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The symbioses between environmental chlamydiae and dictyostelids

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This thesis underwent language and grammar improvement with Chat-GPT-5 following the AI policy of the University of Vienna.

General introduction

Shades of symbiosis

Organisms evolve in constant contact with other organisms. According to the widely accepted definition by de Bar, symbiosis describes the living together of unlike organisms (De Bary, 1879). Based on the effect on the fitness, i.e., reproductive success, of the partners, symbiosis is usually further categorized as mutualistic, parasitic or commensalistic (Bronstein, 1994a). Interactions with reciprocal benefits for the partners, usually exchanging a "service" for a "reward", are termed mutualism (Bronstein, 1994b; Herre et al., 1999). Prominent examples include plant-pollinator interactions, ant-aphid or coral-algae associations (Flatt & Weisser, 2000; Janzen, 1983; Taylor, 1973). Despite the net benefit for both partners, these interactions should be considered reciprocal parasitism, due to the selfish character of the individual partners (Bronstein, 1994b; Herre et al., 1999; Husnik & Keeling, 2019). Accordingly, the origin of mutualistic relationships lies mostly in parasitic interactions - either by a parasitic symbiont (Tso et al., 2018) or an exploitative host (Sørensen et al., 2019) - which are evolving towards mutualism due to ecological shifts (Ewald, 1987; J. L. Sachs et al., 2014). Parasitism is defined as a form of symbiosis in which one organism increases fitness, while the other suffers fitness loss. The importance of parasites to ecosystems lies in not only in their immense abundance (May, 1988) but also their contribution to ecosystem health (Hudson et al., 2006). Historically, parasitism has been a large part of symbiosis research, however, mostly human-centered (Bronstein, 1994b; Mathis & Bronstein, 2020). Commensalism, the symbiotic relationship in which one species receives a net benefit while the second species is on balance unaffected, has lacked attention in comparison to other forms of symbiosis (Mathis & Bronstein, 2020). Briefly, two forms of commensalism are considered to occur in nature (1) the complete absence of fitness effects and (2) the exact balance between costs and benefits on one of the partners, while the other benefits (Mathis & Bronstein, 2020). The concept of commensalism is debated, since "narrow" commensalism requires a "proof of absence" (which is empirically impossible) and "broad" commensalism is subjective and should rather be coined as weak parasitism and mutualism (Zapalski, 2011). The evolution of "apparent commensalism" can emerge if hosts counter parasites with tolerance instead of resistance mechanisms (Miller et al., 2006). While resistance aims to prevent infection or limit its extent, tolerance aims to offset negative fitness effects (Roy & Kirchner, 2000). Symbioses evolve fast and change the fitness outcome for both partners depending on ecological context, e.g., mutualistic associations can break down fast (J. Sachs & Simms, 2006) and parasitic associations can develop to become

mutualistic (Clayton et al., 2012; J. L. Sachs et al., 2014). In that sense, commensalism may be considered a tipping point on an evolutionary time frame (Mathis & Bronstein, 2020). Ultimately, net fitness outcome of a symbiosis is always context dependent and can change fast depending on ecological context.

Life inside an organism: the curious (but frequent) case of endosymbiosis

The role of symbiosis in major evolutionary transitions is especially intriguing in microbiology. Microbial endosymbionts reside directly within host tissues or cells and can provide benefits to their hosts due to their versatile metabolic functions (Wernegreen, 2012). Most strikingly, endosymbiosis has led to the emergence of the eukaryotic cell as the Russian botanist Constantin Mereschkowsky and Lynn Margulis famously claimed in the early 1900's and 1960's, respectively (Archibald, 2015). Today the endosymbiotic origin of many organelles, such as the mitochondria, chloroplasts and plastids is textbook knowledge and topic of numerous reviews (Archibald, 2015; Martin et al., 2015; Vosseberg et al., 2024). Despite the complex and still-debated origins of organelles and the eukaryotic cell, the establishment of endosymbiotic relationships follows surprisingly uniform rules. Briefly, a prokaryote invades a eukaryotic cell and is maintained within. Soon after establishment, the genome of these endosymbionts undergoes massive changes (Clayton et al., 2012). On the one hand, gene loss primarily occurs through deletions and pseudogenization, often mediated by mobile elements and chromosome rearrangements (McCutcheon & Moran, 2012). On the other hand, novel functions are gained via horizontal gene transfer (Drew et al., 2021). These phenomena are accelerated by genetic drift and small population sizes leading to fixation of deleterious mutations (Moran, 1996). If the host depends on symbiont functions, such as the supply of nutrients, it will compensate for these gene losses by adaptations to maintain the symbiont increasing their reciprocal dependency (Bennett & Moran, 2015). For organelles, this "transfer of ancestral functions", e.g., cell division, is obvious (Timmis et al., 2004) and in some endosymbionts this transfer can be supposedly seen in intermediate stages (Maurya et al., 2025). Despite these adaptations, endosymbionts are under the constant threat of being replaced or completely lost (Karnkowska et al., 2016; McCutcheon et al., 2019). Ultimately, endosymbioses can only be maintained if they are regularly transmitted to naïve host cells. The symbiont is either acquired from an environmental source or directly from a parent cell resulting in horizontal or vertical transmission, respectively (Bright & Bulgheresi, 2010). Vertical transmission leads to attenuation, since it ties symbiont fitness to host fitness (Ewald, 1987; Fisher et al., 2017). Nevertheless, even some well-established symbioses rely on

horizontal transmission (Visick et al., 2021) and often transmission modes are mixed (Bright & Bulgheresi, 2010). Many major bacterial human pathogens replicate intracellularly and transmit horizontally. While organisms, like *Mycobacterium tuberculosis*, causing tuberculosis, and *Legionella pneumophila* (Boamah et al., 2017), the cause of Legionnaires disease can thrive and replicate outside of host cells, other pathogens like *Rickettsia* (Salje, 2021) or *Chlamydia* (Elwell et al., 2016) only replicate within host cells and only sustain extracellularly to transmit to new host cells.

Chlamydiae: professional parasites ubiquitous in the environment

Chlamydia trachomatis is the best-known member of the family Chlamydiaceae and has an estimated global prevalence of ~3% (Huai et al., 2020). Depending on the strain, or so-called serovar, *C. trachomatis* infects different human organs. Serovars A to C infect the human eye causing trachoma (Murray & McKay, 2021). Globally, approximately 1.2 million humans suffer from Trachoma, making it the leading cause of infection-related blindness (World Health Organization, 2025). Serovars D to K infect the genital tract, where they often remain asymptomatic but can result in pelvic inflammatory disease, infertility or ectopic pregnancies (Witkin et al., 2017). Other members of the family Chlamydiaceae, which consists of the single genus *Chlamydia*, infect animals, many in close contact to humans and some able to infect humans (Cheong et al., 2019). Zoonosis led to the emergence of *Chlamydia pneumoniae*, which infects the human respiratory system asymptomatically and can evoke community-acquired pneumonia (Bachmann et al., 2014; Myers et al., 2009). Despite decades of research, no vaccine against chlamydiae exists up to this date (Poston, 2024). Chlamydial infections can be cured by using antibiotics, however, asymptomatic infections are often not treated and, like other pathogens, chlamydiae can develop antibiotic resistance (Poston, 2024; Sandoz & Rockey, 2010). Thus, chlamydiae remain a global health issue with potential for novel emerging pathogens.

All members of the Chlamydiaceae share a canonical developmental cycle, transitioning between intracellular and extracellular stages (Elwell et al., 2016; Stelzner et al., 2023). Briefly, an extracellular chlamydial cell, the elementary body (EB), encounters and attaches to a potential host cell. Subsequently, the EB induces the uptake by the host cell using virulence factors injected by the conserved type three secretion system (T3SS). After entry, the EB is located within a host-derived vesicle, the inclusion, and transforms into the replicative form, the reticulate body (RB). The RB depends on metabolites and amino acids scavenged from the host cell, e.g. in the form of nucleotides and carbohydrates (Stelzner

et al., 2023). After several rounds of replication, RBs transform into EBs and exit the host cell via extrusion or host cell lysis. Progression through this developmental program is controlled by conserved regulatory genes. The most prominent is the transcription factor *early upstream ORF* (*euo*), which is expressed in the early and mid-stages and represses development from RBs to EBs (Appa et al., 2024; Hakiem et al., 2023). Another well-known transcription factor is C*tcC*, which, after phosphorylation by the kinase *CtcB*, activates the transcription factor σ_{54} (*RpoN*) (Koo & Stephens, 2003). The transcriptional regulator *ChxR* is conserved across all chlamydiae, however, it is not an essential gene under laboratory condition (Domman & Horn, 2015; Koo et al., 2006; Yang et al., 2017). With increased sampling and molecular techniques more and more bacteria with similar developmental features were isolated and characterized from medical and environmental samples. These were coined "chlamydiae like organisms" or "environmental chlamydiae" and broadened our view on chlamydiae substantially (Collingro et al., 2020; Taylor-Brown et al., 2015). The first described environmental chlamydia was isolated from an aborted bovine fetus and originally missclassified as *Rickettsiales* (Dilbeck et al., 1990). A few years later, it was reclassified as *Waddlia chondrophila* based on 16S rRNA phylogeny, thus, founding the family Waddliaceae (Rurangirwa et al., 1999). Soon after, representatives of the Simkaniaceae and Parachlamydiaceae were isolated from a contaminated HeLa cell culture and from an Acanthamoeba strain taken from nasal mucus, respectively (Amann et al., 1997; Kahane et al., 1993). Subsequent isolates included members of the genera *Protochlamydia* and *Neochlamydia* affiliated to the family Parachlamydiaceae, isolated from free-living amoebae (Collingro et al., 2005; Horn et al., 2000). The individual *Neochlamydia hartmannella* cells are located directly within the cytosol of their host *Vermamoeba vermiformis* and can also infect other amoebae like *Dictyostelium discoideum* (Horn et al., 2000; Skriwan et al., 2002). With the first closed genomes and growing metagenomically assembled genome (MAG) datasets, key similarities and differences of environmental chlamydiae to Chlamydiaceae emerged. The first closed genome from an environmental chlamydiae, namely *Protochlamydia*, revealed shared and diverged features with the Chlamydiaceae like a functioning T3SS and a full TCA cycle, respectively (Horn et al., 2004). Beyond gene content, phylogenomics placed the chlamydiae as sister phylum to the mostly free-living taxa Planctomycetes and Verrucomicrobia (Wagner & Horn, 2006). Since the split of the chlamydial phylum from the Verrucomicrobia around 2 billion years ago, the originally facultatively anaerobic chlamydiae evolved oxygen tolerance and an increased metabolic potential via gene gain (Betts et al., 2018; Köstlbacher et al., 2021). Gene gain led to an increased host range, contradicted by a small conserved gene set due to successive gene loss (Dharamshi et

al., 2023). Today, at least six chlamydial families have isolated representatives, however, more than a thousand additional families are expected to exist (Collingro et al., 2020). The isolation of chlamydiae from environmental sources via amoeba will remain a powerful tool to expand our knowledge about the evolution and diversity of chlamydiae.

