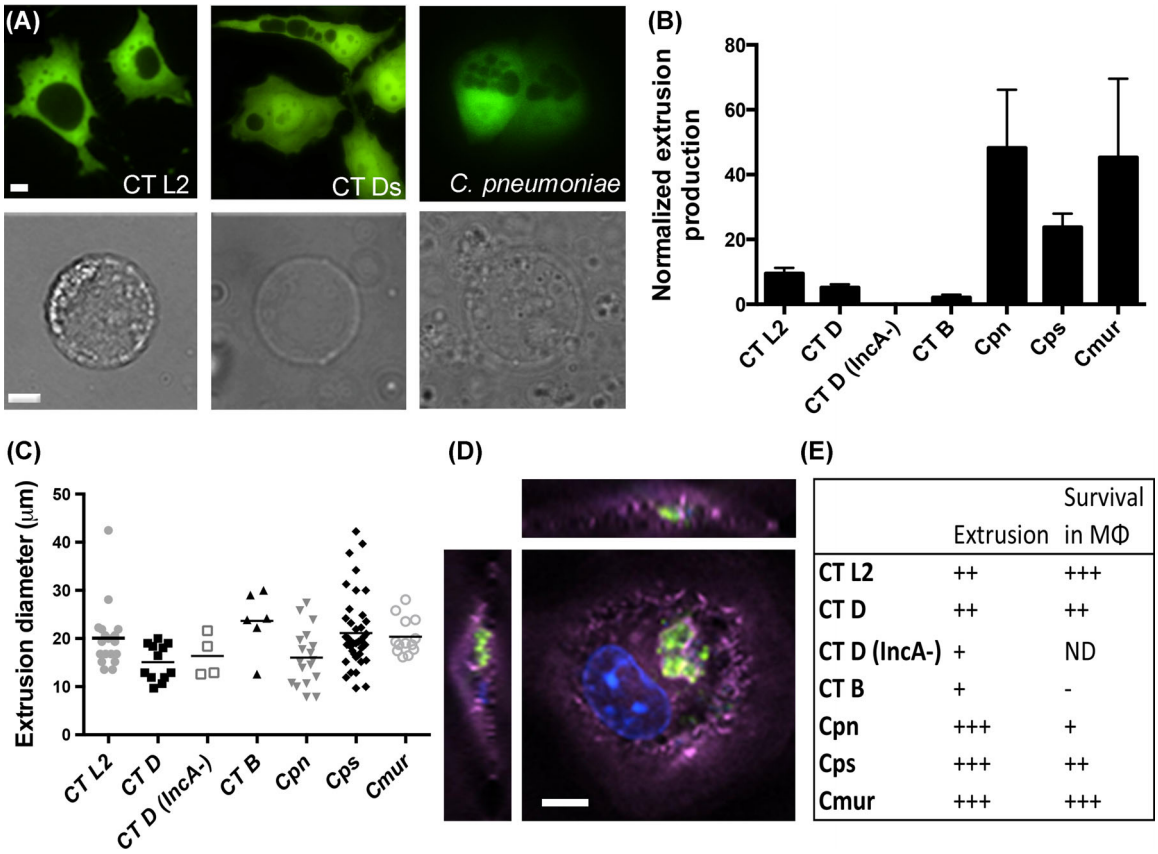

Figure 1. Conservation of extrusion production, size and survival in macrophages across Chlamydiae. (**A**) Inclusion morphology of *Chlamydia* spp.; HeLa cell (GFP), inclusion is shown by absence of GFP, at 60× magnification. Top row: CT L2 (left), CT D^{incA-} (middle) and *C. psittaci* (right). Bottom row: single, isolated extrusions matching top row *Chlamydia* species shown in brightfield at 60× magnification. Scale bar = 5 μm. (**B**) Normalized extrusion production, calculated by counting number of extrusions relative to % of cells infected in original infection. Columns show mean ± SEM. $n = 3$ independent experiments. Statistics were performed by one-way ANOVA analysis using Tukey's multiple comparisons test, and no significance was observed between species or *C. trachomatis* strains. (**C**) Extrusion size graph displaying each extrusion as a single point on the graph. Extrusions counted by Volocity microscope software parameters to measure diameter (μm), $n = 3$ independent experiments. Statistics were performed using one-way ANOVA analysis using Tukey's multiple comparisons test. No significance was observed between species or *C. trachomatis* strain extrusion diameter or spread. (**D**) Extrusion uptake by bone marrow macrophages. CT L2 extrusion (GFP), host cell actin (purple) and nucleus (DAPI) at 60× magnification with z stacking. Scale bar = 5 μm. (E) Summary table displaying normalized extrusion production and survival within macrophages up to 6 h. +++ denotes >100 IFU/field, ++ denotes 50–100 IFU/field, + denotes < 50 IFU/field and – denotes no IFU seen. ND = no data.

displayed multiple inclusions per cell (Fig. 1A, middle, right, respectively).

To determine which *Chlamydia* species and *C. trachomatis* serovars utilize the extrusion exit pathway, all strains were grown in HeLa or Hep2 cells (for *C. pneumoniae*), and extrusions were collected over a time course from 0 to 48 hpi. For all strains, extrusion production was greatest at 48 hpi. (Fig. S1A-F, Supporting Information). Extrusions were collected using a centrifugation protocol (Zuck *et al.* 2016), and counted via microscopy (Fig. 1A and B). Despite the presence of multiple inclusions or varied inclusion morphologies for some strains, the ability to extrude was conserved in all *Chlamydia* species and strains tested. Additionally, all extrusions had similar appearances via brightfield microscopy (Fig 1A).