Dictyostelids: a social lifestyle reserved for kin

Free-living amoebae are unicellular, soil-dwelling eukaryotes which move via pseudopods and feed by phagocytosis (Samba-Louaka et al., 2019). Among them the social amoeba *Dictyostelium discoideum* – a dictyostelid – is a model organism for various cellular processes (Samba-Louaka et al., 2019). Upon nutrient deprivation *D. discoideum* can enter a sophisticated life cycle, already expressed by the last common ancestor of dictyostelids (Kin & Schaap, 2021). This social life cycle starts with a dictyostelid cell secreting a chemoattractant, often cAMP (Konijn et al., 1967), leading to the aggregation of tens of thousands of dictyostelid cells. Once enough cells accumulated in the aggregation center, the cells can form a slug, often coined "pseudoplasmodium" in the early years, in which the individuals keep their integrity (Raper, 1940). The slug migrates towards environmental cues, e.g., light or temperature, before developing into a fruiting body consisting of a stalk and several thousand dormant spores. After dispersal, aided by wild animals, the dormant spores can germinate to form a new dictyostelid population (Guo et al., 2026). Strikingly, all cells that differentiate to form the stalk die in the process and only the spore cells survive the social life cycle.

The dictyostelid social life cycle is a form of extreme division of labor, i.e. self-sacrificing helpers that produce a common good - propagation of reproductive cells - which increases population fitness (Cooper & West, 2018). These systems depend on high relatedness of the participating individuals, because common goods are otherwise exploited by non-relative "cheaters" (Bastiaans et al., 2016). Individuals of *D. discoideum* which contribute less to stalk production and predominantly become spores, are considered as cheaters (Ostrowski, 2019). Facultatively multicellular organisms need to constrain cheaters to preserve the success of the mechanism (Travisano & Velicer, 2004). The clonality of multicellular structures is therefore vital for the propagation of social behavior. Clonality can be ensured by single-cell bottlenecks, e.g., by sexual reproduction in animals or spore cells in dictyostelids (Kuzdzal-Fick et al., 2011). Alternatively, clonality is induced by kin selection, which is the exclusion of distantly related individuals from the participation in division of labor. In *D. discoideum* this function is, at least partly, fulfilled by *TgrB1* and *TgrC1*, fast-evolving proteins presented on the cell surface which interact during aggregation (Hirose et al., 2011; Holland et al., 2025; Katoh-Kurasawa et al., 2024).

Due to their habitat and their dependency on bacteria as a food source, dictyostelids are permanently in close contact with bacteria (Farinholt et al., 2019; Jauslin et al., 2021). Therefore, dictyostelids have evolved sophisticated mechanisms for phagocytosis which are well-studied due to their parallels to human immune cells (Chen et al., 2007; Dunn et al., 2018). However, the frequent uptake of bacteria renders dictyostelids also susceptible to bacteria like *Legionella and Mycobacterium*, which have evolved ways to escape the phagosome and thrive within the dictyostelid cell (Bozzaro & Eichinger, 2011). Multicellular stages induce the generation of sentinel cells, which sequester toxins and bacterial pathogens to minimize negative impacts on spore production (Chen et al., 2007). Endosymbiotic *Burkholderia* spp. reduce the number of sentinel cells within the multicellular host stages, while providing protection against toxins (Brock et al., 2016). Nevertheless, infection of *D. discoideum* with *Burkholderia* decreases spore production under food rich conditions (DiSalvo et al., 2015). However, the same *Burkholderia* species induces the carry-over of food bacteria into host spores, ensuring food supply at germination (Brock et al., 2011; DiSalvo et al., 2015). Additionally, *D. discoideum* benefits from *Burkholderia* symbionts by reducing the spore production of aposymbiotic *D. discoideum* during cocultivation (Brock et al., 2013). Taken together, the *D. discoideum*-*Burkholderia* symbioses underline that the net benefit of a symbiosis is context dependent and can hardly be captured with a single variable. The discovery of environmental chlamydiae and *Amoebophilus* species associated with wild dictyostelids is an opportunity to further expand the knowledge on dictyostelid-bacteria symbioses (Haselkorn et al., 2021).

Aims of this study

The chapters presented in this thesis explore the influence of facultative multicellularity in amoebae on their chlamydial symbionts. The dictyostelid social life cycle poses challenges to chlamydiae such as extensive host cell modulation but offers opportunities for transmission unprecedented in other amoebae, such as tight cell-to-cell contacts and exchange of cytosol between host cells. I focused on three main questions answered in three separate chapters: 1) Have chlamydial development and transmission adapted to facultative host multicellularity? 2) Are adaptations common among dictyostelid-associated chlamydiae? 3) Can the associated chlamydiae provide a fitness advantage in competition with naïve dictyostelids?

In the first chapter, I describe a *D. giganteum* isolate naturally infected with a yet uncharacterized chlamydial species. I aimed to elucidate the chlamydial adaptations with a diverse set of methods to obtain a holistic view of this symbiosis. By cell culture

experiments, I showed the lack of pathogenicity of the symbionts. While fluorescence *in-situ* hybridization (FISH) confirmed the presence of chlamydiae in all stages of the host life cycle, I did not detect chlamydiae in the cell culture supernatant using digital PCR. This was in line with the lack of morphological variation seen using electron microscopy and the loss of important genes for development and extracellular survival. Lastly, I showed the importance of host cell-cell contact for transmission of chlamydiae. I concluded that the chlamydial symbiont adapted a way to utilize host aggregation for horizontal transmission.

In the second chapter, I aimed to understand whether the findings from chapter one can be generalized for other dictyostelid-associated chlamydiae. Thankfully, my co-authors provided environmental isolates spanning multiple dictyostelid hosts and chlamydial lineages. Additionally, I was supplied with two additional closed genomes derived from dictyostelid-associated chlamydiae. Taken together, I collected seven isolates comprising two chlamydial families, *Rhabdochlamydiaceae* and *Plastochlamydiaceae*. Comparative genomics confirmed the loss of regulatory and metabolic genes in all dictyostelid-associated chlamydiae. The representative isolates of both chlamydial families, exhibited similar adaptations of development and transmission, based on cell culture experiments, fluorescence microscopy and gene expression analysis. The strength of these adaptations however suggested that the two families were at different stages in their transition to a more attenuated endosymbiotic lifestyle. Additionally, despite phenotypic convergence, the specific sets of lost regulatory genes differed.

Given the apparent absence of chlamydial infection on dictyostelids, I aimed to study the role of tolerance and intraspecies competition. Fluorescence microscopy and flow cytometry (fluorescence-based cell counting) was used to test whether dictyostelid-associated chlamydiae alter host fitness when co-cultured with naïve competitor strains. In parallel I monitored chlamydial transmission to the naïve competitor strains.

The overarching goal of this thesis is to understand the adaptations of chlamydiae confronted with facultative multicellularity in the form of a sophisticated host life cycle. Ultimately, I aim to describe how these adaptations shape symbiont transmission, symbiont genomes and host ecology.

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Title: The adaptation of chlamydiae to facultative host multicellularity

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Summary

The phylum Chlamydiota consists of obligate intracellular bacteria comprising the human pathogen *Chlamydia trachomatis* and a large variety of species infecting animals and protists. Despite their diversity, a feature shared by all known chlamydiae is their biphasic developmental cycle, consisting of intra- and extracellular stages with substantial differences in morphology and physiology. Here we report the isolation of a social amoeba, *Dictyostelium giganteum,* naturally infected with a chlamydial symbiont. The social life cycle of dictyostelids is characterized by multicellular stages through aggregation of vegetative trophozoites, leading to the development of multicellular fruiting bodies and the formation of spores. Although dictyostelids undergo symbioses with various bacteria, chlamydiae have only recently been found to be associated with these amoebae. The chlamydial symbiont identified here represents a novel species, *Reclusachlamydia socialis,* and is retained in all stages of the host's social life cycle. Notably, the symbiont lacks a detectable extracellular form. Combining fluorescence microscopy and quantitative PCR we show that transmission is entirely dependent on cell-to-cell contact during the host aggregation stage. The absence of an extracellular stage is further supported by transmission electron microscopy and the lack of genes essential for chlamydial developmental cycle regulation and extracellular survival. This variation of a highly conserved developmental feature that evolved more than a billion years ago illustrates the remarkable adaptability of chlamydiae. This study adds to our understanding of endosymbiosis in the face of facultative host multicellularity.

Keywords

Dictyostelium giganteum, dictyostelids, social amoeba, social life cycle, cell-to-cell transmission, endosymbiont, *Chlamydia*, *Rhabdochlamydia*, developmental cycle, eukaryotic-like proteins

Highlights

- A chlamydial symbiont of dictyostelids is retained throughout its host's social lifecycle.
- Symbiont lacks the classical chlamydial developmental cycle and an extracellular form.

- The symbiont's genome lacks vital genes for extracellular survival.
- Spreads by cell-to-cell contact as adaptation to facultative host multicellularity.

Introduction

The phylum Chlamydiota is a group of obligate intracellular bacteria best known for major animal and human pathogens such as *Chlamydia trachomatis*. This species causes the ocular disease trachoma affecting 1.5 million humans globally [1] and infects the human genital tract at an estimated worldwide prevalence of 3% [2]. The last three decades have expanded our knowledge of the Chlamydiota beyond those pathogens to the so-called environmental chlamydiae. These chlamydiae are found in diverse eukaryotic hosts and environments, natural and engineered, with molecular data suggesting the existence of hundreds of chlamydial families [3]. The most characteristic feature of known chlamydiae is their biphasic developmental cycle. This lifestyle is best described for *C. trachomatis* and other members of the family Chlamydiaceae but highly conserved among environmental chlamydiae [4–6]. Briefly, an extracellular stage, the elementary body (EB), enters a host cell. Within a host-derived vacuole, termed inclusion, the EB radically changes its morphology and metabolism, and transforms into a reticulate body (RB). After multiple rounds of replication as RBs, the chlamydial progeny differentiates back to EBs and exits the host cell, via lysis or extrusion, to infect new hosts and start a new developmental cycle. Due to the challenging extracellular environment EBs possess a more rigid cell envelope and densely packed chromatin compared to their RB counterparts [7–9]. The progression through this developmental program is well-orchestrated by a set of conserved chlamydiae-specific transcription factors [10–12] and metabolic pathways to meet the phase-specific needs of energy parasitism and energy conservation [6,13]. Reconstruction of the genomic repertoire of the last common ancestor of the chlamydiae revealed that already 1-2 billion years ago, these bacteria were obligate endosymbionts equipped with the hallmark genes of an intracellular lifestyle and the chlamydial biphasic developmental cycle [14].

The strictly host-associated lifestyle has been hampering the isolation of environmental chlamydiae, and we thus lack isolates for the vast majority of chlamydial lineages [3,15,16]. Yet, a source of chlamydial isolates in the past was the paraphyletic group of the free-living amoebae. These unicellular eukaryotes move via pseudopods and are ubiquitous in the environment [17]. One of the most intensely studied amoebae is the cellular slime mold *Dictyostelium discoideum*, affiliated to the clade Dictyostelia, i.e., dictyostelids, rooted within the Amoebozoa [18,19]. Undoubtedly, the characteristic drawing the most attention to

this microorganism is its social life cycle first described in the original report about *D. discoideum* [20] and known to occur in different variations in all dictyostelids studied so far [18]. Briefly, the social life cycle involves the aggregation of tens of thousands of vegetative amoeba cells (trophozoites) upon nutrient deprivation to form a multicellular structure, the pseudoplasmodium, resembling a slug. This slug migrates towards light sources and other environmental cues to subsequently transforms into a multicellular fruiting body visible with the naked eye. The fruiting body is made up by a stalk lifting up spores ready to be dispersed in the environment and to found new populations [21]. Despite its complexity, similar forms of facultative multicellularity have evolved at least seven times independently in amoeba [22]. *D. discoideum* shows complex interaction patterns with facultative bacterial symbionts to ensure nutrient availability [23] and serves as a model to study host-microbe interaction with several human pathogens [24,25]. Yet, the first molecular evidences of chlamydiae related to the families Parachlamydiaceae, Simkaniaceae, and Rhabdochlamydiaceae naturally infecting *D. discoideum* and other dictyostelids were only reported recently [26,27].

Here, we report on the isolation and characterization of *Dictyostelium giganteum* PALH, a close relative of *D. discoideum*, harboring a chlamydial symbiont remarkably well-adapted to the social life cycle of its amoeba host. The symbiont remains within the host cell throughout the entire dictyostelid social life cycle and depends on the host's aggregation stage for the infection of naïve amoeba. This adaptation is also reflected in the symbiont genome by the loss of highly conserved genes involved in the canonical biphasic developmental cycle of chlamydiae.

Results and Discussion

A chlamydial symbiont stably infecting *Dictyostelium giganteum*

In an effort to isolate novel environmental chlamydiae, the amoeba isolate *D. giganteum* PALH was recovered from forest soil (Figure 1A) and identified based on its partial 18S rRNA gene sequence (100% sequence similarity to known *D. giganteum* isolates; Data S1). Either on plates or in liquid cultures *D. giganteum* PALH was able to aggregate and enter the social life cycle. However, fruiting bodies, and thus spores, only formed on agar plates resembling what is known from previous reports about this species (Figure 1CD) [28]. By performing fluorescence in situ hybridization (FISH) with chlamydiae-specific probes, the presence of chlamydial symbionts in *D. giganteum* PALH trophozoites and

spores was readily visible (Figure 1BE). Despite a chlamydial prevalence of 100% in *D. giganteum* PALH, the number of chlamydial cells counted per trophozoite and spore hardly exceeded 10 and 5, respectively (Figure 1BE). This stands in stark contrast to other chlamydial species like *Parachlamydia acanthamoebae* [29], *Simkania negevensis* [30], *Rhabdochlamydia porcellionis* [31] or *C. trachomatis* [13], which are found in large inclusions harboring dozens of bacterial cells. To our knowledge, only the dinoflagellate symbiont *Algichlamydia australiensis*, found in single-cell inclusions, exhibits a similarly low number of chlamydial cells per host cell [32]. The observed stable, low level of infection indicates that the replication of the chlamydial symbiont of *D. giganteum* is tightly controlled either by the symbiont or the host.

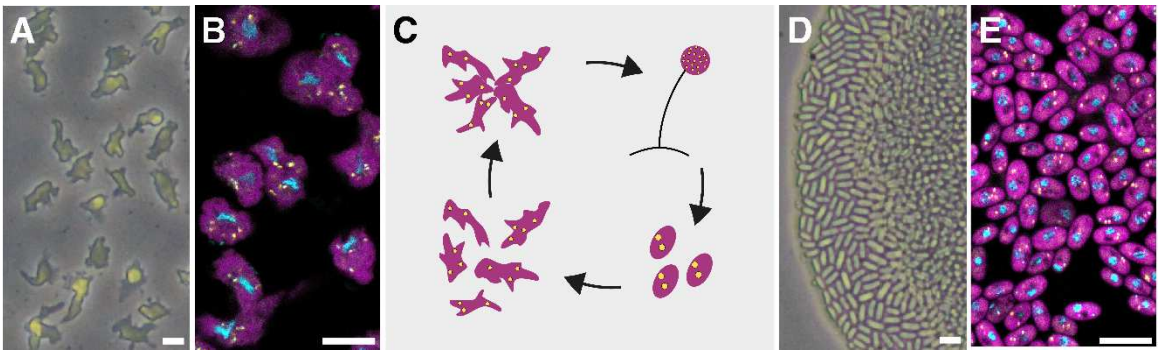

Figure 1: D. giganteum PALH Naturally Containing Chlamydial Symbionts. Light microscopy images and fluorescence in situ hybridization images of trophozoites (A, B) and spores (D, E); nuclei are shown in cyan, amoebae in magenta, and chlamydiae in yellow. Bars, 10 µm. A scheme illustrating the presence of the symbiont during the social life cycle stages of its dictyostelid host is depicted in (C); infected amoeba trophozoites and spores are shown in magenta, chlamydial symbionts in yellow.

Apart from the chlamydial symbiont, we did not detect other bacteria, including food bacteria, stably associated with *D. giganteum* PALH. Yet, *D. discoideum* are known to interact with food bacteria and symbionts in manifold ways throughout their entire life cycle [33]. In the vegetative trophozoite stage the amoeba exploit distinct pathways to discriminate between different types of food bacteria [34] and are able to induce endosymbiosis with food bacteria by the secretion of lectins [35]. During multicellular stages, specialized sentinel cells are responsible for clearing pathogens via phagocytosis or extracellular traps [36,37]. In addition, *D. discoideum* may show a farming phenotype, in which food and non-food bacteria are maintained throughout the social life cycle [23]. This behavior is controlled by facultative intracellular *Burkholderia* spp. symbionts, which can be present in high numbers in both trophozoites and spores [38–40]. Further, obligate intracellular *Amoebophilus* and *Procabacter* species, as well as members of the Chlamydiota have been detected as natural symbionts inside *D. discoideum* spores with no measurable fitness costs for the host [41]. In contrast, when *D. discoideum* was artificially infected with environmental

chlamydiae in the past, they either prevented spore formation or were lost during spore development [42,43]. The natural chlamydial symbiont of *D. giganteum* identified here and its retention throughout the social life cycle, thus, point to a well-established symbiosis and a high degree of reciprocal adaptation of both partners.

Electron microscopy reveals peculiar symbiont morphology

To elucidate the morphological features of the symbiont and its location within the amoeba host cells, we cryofixed *D. giganteum* PALH trophozoites and spores and analyzed them at the transmission electron microscope. In both trophozoites and spores, the bacterial symbionts showed a similar appearance and could be identified based on their size between 300 and 800 nm, a Gram-negative type cell wall, and the ribosomes visible in the cytoplasm (Figure 2). Unlike most chlamydiae, the symbionts were located directly in the host cytosol and not surrounded by an inclusion membrane. In other chlamydiae, this host-derived membrane is formed after host cell entry and serves to protect the bacteria from the host immune response and phagocytosis [13]. Although wide-spread among the Chlamydiota, inclusions are not seen for all environmental chlamydiae or host cells [3,42,44]. For *Neochlamydia sp.* S13, a symbiont of *Acanthamoeba* sp., this phenotype is accompanied by a lack of distinguishable EBs [44].

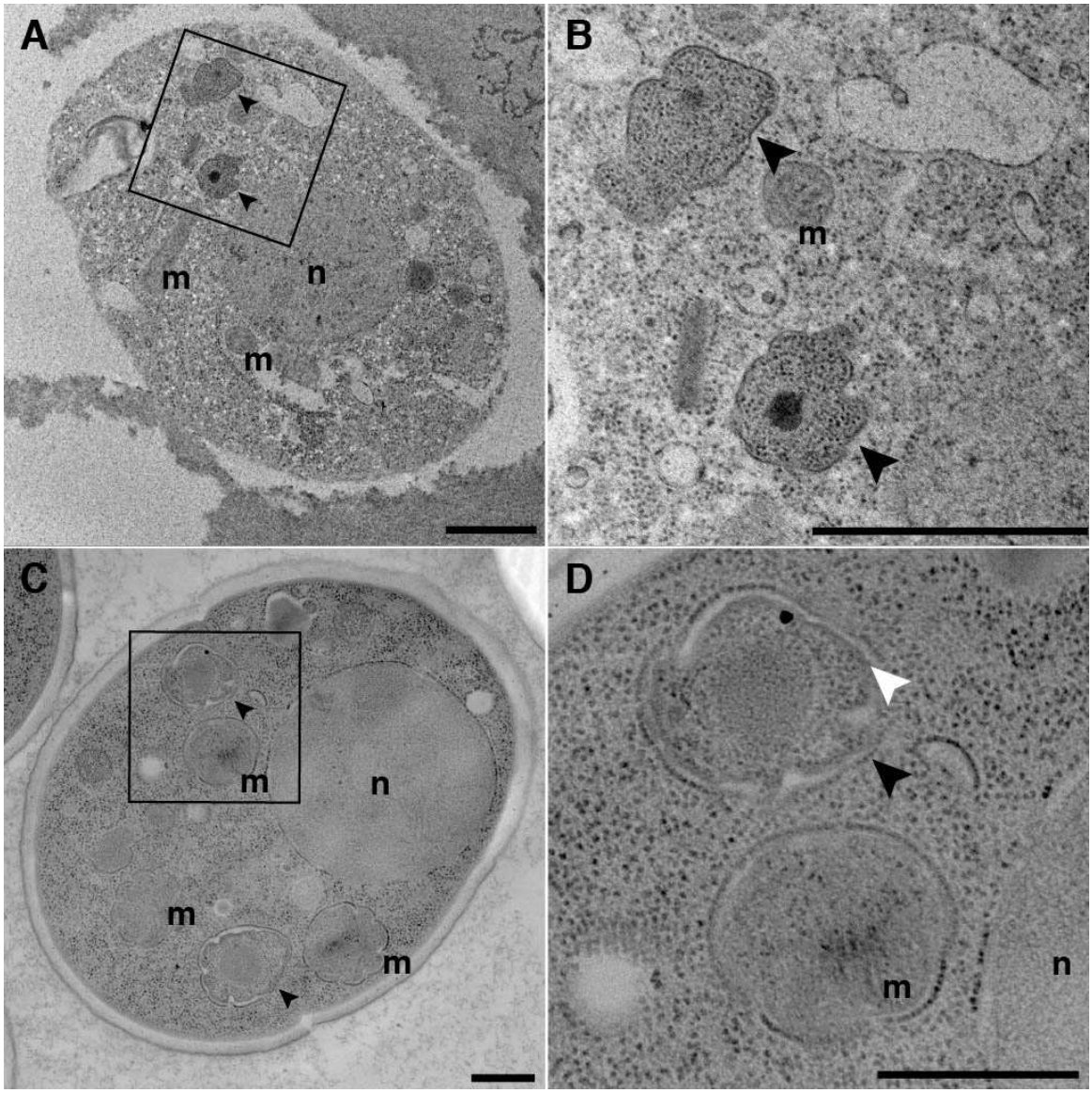

Figure 2: The Chlamydial Symbiont *Reclusachlamydia socialis* in *D. giganteum* PALH. Transmission electron microscopy images of cryofixed and freeze-substituted amoeba trophozoites and spores are shown. Overviews of a single amoebae trophozoite **(A)** and spore **(C)** harboring chlamydial symbionts (black arrowheads). Rectangles indicate the area shown in detail in **(B)** and **(D)**. The chlamydial symbionts show amorphous cell shapes, contain ribosomes and a Gram-negative type cell envelope (white arrowhead). Within the bacterial cells, nucleoid-like structures of varying electron density could be seen in 14% (n=65) and 22% (n=18) of all symbionts within trophozoites and spores, respectively. This difference is statistically not significant (Z-test; p=0.62). Note that different staining methods were used for trophozoite and spore samples, respectively. Bars, 500 nm; n, amoeba nucleus; m, mitochondria.