Following confirmation that extrusion is a conserved exit mechanism among all *Chlamydia* species tested, we next sought to determine any differences in relative extrusion production. We collected extrusions from each strain at 48 hpi, enumerated extrusions by microscopy, and normalized extrusion quantity to the infection level of the monolayer that extrusions were collected from. Extrusion production differed among *Chlamydia* species but these differences were not statistically significant (Fig 1B; Fig. S1, Supporting Information). There was robust extrusion production in *C. pneumoniae* and *C. muridarum* but little extrusion production by the ocular strain CT B/Jali20 as previously reported (Fig. 1B; Lutter *et al.* 2013). Interestingly, *C. trachomatis* D^{-incA} did not extrude at 48 hpi; however, further experiments showed delayed extrusion production, peaking at 72 hpi. This delay could be due to attenuated growth, as previously shown for IncA mutants (Xia *et al.* 2005). Alternatively, IncA could be required for normal extrusion kinetics, but other compensatory mechanisms are active after 48 hpi. The size of extrusions was also examined, and extrusion size of all *Chlamydia* species was conserved, at about ~20 μm (Fig. 1C). We found no statistically significant difference in the mean or range of extrusion diameter among the strains tested, including the *C. pneumoniae* and CT D^{-incA} species that harbor, on average, multiple and much smaller inclusions. Considering the substantial variation in developmental cycle among species, e.g. *C. pneumoniae* and *C. muridarum*, the absence of significant differences in extrusion sizes or numbers is intriguing. All *Chlamydia* species continually shed extrusions (Fig. S1, Supporting Information), possibly prior to inclusion fusion for *C. pneumoniae*, ultimately producing multiple extrusions from the same infected cell, though this has not been directly assessed.

The data for extrusion's survival benefits to *Chlamydia*, coupled with the conservation of extrusion in a diverse set of *Chlamydia* species, suggest that this mechanism plays an important part in pathogenesis. In a previous study (Zuck *et al.* 2016), we demonstrated that extrusions are engulfed by primary bone marrow-derived macrophages from C57Bl/6 mice, and subsequently survive within macrophages much longer than free *Chlamydia* elementary bodies (EB). To determine if this is a conserved survival strategy, we examined uptake of extrusions within macrophages. Extrusions were collected and co-cultured with macrophages for 1 h at 37°C, then thoroughly rinsed and analyzed via immunofluorescence. Fluorescence microscopy revealed that all *C. trachomatis* serovars and *Chlamydia* species tested were capable of being engulfed by macrophages. Figure 1D shows a CT L2 extrusion within a macrophage at 1 hpi. The majority of extrusions were intact upon engulfment, but some engulfed extrusions displayed GFP fluorescence localized into several distinct foci or scattered throughout the cytoplasm of the host macrophage. This range in morphology was characteristic of all strains observed (Fig. S2, Supporting Information).

Lastly, we characterized the extrusion-mediated enhancement of *Chlamydia* survival within macrophages. We co-cultured primary macrophages with extrusions, rinsed, then incubated macrophages at 37°C for 6 h to measure short-term survival of extrusion-delivered *Chlamydia*. Notably, all extrusions were engulfed and facilitated short-term survival of their bacteria within macrophages (Fig. 1D and E). Survival of CT B/Jali20 within macrophages was not detected, but could be a result of our inability to harvest high numbers of extrusions for this strain. Survival of *C. pneumoniae* in macrophages was surprisingly low, considering previously published data demonstrating the ability of free EB to infect macrophages (Gaydos *et al.* 1996; Redecke *et al.* 1998; Marangoni *et al.* 2014). These data demonstrate the conservation of the extrusion exit mechanism across a diverse set of *C. trachomatis* serovars and *Chlamydia* species.

Our results reveal that extrusion is a broadly conserved exit mechanism within Chlamydiae. Among the species and serovars tested, there was no significant difference in the ability to extrude or the size of extrusions between Chlamydiae. The absence of functional proteins CT228 and IncA hindered extrusion production, though this effect was not statistically significant. These genes may be involved in extrusion formation, but are not absolute requirements for this exit mechanism. Given the natural history of Chlamydiae as obligate intracellular bacteria, the evolution of promoting exit in an extracellular compartment makes sense as a pathogenesis strategy. It was recently shown that there are tangible benefits for *Chlamydia* that exit within extrusions (Zuck *et al.* 2016), including enhanced survival in the extracellular environment compared to free EB. The extrusion compartment may function as an 'intracellular-like' environment, retaining vital nutrients and protecting against extracellular damage. The broad conservation of extrusion suggests that this exit strategy is universally beneficial to *Chlamydia* species in a common stage of pathogenesis.

Additionally, our data indicate that uptake of extrusions into primary macrophages is conserved among Chlamydiae. Spread of *Chlamydia* within the host from the original infection site is a pathogenesis trait for several species including *C. trachomatis* and *C. pneumoniae* (Moazed *et al.* 1998; Darville and Hiltke 2010). However, as a non-motile bacterium, the mechanism by which *Chlamydia* spreads in its host is not well understood. Migration of other non-motile bacteria can be assisted by hitching a ride in a host phagocyte, as shown for *Mycobacterium marinum, Neisseria gonorrhoeae* and *Staphylococcus aureus* (Davis and Ramakrishnan 2009; Thwaites and Gant 2011; Criss and Seifert 2012). Extrusion, and the conservation of this mechanism throughout the *Chlamydia* species tested, is a strategy to allow survival within phagocytic cells, perhaps to sustain *Chlamydia* until they disseminate to new mucosal tissues or distant sites.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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