The chlamydial symbionts in *D. giganteum* show a peculiar, strongly wrinkled, almost flower- or clover-like shape, in many cases containing a nucleoid-like structure of varying electron density (Figure 2). Prominent, electron-dense nucleoid-like structures are a characteristic feature of chlamydial EBs [9,42,45,46]. In *C. trachomatis,* chromatin condensation is mediated by at least two histone-like proteins, HctA and HctB (formerly called Hc1 and

Hc2, respectively), and is involved in the regulation of replication and transcription [47–49]. However, in contrast to classical chlamydial EBs with their rigid and thickened cell envelope, the *D. giganteum* symbionts are of irregular shape, even more pronounced than observed for RBs of some environmental chlamydiae [9]. This polymorphic cell shape suggests a high degree of cell envelope flexibility and a lack of rigid, stabilizing structures. The symbiont morphology is also different from described aberrant bodies, a persistent cell form induced by tryptophan depletion or peptidoglycan-targeting antibiotics in *Chlamydia* species [50]. To our knowledge, the only chlamydial species with a comparable cell morphology was described for a chlamydial symbiont of *D. discoideum*, visualized in spores after chemical fixation [41].

Symbiont genome features and phylogenetic relationship

To investigate if the observed phenotypic traits are reflected in the symbiont's genome, we combined long and short read sequencing and obtained a closed circular genome of 1.74 Mbp with an average G+C content of 38.4%. This is well within the expected range for environmental chlamydiae [14,16,51] (Figure S1). No plasmid was recovered. The genome contains one ribosomal RNA operon, 36 tRNAs and 1236 coding sequences, the latter with an average length of 1251 bp (Table S1). We next constructed a phylogenetic tree using an alignment of 43 conserved marker proteins (Data S2) generated from a data set of high-quality Chlamydiota genomes (Table S2). The maximum likelihood tree placed the chlamydial symbiont in the family Rhabdochlamydiaceae, proposed previously to represent the most species-rich family within the Chlamydiota (Figure 3A, Figure S1, Data S3)[52]. Members of this family were detected in arthropods such as ticks [53], spiders [54,55], and terrestrial isopods [31], all affiliated to the genus *Rhabdochlamydia*. Additionally, the genera *Sacchlamyda* and *Renichlamydia* were proposed to be associated with brown algae and fish, respectively, based on molecular data [56,57]. Average amino acid identity (AAI) values of less than 60% with other members of the Rhabdochlamydiaceae support the classification of the symbiont as a new genus within this family (Figure S2) [58,59]. The symbiont forms a monophyletic clade with a metagenome assembled genome (MAG Gw_Bodden_bin_68) originating from wastewater, which is however only moderately related (60% AAI, Figure 3A, Figures S1, S2). We thus propose the novel name *Reclusachlamydia socialis* for the chlamydial symbiont of *D. giganteum* PALH (see Text S1 for a formal species description).

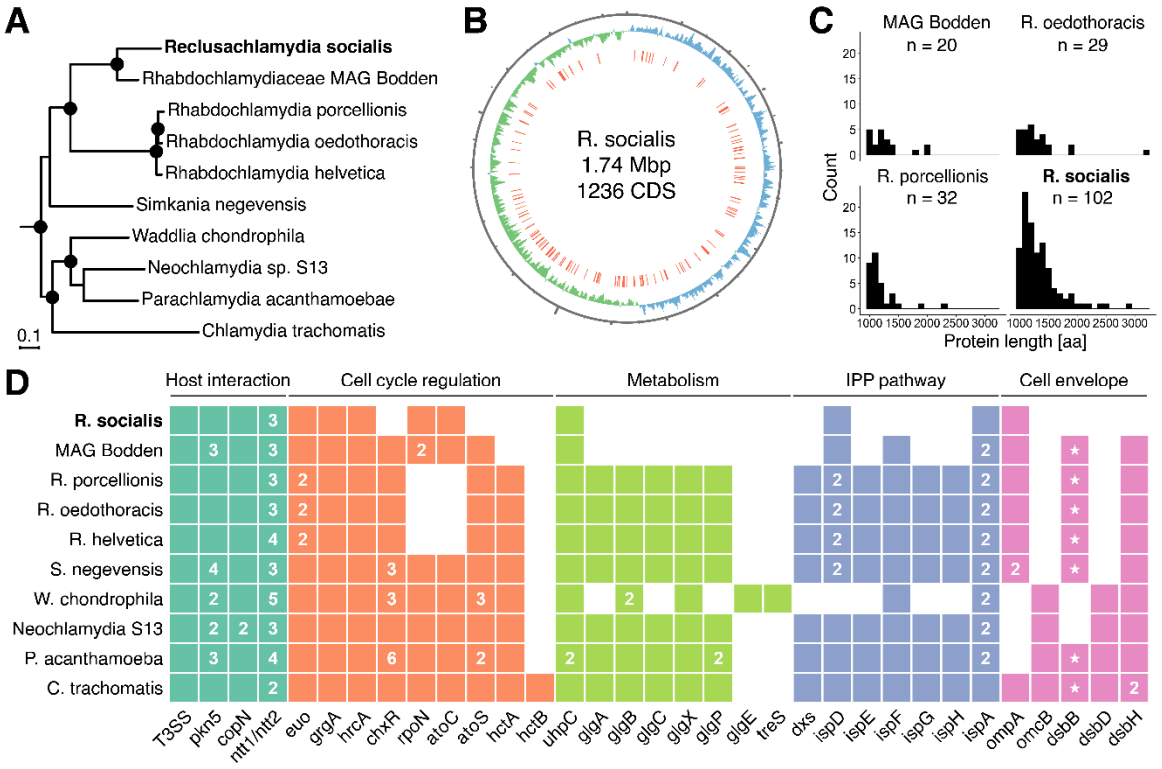

Figure 3: Genome Features of the *D. giganteum* Symbiont *R. socialis*. (A) Phylogenetic relationship of *R. socialis* with select members of the family *Rhabdochlamydiaceae* and the Chlamydiota based on maximum likelihood phylogenetic analysis of 43 conserved marker genes. A pruned tree is shown, the full tree is available as Data S3. *R. socialis* is the first member of the novel genus *Reclusachlamydia* and represents a sister branch of a metagenome assembled genome (MAG) from a wastewater sample. Members of the genus *Rhabdochlamydia* are the closest described and cultured relatives of *R. socialis* (Figure S1). The scale bar represents 0.1 substitutions per site. Black dots indicate ultrafast bootstrap values >90%. **(B)** Genomic map of *R. socialis*. Coordinates in black, GC skew in green and blue; the orange bars denote the position of genes encoding large proteins (>1000 aa). **(C)** Length distribution and absolute number (n) of proteins longer than 1000 aa encoded in the genomes of selected members of the *Rhabdochlamydiaceae*. **(D)** Presence or absence of hallmark chlamydial genes in *R. socialis* and representative Chlamydiota species. Presence and the number of orthologs is indicated by a tile and the depicted number, respectively. Genes which are represented by more than one orthogroup are marked with a star. The orthologous groups containing the T3SS genes *sctJ*, *sctV*, *sctS* and *pknD* were chosen to represent the presence of a functional T3SS. Gene annotation and protein names are available in Table S5.

Large eukaryote-like proteins of *Reclusachlamydia socialis*

Upon initial inspection of the *R. socialis* genome, we noticed that 102 of the total 1236 genes (8.3%) encode unusually large proteins (> 1000 amino acids), which is unexpected for small genomes and more than three times the amount reported for other select members of the Rhabdochlamydiaceae (Figure 3BC). Of these, 91 were annotated as hypothetical proteins but we identified tetratricopeptide repeats, ankyrin repeats and L domain-like stretches in 48%, 14% and 11% of the proteins, respectively (Table S1, see methods). These domains are often part of so-called eukaryote-like proteins, which are found primarily encoded in the genomes of eukaryotes and host-associated bacteria but

rarely in free-living microbes. They are assumed to be secreted to the host cytosol and may interfere with host cellular processes such as cell cycle control, ubiquitination and phagocytosis [60–62]. The importance of eukaryote-like proteins for bacteria replicating in amoebae is underlined by their high prevalence across several bacterial phyla [61,63].

Additionally, we identified protein prenyl transferase domains in 8 of the 102 large proteins, and 14 in total (Table S1). Conversely, none of the related Rhabdochlamydiaceae or the Chlamydiota representatives analysed (Figure 3A) encodes proteins with prenyl transferase domains. The only exception is the amoeba symbiont *Parachlamydia acanthamoebae,* with one predicted protein containing this domain. Prenyl transferases are involved in the post translational modification of proteins in eukaryotes, by specifically binding to CAAX motifs at the N-terminal end of proteins and attaching isoprenoids to the cysteine residue, which leads to the localization of the modified proteins to lipids and membranes [64]. This CAAX motif is often found in members of the Ras superfamily of small GTPases. Small host GTPases are recruited and used for manipulation of cellular pathways by chlamydial pathogens (reviewed in [65]). In *D. discoideum* this superfamily includes Rac proteins, such as RacG, RacJ, and RacH [66]. RacH has been shown to influence bacterial infections in *D. discoideum* in at least two, contradicting ways. Firstly, it is a driver of the acidification of phagosomes, thus deletion of *racH* enhances intracellular growth of *Legionella pneumophila* and *Mycobacterium marinum* in *D. discoideum* [67,68]. Conversely, RacH mediates the non-lytic transmission of *M. marinum* via ejectosomes during cell-to-cell contact [69,70]. Eight out of the 14 putative prenyl transferases encoded in the *R. socialis* genome are predicted to be secreted by the type III secretion system (T3SS) and therefore likely function within the amoeba host cell. The manipulation of host Ras GTPases by symbiont-encoded prenyl transferases could be an efficient way of host-symbiont interaction.

R. socialis lacks vital genes for extracellular survival

Similar to other obligate endosymbionts, chlamydiae experienced substantial gene loss during the millions of years of evolution as strictly host-associated bacteria. Yet, their core genome is remarkably conserved [14,16]. Many of the core genes reflect the obligate intracellular lifestyle and are involved in the interaction with the eukaryotic host cell. The most prominent feature is the chlamydial T3SS and its affiliated effectors [71]. The presence of all relevant structural genes and some well-conserved effectors, such as the T3SS sensor protein CopN and the pseudokinase Pkn5 in the genome of *R. socialis* suggest a functional type III secretion system. Similarly, the two nucleotide transporters NTT1 and

NTT2, as well as the glucose-6-phosphate transporter UhpC, involved in energy parasitism, nucleotide, and glucose acquisition from the host cell [6,13,72] are encoded in the genome of *R. socialis* (Figure 3D, Table S1).

Of note, *R. socialis* lacks all of the known pathways for isoprenoid synthesis. The deoxyxylulose 5-phosphate (DXP) pathway is virtually absent although it is well conserved across the domain Bacteria including the Chlamydiota (Figure 3D). Additionally, none of the genes of the alternative mevalonic acid (MVA) pathway used by the chlamydial species *Waddlia chondrophila* and generally found in mammals and yeast [73,74] could be detected in *R. socialis*. Isoprenoids are essential cellular components involved in critical functions such as electron transport and peptidoglycan synthesis [75]. A dependency on host-derived isoprenoid precursors has been reported for the obligate intracellular pathogen *Rickettisa parkerii* [76] but represents a novel type of parasitism among chlamydiae.

Progression through the bi-phasic developmental cycle is a tightly regulated process in all known members of the *Chlamydiota*. The best-known major regulator, early upstream ORF (EUO), is expressed during the initial infection stage, and its downregulation coincides with transformation of RBs to EBs [77]. This process further relies on the cell cycle regulators GrgA and HrcA [78], all of which are encoded in the genome of *R. socialis* (Figure 3D, Table S1). The σ54 transcription factor (RpoN) that regulates genes involved in the RB-to-EB transition in *C. trachomatis* [79,80] is also present. Conversely, the genome of *R. socialis* lacks the gene for AtoS, part of the two-component signal transduction system AtoS/AtoC activating σ54 [79]. This coincides with the loss of ChxR, a transcriptional regulator of mid- and late cycle genes functioning in, e.g., glycogen and nucleotide metabolism, modification of the inclusion membrane, and the T3SS [10,81].

The extracellular EB stage of chlamydiae demands protection and condensation of the DNA, which is facilitated by two histone-like proteins HctA and HctB in *C. trachomatis* [47–49]. In contrast to *hctB*, *hctA* is conserved across most environmental chlamydiae [16]. Yet, the genome of *R. socialis* lacks both genes (Figure 3D, Table S1). It is thus either unable to pack its chromatin or employs a yet undescribed mechanism, as suggested by transmission electron microscopy (Figure 2). To release chromatin from both HctA and HctB, *C. trachomatis* depends on the expression of IspE, a central enzyme of the DXP pathway missing in the *R. socialis* genome [49,82].

The extracellular EB stage relies on efficient energy storage mediated by glycogen. Glycogen synthesis is thus considered a hallmark of chlamydiae. All known chlamydiae encode one of two glycogen synthesis pathways, i.e., the conventional GlgC-dependent pathway or the trehalose-dependent GlgE-pathway [83,84]. Yet, none of the enzymes of these

pathways is encoded in the *R. socialis* genome (Figure 3D). This suggests the lack of the capability to produce and utilize this storage compound, which should severely impact the symbiont's ability to survive outside the host cell.

Survival of extracellular EBs further depends on a rigid cell envelope, in which the Chlamydia Outer Membrane Complex (COMC) and crosslinking of membrane proteins provides structural stability and facilitates the entry of EBs into the host cell [13]. The most prominent COMC proteins are the major outer membrane protein (MOMP) and the cysteine-rich proteins OmcA and OmcB [85]. *R. socialis* encodes a MOMP, yet lacks the other conserved outer membrane proteins. This is consistent with the lack of disulfide oxidoreductases, such as DsbA and DsbB [14,86], which catalyze disulfide bond formation and crosslinking of membrane proteins (Figure 3D, Table S5). Further, polymorphic outer membrane proteins involved in adhesion of EBs to host cells [87] and the highly variable inclusion membrane proteins are absent in *R. socialis*, the latter of which is consistent with the absence of an inclusion membrane around the symbionts in *D. giganteum* (Table S5).

We noted that the closest relative of *R. socialis*, the *Rhabdochlamydiaceae* MAG Gw_Bodden_bin_68 (Bodden) (Figure 3A), seems to exhibit a similar pattern of gene loss, suggesting that this is a common feature of this clade (Figure 3D). The lack of detectable extracellular chlamydiae and impaired capability to infect new host cells was observed previously for the *Acanthamoeba* symbiont *Neochlamydia* sp. S13 [88]. Yet, its genetic repertoire with respect to the genes involved in EB formation and extracellular survival is largely consistent with other Chlamydiota and differs substantially from the pattern observed in *R. socialis* (Figure 3D). Genome reduction is a process common in endosymbiosis, however, the genes lost in *R. socialis* compared to other chlamydiae indicate a reduced role or complete loss of the extracellular phase of the canonical chlamydial developmental cycle.

Transmission of *R. socialis* is independent of extracellular chlamydiae

The absence of hallmark genes involved in the biphasic chlamydial developmental cycle, particularly in the formation of the extracellular EB stage, led us to investigate how *R. socialis* spreads and establishes new infections. To this end, we used rifampicin to generate an aposymbiotic *D. giganteum* PALH culture. We subsequently tested whether it was possible to reinfect symbiont-free amoeba using supernatant from infected amoeba cultures or lysed symbiont-containing amoeba cells. Despite following well-established protocols[89], reinfection of aposymbiotic amoeba failed repeatedly (data not shown). We

thus resorted to survey and compare infection dynamics of *R. socialis* in aposymbiotic, symbiotic, and mixed (1:5; symbiotic:aposymbiotic) *D. giganteum* populations. We monitored amoeba and symbiont total abundance in both culture supernatant and cellular fraction, as well as *R. socialis* prevalence.

D. giganteum cell numbers increased by a factor of ~230 in all populations during the course of the experiment and were not significantly different between aposymbiotic, symbiotic, and mixed populations 8, 24, 32, 48 and 72 hours after inoculation (ANOVA; $p > 0.1$; at all time points; Figure 4A, Table S3). The absence of cytopathic effects and nearly identical growth in the presence and absence of *R. socialis* indicates that the symbiont does not impose considerable fitness costs on its amoeba host under the monoxenic culture conditions used. This was also reflected in the maximum growth rate of all populations, which was not significantly affected by the presence of the symbiont (Figure S3).

Consistent with host cell growth, the number of chlamydial genome copies measured by digital PCR increased continuously in the cellular fraction of symbiotic and mixed populations over the course of the experiment and reached similar levels in both after 72 h incubation (Figure 4B, Table S4). In contrast, the number of chlamydial genome copies detected at this time point in the supernatant of symbiotic and mixed populations did not differ significantly from the aposymbiotic population (ANOVA; $p = 0.173$; Figure 4B, Table S4). This indicates the absence of extracellular chlamydiae in the culture medium of all populations. A control experiment with a chlamydial symbiont known to produce extracellular EBs showed that we cannot completely rule out the presence of any EBs in the supernatant with our assay (Figure S4). Yet, the number of extracellular EBs would be low and orders of magnitude less than in the cellular fraction or observed for other chlamydiae. In parallel to the quantification of extracellular chlamydiae in the supernatant, we used FISH to monitor the prevalence of intracellular *R. socialis* in the mixed amoeba populations (Figure 4C). Here, we observed a significant increase from 29.9% infected amoeba cells at 48 h to 39.5% at 72 h (t-test; $p = 0.000498$; Figure 4D), with a standard deviation (SD) of 4.8% and 4.5%, respectively.

Taken together, the increase of *R. socialis* cell numbers and prevalence in the mixed populations in the absence of detectable extracellular chlamydiae suggests that the infection of new host cells is independent of an extracellular stage of *R. socialis*. This leaves cell-to-cell transmission as the only option for *R. socialis* to infect naive amoeba host cells. This infection route would demand intimate contact of individual amoeba cells, a behavior frequently observed in dictyostelids, especially during the initiation of the

multicellular stages [18]. The frequency of such intimate contacts between *D. giganteum* trophozoites could readily be seen by light microscopy and increased over the course of the experiment, i.e., with increasing cell densities (data not shown).

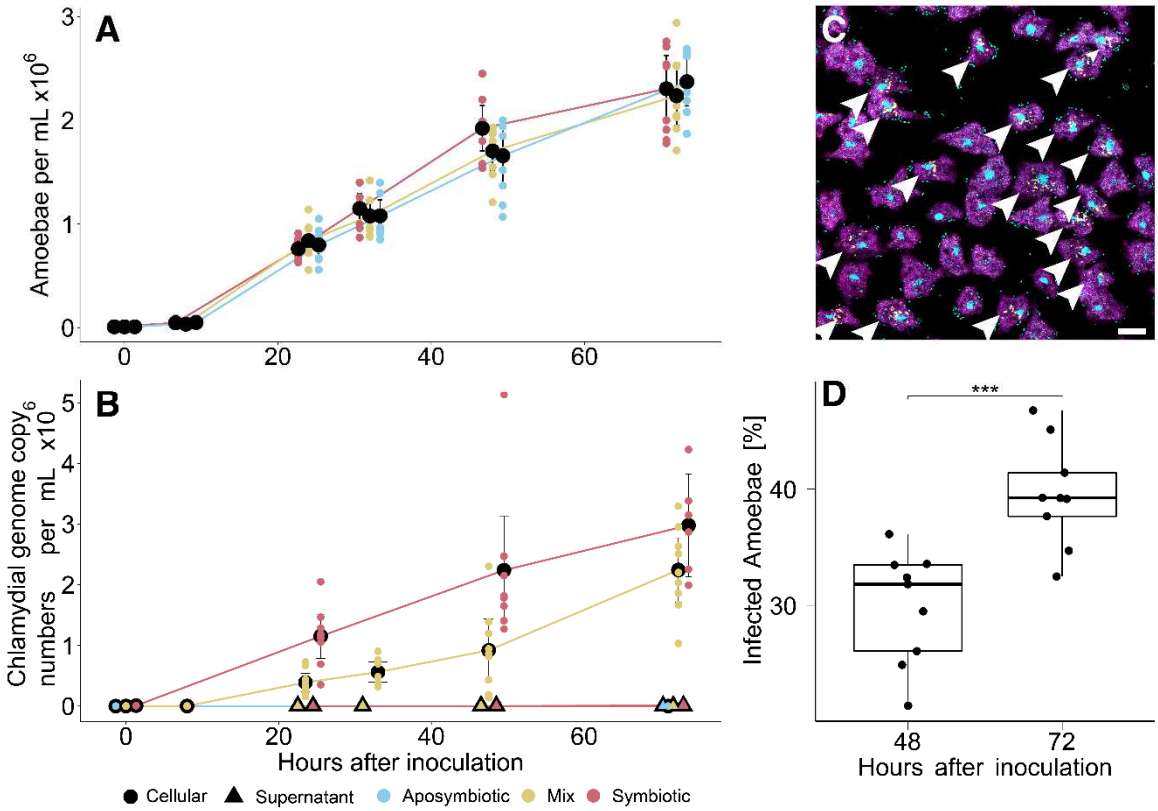

Figure 4: Transmission of *R. socialis* is Independent of Extracellular Chlamydiae. Symbiotic (red), aposymbiotic (blue) or mixed (yellow; 1:5, symbiotic:aposymbiotic) *D. giganteum* populations were allowed to grow in the presence of an excess of *K. aerogenes* as a food source and sampled at 8, 24, 32, 48, and 72 h. Amoeba cell numbers were monitored using a cell counter **(A)**. Chlamydial genome copy numbers were determined by dPCR in cellular fractions (circles) and supernatant (triangles), respectively **(B)**. Mean values or replicates are shown in black or color, respectively. Amoeba growth rate was not significantly affected by the presence of *R. socialis* ($P > 0.05$; ANOVA; Figure S3). Chlamydial prevalence in mixed populations was quantified 48 and 72 hours after inoculation by FISH **(C)** and increased significantly during this period (***, $P < 0.001$, t-test) **(D)**. Each dot represents a replicate. Boxes and error bars depict the interquartile range and the 95% confidence interval, respectively. In the fluorescence images, amoebae are depicted in magenta, chlamydiae in yellow, nuclei and food bacteria *K. aerogenes* in cyan. Infected amoebae are marked with white arrowheads. Bar, 10 µm.

Infection depends on cell-to-cell contact between host cells

Exploiting the direct contact between host cells for transmission is a behavior known from human and amoeba pathogens, such as *Listeria* and *Mycobacteria* species [70,90]. To investigate the importance of cell-to-cell contact for the transmission of *R. socialis* we co-incubated symbiotic and aposymbiotic *D. giganteum* populations under conditions leading to rapid amoeba aggregation and determined chlamydial prevalence after a short incubation period (Figure 5AC). To distinguish between originally symbiotic and

aposymbiotic populations, amoebae were stained differentially using fluorescent live stains prior to co-incubation. A second set of cultures was set up identically, except that the populations were separated by a cell culture insert including a filter membrane. This filter is permeable for chlamydiae but efficiently inhibits migration of amoebae and thus hampers cell-to-cell contact between symbiotic and aposymbiotic amoeba (Figure 5BD). The size-selective retention of amoebae by cell culture inserts has been used for the isolation of chlamydiae in the past [29]. Hence, the cell culture inserts were no barrier for EBs of the chlamydial symbiont *P. acanthamoebae* infecting *Acanthamoeba terricola (*formerly *A. castellanii* Neff) used as positive control for the extracellular transmission route in our experiment (Figure S5). At the end of the incubation period, we removed the cell culture insert containing the symbiotic amoeba. We then sampled the originally aposymbiotic population and examined the prevalence of *R. socialis* using FISH (Figure 5CD). Originally aposymbiotic *D. giganteum* cells that were allowed to freely interact with their symbiotic counterparts showed a chlamydial prevalence of 35.4% (SD=5.6%), which can be explained by the transmission of *R. socialis* between these populations. Conversely, chlamydial prevalence in originally aposymbiotic amoeba physically separated from the symbiotic population was with 3.5% extremely low (SD=1.8%; t-test; $p < 0.0000001$; Figure 5E). In the absence of detectable extracellular EBs, this low percentage of newly infected amoeba most likely resulted from direct interactions with the low number of symbiotic amoeba, which still passed the filter (1.7% of all counted amoebae). We thus concluded that transmission of *R. socialis* is dependent on physical contact between its host cells.

For known chlamydiae, the infection of new host cells primarily depends on the uptake of extracellular EBs. Yet, if uptake is blocked experimentally, direct cell-to-cell transmission has been reported for *C. trachomatis* through tunneling nanotubes in human cell lines [91]. Similar to tunneling nanotubes, *D. discoideum* exhibits temporary cell fusion, particularly during aggregation, called anastomosis [92]. These structures facilitate the exchange of cytoplasm and even mitochondria between neighboring cells, albeit at a low rate [93]. *R. socialis* hijacking *Dictyostelium* anastomosis during the social life cycle could thus represent a route for cell-to-cell transmission. Other conceivable transmission routes include trogocytosis (cell nibbling) or predation of infected amoeba by phagocytosis [94]. We found no indications for these, such as double stained amoeba cells, in our experiments. The mechanism of transmission thus remains to be elucidated.

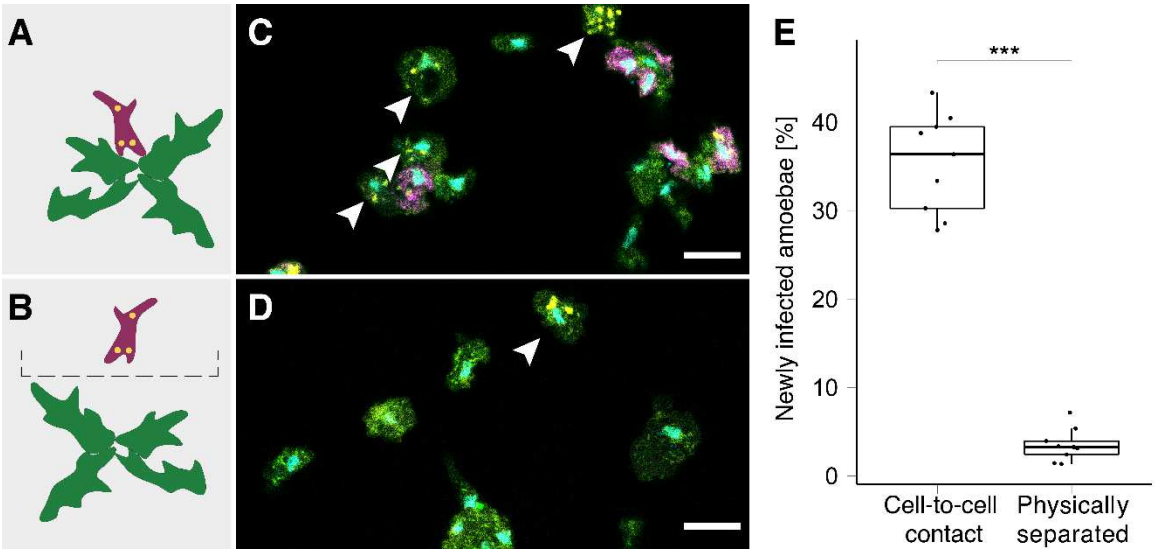

Figure 5: Transmission of R. socialis Requires Cell-to-Cell Contact Between Amoeba Host Cells D. giganteum PALH populations were differentially stained with CellTracker dyes and incubated for 48h, followed by FISH to visualize R. socialis. Originally aposymbiotic amoeba (green) and symbiotic amoeba (magenta, chlamydiae in yellow) could either interact freely **(A, C)** or were separated physically by a filter membrane permeable for bacteria **(B, D)**. At the end of the incubation period, the number of amoebae newly infected with R. socialis was determined by fluorescence microscopy **(C, D)**. P*opulations of* D. giganteum in which the amoebae were allowed to interact *showed a significantly higher prevalence of chlamydiae in originally aposymbiotic cells than the physically separated populations* ***(E)*** *(***, P < 0.001, t-test).* Each dot represents a replicate. Boxes and error bars depict the interquartile range and the 95% confidence interval, respectively. In the fluorescence images, newly infected amoebae are marked with white arrowheads. Bars, 10 µm.

Conclusion

The chlamydiae represent one of the evolutionary oldest groups of strictly host-associated bacteria. Their ability to infect eukaryotic cells and their intracellular lifestyle can be traced back to a last common ancestor that lived around 1 billion years ago [14]. Here we show that one of the most conserved features of all known chlamydiae, their biphasic developmental cycle including an infective extracellular stage, shows remarkable variations in a novel chlamydial species, *R. socialis*, that infects the social amoeba *D. giganteum*.

We present evidence indicating that the chlamydial symbiont *R. socialis* does not have an extracellular EB stage: The symbiont lacks (i) morphologically clearly distinct developmental forms, (ii) genes for extracellular survival and developmental cycle regulation, and (iii) detectable extracellular cells. The symbiont is retained through the dictyostelid social life cycle in an infected amoeba population and is dependent on cell-to-cell contact between host cells for the infection of naïve amoeba.

This represents a remarkable adaptation of a chlamydial symbiont to its host and raises the question of evolutionary causes and consequences. It is tempting to speculate that the long-term co-existence of *R. socialis* with a unicellular host frequently undergoing multicellular stages might have relaxed the dependency on an extracellular chlamydial

form. This would in turn have favored an alternative transmission route in which the symbionts eventually rely on aggregation of their host cells. While the mechanism facilitating direct cell-to-cell transmission is still unclear, *R. socialis* notably encodes putative effector proteins, such as prenyl transferases, with the potential to manipulate host cellular processes involved in the cell-to-cell transmission of *M. marinum* in *D. discoideum* [69,70,95].

The social life cycle of dictyostelids poses a barrier for maladapted intracellular bacteria [36,37,43]. *R. socialis*, however, is well able to persist during the social life cycle and the spore stage. This type of vertical transmission over extended evolutionary time periods is known to lead to reduced virulence of chlamydiae [89]. More generally, vertical transmission increases cooperation between symbiotic partners [96], often resulting in obligate symbioses in which one or both partners strongly depend on the other [97]. Consistent with a previous report about diverse chlamydial and *Amoebophilus* symbionts in dictyostelids [41], we did not observe an effect of *R. socialis* on amoeba fitness with respect to host growth rate, yet host fitness is strongly context dependent [98]. Bacterial symbionts of microbial eukaryotes can function in nutritional or protective symbiosis, the latter of which can be facilitated by biosynthetic gene clusters that are widespread in environmental chlamydiae [99–101]. Defensive symbiosis was described for members of the *Parachlamydiaceae,* which protect their amoeba host against potentially lethal *Legionella* and giant virus infections [102,103]. In dictyostelids, the symbioses between *D. discoideum* and *Paraburkholderia* species leads to decreased host growth in vegetative cells but increased probability of spore survival after germination [23,104–107].

The microbiome of dictyostelids in the wild is diverse and includes among other bacteria various chlamydial lineages, including members of the *Rhabdochlamydiaceae* [26,27,41]. Studying these symbionts will help to further understand microbe-host interactions and evolutionary consequences of facultative host multicellularity. Given that aggregative multicellularity has evolved independently several times in different eukaryotic lineages, [95], studying these organisms will further elucidate how common lifestyle and infection strategy adaptations are among bacterial symbionts.

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Author contributions

Conceptualization, Methodology: L.H., P.A., M.H.; Investigation, L.H., P.A., N.C., A.C.; Resources, Data Curation, A.C.; Writing – Original Draft, L.H.; Writing – Review & Editing, all authors. Funding Acquisition, M.H., A.C.

Declaration of interests

The authors declare no competing interests.

Supplemental information

Document S1. Text S1, Figures S1–S5

Data S1. Fasta file containing the 18S rRNA Sequence of *Dictyostelium giganteum* PALH.

Data S2. Fasta file containing the concatenated protein alignment produced by CheckM.

Data S3. Phylogenetic tree in Newick tree format calculated with genomes listed in Table S2.

Table S1. Excel file containing comprehensive information on the annotation of *R. socialis*.

Table S2. Excel file containing information on the genomes used for calculating the phylogenetic tree and orthogroups.

Table S3. Excel file containing raw data of Figure 4A.

Table S4. Excel file containing raw data of Figure 4B.

Table S5. Excel file containing orthogroups and orthogroup annotation.

STAR Methods

Isolation and maintenance of amoeba and bacteria

D. giganteum PALH was isolated from soil samples from Harvard Forest, Massachusetts, USA. An aliquot was placed on non-nutrient agar (NNA) plates (1 g/L sodium citrate, 0.4 mM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM KH_2PO_4, 15 g/L agar) and overlayed with a suspension of live *E. coli*. After incubation at 20°C and the onset of amoebae growth, trophozoites were picked from the NNA plate and transferred to a fresh *E. coli*-covered NNA plate. Eukaryotic primers TAReuk454FWD (5′-CCAGCASCYGCGGTAATTCC-3′) and TAReukREV3 (5′-ACTTTCGTTCTTGATYRA-3′) were used to amplify and sequence an approximately 290 bp region of the eukaryotic 18S rRNA gene [108].

For long term storage, *D. giganteum* PALH spores were generated on SM/5 plates (2 g/L glucose, 2 g/L bacto peptone, 0.2 g/L yeast extract, 0.1g/L MgSO4, 1.9 g/L KH_2PO_4, 1.0 g/L K_2HPO_4, 15 g/L agar), collected in Page's amoeba saline buffer (PAS; 1 g/L sodium citrate, 0.4 mM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM KH_2PO_4, in MiliQ water) containing 25% glycogen and stored at -80°C. To perform experiments *D. giganteum* PALH were inoculated in PAS buffer supplemented with *E. coli* or *K. aerogenes* as food bacteria in 25 cm² cell culture flasks at 22°C. Bacteria were inoculated in 100 mL LB-Medium (10 g/L tryptone, 5 g/L yeast-extract, 10 g/L NaCl) and incubated overnight at 37°C and 180 rpm. Subsequently, the bacterial cultures were centrifuged at 5,000 x g for 5 min, resuspended in PAS buffer to a final concentration of $2.5x10^{10}$ cells per mL and stored at 4°C.

Generation of aposymbiotic amoeba

NNA plates were overlayed with 200 µL rifampicin (c=2.5 mg/mL) and 200 µL *E. coli* ($2.5x10^{10}$ cells/mL). A single *D. giganteum* PALH fruiting body was picked with a sterile pipette tip and placed in the middle of a freshly prepared plate, which was allowed to dry and then incubated at 20°C until new fruiting bodies were formed. Fruiting bodies picked from this plate were used to repeat the procedure twice. The loss of the symbiont was confirmed by chlamydiae-specific FISH and PCR. Aposymbiotic *D. giganteum* PALH were subsequently propagated using the conditions described above.

Transmission electron microscopy

D. giganteum PALH trophozoites infected with *R. socialis* were grown in 25 cm² flasks in PAS buffer supplemented with *E. coli*. At the phase of nascent aggregation, the trophozoites were harvested by centrifugation at 1500 x g for 3 min. Spores infected with *R. socialis* were grown on SM/5 plates, collected with PAS buffer in the plate lids and centrifuged at 1500 x g for 3 min. Trophozoite and spore pellets were transferred into high-pressure freezing type A carriers (3 mm in diameter, 200 µm in depth) and covered with 20% bovine serum albumin (BSA) and type B carriers (Leica Microsystems, Austria). Before use, the inner surfaces of the carriers were coated with 1-hexadecene. The mounted sample carrier sandwiches were then frozen at approximately 2000 bar using the HPM100 high-pressure freezer (Leica Microsystems, Austria). Freeze substitution was performed in an automated freeze substitution system AFS2 (Leica Microsystems, Austria). Carriers containing high-pressure frozen samples were placed on 1 mL liquid nitrogen frozen 1% OsO_4 in acetone in 2 mL cryotubes. The following temperature program was used for freeze substitution under agitation: 2 h from -105°C to -95°C, 40 h at -95°C, 2 h from -95°C to -90°C, 5 h at -90°C, 2 h from -90°C to -60°C, 2 h at -60°C, 4 h from -60°C to 20°C. Samples were infiltrated with mixtures of 100% acetone and epoxy resin Agar 100 according to the following schedule: 3:1 for 1 h, 1:1 for 1.5 h, 1:3 overnight. The samples were then transferred into silicon embedding molds and infiltrated with low-viscosity resin for 4 h. The resin was polymerized in an oven at 63°C for 28 h. Ultrathin sections of 70–90 nm thickness were prepared with a Leica ultramicrotome (EM UC7, Leica Microsystems, Austria) using a diamond knife (Diatome, Nidau, Switzerland) and mounted on 200 mesh copper grids. The grids holding the ultrathin sections were mounted on a grid holder and single drop stained with 2% gadolinium (III) acetate for 25 min (trophozoites) or 4% neodymium (III) acetate hydrate for 50 min (spores). Subsequently, the samples were rinsed and immersed in water, single drop post-stained with 3% lead citrate for 8 min (in a covered petri dish with NAOH pellets to scavenge lead carbonate) and then washed again with water. The stained ultrathin sections were examined with a Zeiss Libra 120 transmission electron microscope.

Fluorescence *in situ* hybridization

Aliquots of *D. giganteum* cell suspensions were placed on teflon-coated microscope slides and incubated for 15 minutes at room temperature to allow attachment of the trophozoites. The PAS buffer droplet was then replaced with 20 µL of 4% (w/v) paraformaldehyde (PFA) in PAS buffer and incubated for 10 min at room temperature. Fixation was terminated by removing PFA/PAS and briefly washing each well with 50 µL water. The fixed cells were

covered with 10 µL of hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 25% formamide) mixed with 1 µL of the respective probes and hybridized at 46°C for 2 h in an atmosphere saturated with hybridization buffer. The slides were then incubated in washing buffer (20 mM Tris-HCl, 0.25 mM EDTA and 0.149 M NaCl) at 48°C for 15 min and washed in ice-cold water for 10 s. To detect the chlamydial symbionts two Chlamydiota-specific probes each 5'-labelled with the fluorescent dye Cy3 were used simultaneously to increase fluorescence signal intensity (Chla-282, 5'- CTC AAT CCG CCT AGA CGT -3'[109], and Chls-523, 5'- CCTCCGTATTACCGCAGC -3'[110]). For the detection of amoeba the probe Euk-516 (5'- GGA GGG CAA GTC TGG T -3') 5'-labelled with Cy5 was used [111]. To stain DNA, all wells were covered with 10 µL 4′,6-diamidino-2-phenylindole (DAPI) in deionized water (c = 1 µg / mL) for 3 min and subsequently washed for 7 min in 99% ethanol. Citifluor AF1 (Electron Microscopy Sciences, USA) was used to mount a coverslip. Slides were analyzed using a Leica TCS SP8 X confocal laser scanning microscope equipped with a 93x glycerol objective and the Leica application suite X software (v3.7.6).

Growth and infection experiments

To assess the maximum growth rates of symbiotic, aposymbiotic, and mixed (1:5, symbiotic:aposymbiotic; following Hagedorn et al.[70]) *D. giganteum* PALH populations, trophozoites in log phase were incubated at a starting cell concentration of 10^4 cells per mL in PAS buffer supplemented with *K. aerogenes* in 25 cm^2 cell culture flasks (Fisher Scientific Austria GmbH, Austria) at 22°C. Over the course of the experiment 8.5 x 10^9 *K. aerogenes* cells were added to each flask to ensure constant growth of the amoebae. After 8, 24, 32, 48, and 72 h, amoeba cells were detached from the bottom of the cell culture flasks using cell scrapers. Aliquots were taken and frozen for subsequent DNA extraction (250 µL) and measurement of amoeba concentrations (10 µL) using the LUNA-FX7™ Automated Cell Counter (Logos Biosystems, South Korea). For microscopy, 40 µL aliquots were applied to microscope slides and allowed to attach prior to chemical fixation and FISH with chlamydiae- and eukaryote-specific probes. To analyse the supernatant for extracellular chlamydiae, 1 mL aliquots of the supernatant were taken, homogenized using 25 Gauge syringe needles to break possible bacterial cell aggregates, and filtered through 1.2 µm filters to remove amoebae. Samples were frozen for subsequent DNA extraction and digital PCR.

To study the effect of physical contact between amoeba host cells on symbiont transmission, log phase symbiotic and aposymbiotic trophozoites were washed to remove food bacteria and stained with CellTracker deep red and green, respectively, according to

the manufacturer's protocol (Thermo Fisher Scientific Inc., USA). Trophozoites were then inoculated at a concentration of $5x10^5$ cells/mL and a ratio of 1:5 of symbiotic to aposymbiotic amoeba either mixed in 12-well plates or separated using Nunc™ Cell Culture Inserts in Carrier Plate Systems with a pore size of 3 µm (Thermo Fisher Scientific Inc., USA). After 48 h of incubation at 22°C, amoebae were harvested from the wells and transferred to a microscope slide. Chlamydial prevalence was assessed by FISH followed by DAPI staining. Additionally, the number of amoebae which passed the cell culture insert was assessed using the fluorescence derived from CellTracker. Positive controls consisting of *Acanthamoeba terricola (*formerly *A. castellanii* Neff) infected with *P. acanthamoebae* were also assessed but the amoebae were derived from cultures grown in TSY medium (30 g/L trypticase soy broth, 30 g/L yeast extract). Infection with *P. acanthamoebae* was performed 48 h before the start of the co-incubation, at a multiplicity of infection (MOI) of 200. Chlamydiae and amoebae were mixed, centrifuged at 150 x g for 15 min, and the supernatant was exchanged after 3 h to remove excess chlamydiae.

Each experiment was repeated three times, each repeat was performed in triplicates, except for the control with *A. terricola* which was performed once in triplicates.

Symbiont quantification by droplet digital PCR

Aliquots of frozen amoeba cells were thawed, centrifuged at 10,000 x g for 10 min and resuspended in 200 µL PAS buffer. DNA was then extracted using the QIAamp 96 Virus QIAcube HT Kit (QIAGEN, NL) according to the manufacturer's protocol. The efficiency of the extraction was tested using the Qubit system (Thermo Fisher Scientific Inc., USA). Digital PCR was performed using the QX200 Droplet Digital PCR System (Biorad, USA). The final PCR reaction mixture contained 1x QX200™ ddPCR™ EvaGreen Supermix, SigF2 (5'-CRGCGTGGATGAGGCAT-3') and SigR2 (5'-TCAGTCCCARTGTTGGC-3') chlamydiae-specific primers (0.18 pM) [112], 2 µL of the respective DNA sample and ddH_2O in a total volume of 22 µL. Droplet generation was performed according to the manufacturer's protocol. PCR was performed with the following steps: enzyme activation at 95°C for 5 min, 40 steps of denaturation at 95°C for 30 s and annealing at 59°C for 1 min, signal stabilization at 4°C for 5 min, and a final step at 90°C for 5 min. The droplet suspension was subsequently analyzed using the QX200 droplet reader (BioRad, USA) combined with the QX Manager standard edition software (v 1.2). To distinguish positive from negative droplets, a fluorescence amplitude threshold of 6000 was set uniformly for all samples.

Genome sequencing and assembly

To produce high numbers of *R. socialis*, symbiotic *D. giganteum* PALH cultures were incubated in PAS buffer supplemented with *E. coli* in twelve 175 cm² cell culture flasks until confluent cell layers were obtained. The supernatant was replaced with fresh PAS buffer without food bacteria, and the amoebae were detached using a cell scraper. The detached amoebae were transferred to 50 mL tubes and centrifuged at 5000 x g for 10 minutes. The resulting pellet was resuspended in PAS buffer, transferred to 2 mL Eppendorf tubes prefilled with 0.5 mL glass beads (0.25-0.5 mm diameter) and vortexed horizontally at 2700 x g for 2 min. The disrupted amoeba cells were further homogenized using a 25 Gauge needle and passed sequentially through 5 µm and 1.2 µm pore size filters to remove host cell debris and nuclei. The Wizard® HMW DNA Extraction Kit (Promega, USA) was then used to extract DNA for long read sequencing according to the manufacturer's protocol. Library preparation was performed at the Vienna Bio Center Core Facilities using the Multiplex Ligation Sequencing Kit and the Native Barcoding Kit (Oxford Nanopore Technologies, Oxford, UK). Long-read sequencing was performed on an ONT (Oxford Nanopore Technologies, Oxford, UK) Flongle system. Libraries for short read sequencing were prepared using the NEBNext Ultra II FS DNA Library Prep Kit for Illumina, pooled equimolarly, and sequenced using an Illumina MiSeq instrument (2 x 300bp, 600 cycles).

Short reads were trimmed using cutadapt 3.5 [113] to avoid adapter contamination and low quality sequences. Long reads were trimmed using Porechop 0.2.4 [114] and corrected using Canu 2.0 [115]. To assemble the *R. socialis* genome, all reads were used in a hybrid approach using Unicycler 0.4.8 [116]. The resulting single chlamydial contig was then rotated and the origin of replication determined using Ori-finder 2022 [117] and originx [118].

Annotation, comparative genomics, and phylogenetic analysis

Gene calling and annotation was performed using bakta 1.9.1 [119]. The coding sequences called by bakta were the basis for the subsequent comparative genome analysis. InterProScan 5 [120] was used for protein domain identification based on the superfamily 1.75 database [121]. The superfamilies referred to and indicated in Table S1 are TPR-like (SSF48452), Ankyrin repeat (SSF48403), L domain-like (SSF52058) and protein prenyltransferase (SSF48439). The prediction of effector proteins was performed using EffectiveT3 and is indicated in Table S1 [122]. To get further insights in the metabolic pathways encoded in the chlamydial genome eggNOG mapper v2.1.12 [123] was used and the results included in Table S1. AAI was calcuated using the enve-omics tool box with minimum alignment length 0 aa, minimum identity 20% and minimum score 0 bits

(http://enve-omics.ce.gatech.edu/aai/) [124]. To understand patterns of gene loss and gain, a comprehensive dataset of 174 chlamydial genomes and high-quality MAGs (Table S2) was used in orthofinder 2.5.5 [125] with default settings to construct groups of orthologous genes (Table S5). Protein annotations of *C. trachomatis* A/HAR-13, *R. porcellionis* 15C, *S. negevensis* Z and *W. chondrophila* WSU 86-1044 from the Genbank were used to link orthogroups to predicted protein function (Table S2, Table S5). Synteny regions, regions coding for long proteins, and the GC skew were visualized using circos v0.69-9 [126].

For phylogenomics, CheckM [127] was used to produce a concatenated alignment of 43 marker proteins (as in Supplemental Table S6 of the original publication [127]) including the homologs of 171 chlamydial and 89 Planctomycetota-Verrucomicrobiota outgroup representatives (Table S2). This alignment (Data S2) was passed to IQ-TREE 2.2.6 to compute a maximum likelihood tree [128]. Modelfinder plus [129] selected LG+C20+R4 as the optimal model, and the final tree was calculated with ultrafast bootstraps [130], and the (Shimodaira-Hasegawa) SH-like approximate likelihood ratio test [131] with 1000 replicates each. The resulting phylogenetic tree (Data S3) was then visualized and pruned (Figure 3A, Figure S1) to show only relevant phyla using iTOL v7[132].

Data visualization

The plots depicting length distribution of proteins, presence or absence of genes in *R. socialis* and time course experiments were designed using the packages ggplot2 [133], cowplot [134] and ggpubr [135] in the RStudio Statistical Software (2024.09.0) [136]. Phylogenetic trees were visualized using iTOL v7[132]. The visualization of the closed *R. socialis* genome was designed using circos v0.69-9 [126]. Fluorescence microscopy images were edited using the Leica Application Suite X 3.7.6.25597. Figures were merged and edited in Adobe Illustrator.

Statistical Analysis

All statistical analyses in this study were performed using the package rstatix [137] in the RStudio Statistical Software (2024.09.0) [136].

Data availability

The *R. socialis* genome sequence and annotation is available under Bioproject no. PRJEB79376, accession no. ERZ24898538.

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Convergent evolution shaped adaptation of chlamydiae to dictyostelid hosts

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Abstract

Chlamydiae are obligate endosymbionts widely known for their potential to infect and cause disease in humans. The success of chlamydiae as pathogens lies within their adaptations to intracellular life and their conserved biphasic developmental cycle. These adaptations enable chlamydiae to infect multicellular hosts, like animals, and unicellular hosts, namely protists. Many protists, however, evolved the ability to form multicellular structures facultatively. The most prominent examples of this behavior come from dictyostelids, often referred to as social amoebae. In the wild, dictyostelids are associated with various endosymbionts including chlamydiae. The only dictyostelid-associated chlamydial symbiont characterized so far has lost key features of the canonical chlamydial developmental cycle, likely as an adaptation to the facultative multicellularity of the host. Here, we characterized seven dictyostelid isolates harboring symbionts affiliated to two distantly related chlamydial families. Genome sequencing revealed the presence of biosynthetic pathways likely acquired independently by symbionts from both families by horizontal gene transfer, including the thiamine salvage pathway and the eukaryotic mevalonate pathway, respectively. All symbiont genomes experienced massive yet lineage-specific gene loss, especially of genes regulating the developmental cycle. This is reflected in the lack of distinct morphological stages during the host social life cycle and marked differences in gene expression dynamics compared to chlamydiae that exhibit the canonical chlamydial developmental cycle. Despite this divergence, the symbionts from both chlamydial families are able to infect naïve dictyostelids. Together, our findings demonstrate that facultative host multicellularity can drive convergent evolutionary reduction of a developmental program conserved for over a billion years, thereby highlighting the remarkable plasticity of chlamydial symbionts.

Introduction

The intracellular lifestyle of chlamydiae requires a well-orchestrated developmental cycle

The phylum *Chlamydiota* is best known for the species *Chlamydia trachomatis*, a severe human pathogen. Worldwide, more than 1.2 million people suffer from chlamydial eye infections, the main cause of infection related blindness (World Health Organization, 2025). Chlamydial infections of the genital tract occur with a global prevalence of 2.9% and can result in pelvic inflammatory disease and ectopic pregnancies (Huai et al., 2020).

The emergence of molecular techniques, has provided accumulating evidence of chlamydial diversity (Taylor-Brown et al., 2015). Including *Chlamydiceae*, the family that comprises all chlamydial human pathogens, representatives of six chlamydial families have been isolated to date, but more than 1400 families were predicted to exist (Collingro et al., 2020). These so-called environmental chlamydiae have been found in diverse habitats and hosts including animals and protists (Collingro et al., 2020).

All chlamydiae are obligate intracellular symbionts that depend entirely on their host for survival and development (Horn, 2008). However, to spread through a host population, they must leave their host cell and survive extracellularly until they can infect a new host. To meet these conflicting demands, chlamydiae undergo a characteristic and evolutionary ancient developmental cycle that can be traced back to their last common ancestor, around a billion years ago (Dharamshi et al., 2023). Briefly, the extracellular chlamydial stage, known as elementary body (EB), is taken up by a host cell, where it transforms into a so-called reticulate body (RB). The RB then multiplies several times before it develops back into an EB and leaves the host cell (Stelzner et al., 2023). The substantial morphological and physiological changes accompanying these events are orchestrated by a conserved gene expression program. Some regulators such as the early upstream ORF (*euo*) gene which forms part of a complex interaction network involving *grgA* and *hrcA* (Appa et al., 2024; Lu et al., 2024; Rosario & Tan, 2012) are found in virtually every chlamydial genome, however, other regulatory elements are highly diverse and can vary in function even between closely related species (Domman & Horn, 2015). Similarly well conserved is the type III secretion system (T3SS) mediating infection and interactions with the host (Dharamshi et al., 2023) during all stages of development (Elizabeth A. Rucks, 2023). In *Protochlamydia amoebophila,* a symbiont of the soil protist *Acanthamoeba*, transcription of effectors and structural genes of the T3SS is upregulated during the early stages of infection, followed by the upregulation of eukaryotic-like effector proteins, presumably for niche development (König et al., 2017). The chlamydial developmental cycle also requires regulation of the metabolism. Chlamydial metabolism during intracellular stages mainly relies on amino acids, nucleotides and lipids derived from the host. Conversely, endogenous energy production becomes more important during extracellular life stages (König et al., 2017; Omsland et al., 2014), e.g., via glycogen degradation, which is synthesized in advance via one of two conserved pathways (Colpaert et al., 2021). The alternative glycogen synthesis pathway is just one example of the metabolic variation encoded in environmental chlamydiae, compared to *Chlamydiaceae* (Köstlbacher et al., 2021). In addition to glycolysis, which is utilized by all chlamydiae, only some environmental chlamydiae can generate energy via the citrate

(TCA) cycle, a flexible electron transport chain and possess (often incomplete) pathways for amino acid formation (Köstlbacher et al., 2021; Omsland et al., 2014). Studying environmental chlamydiae has revealed which of these pathways and regulatory networks are conserved among chlamydiae and which genes are potentially negligible.

The dictyostelid social life cycle and its influence on the microbiome

The majority of isolates of environmental chlamydiae are symbionts of amoebae (Taylor-Brown et al., 2015). Amoebae are a paraphyletic group of unicellular eukaryotes that move via pseudopods, feed by phagocytosis, and are ubiquitous in the environment (Samba-Louaka et al., 2019). One of the best-studied amoebae to this date is the cellular slime mould *Dictyostelium discoideum*, mainly due to its ability to form multicellular structures during a social life cycle in response to environmental stress. Briefly, *D. discoideum* cells secrete cAMP as a signaling molecule to initiate the aggregation of tens of thousands of single cells, which ultimately develop into a fruiting body, consisting of dormant spores supported by a stalk (Kessin, 2001). All members of the phylum *Dictyostelia*, i.e., dictyostelids, can undergo this social life cycle (Kang et al., 2017; Romeralo et al., 2012). Additionally, *D. discoideum* is a prominent model system for studying cellular processes, such as the interaction with intracellular pathogens (Bozzaro & Eichinger, 2011; Dunn et al., 2018; Kessin, 2001). In recent years, more light has been shed on the interactions between *D. discoideum* and its natural microbiome. Endosymbiotic *Burkholderia* spp. can induce a so-called “farming phenotype”, enabling *D. discoideum* to carry food bacteria within their fruiting bodies (DiSalvo et al., 2015). After dispersing to nutrient-poor habitats, spores can increase their fitness by consuming these food bacteria (Brock et al., 2011). By screening hundreds of natural isolates, the ubiquitous association of wild *D. discoideum* and *D. giganteum* with environmental chlamydiae was shown recently (Haselkorn et al., 2021; Liu et al., 2024). The in-depth analysis of a chlamydial symbiont of *D. giganteum* revealed the lack of developmental cycle features and a primary mode of transmission via host cell-to-cell contact (Helmlinger et al., 2025).

Here, we show that adaptations to facultative host multicellularity are much more widespread among environmental chlamydiae. Studying a collection of dictyostelid strains with respect to developmental cycle, host interaction, and genome evolution of their symbionts revealed striking convergence between diverse chlamydiae infecting social amoebae.

Results

Chlamydial symbionts of dictyostelids belong to two separate clades

To gain a comprehensive understanding of dictyostelid-associated chlamydiae we assembled eight isolates from recent studies (Haselkorn et al., 2021; Helmlinger et al., 2025; Holland et al., 2025) and new collections. These isolates were sampled across the USA and Costa Rica spanning almost 4,000 kilometers (Figure 1A, Table S1) and cover a broad range of species (Figure 1B). Using fluorescence *in-situ* hybridisation (FISH), we demonstrated stable infection with chlamydial symbionts in spores and trophozoites for all six strains tested, including one isolate studied previously (Figure S1) (Helmlinger et al., 2025). Subsequently, we determined the complete genome sequences of the seven new chlamydial isolates. To study their relationships within the phylum Chlamydiota, we aligned 46 core genes from 262 chlamydial genomes and 111 additional bacterial genomes (Table S2) and calculated a maximum likelihood phylogenetic tree (Figure 1B, Figure S2). The dictyostelid symbionts grouped with two phylogenetically divergent chlamydial families. Four isolates belong to the family Rhabdochlamydiaceae, which includes the recently described *Reclusachlamydia socialis*. Three isolates formed a yet undescribed family of the *Chlamydiota* (Figure 1B). Average amino acid identity (AAI) and average nucleotide identity (ANI) suggest that the *Rhabdochlamydiaceae* isolates contain a novel *R. socialis* strain, two strains of the novel species *Reclusachlamydia dictyostelii*, and a member of a novel genus, *Isochlamydia aureostipeii* (Table S3). The closest described relative to this clade is the metagenome assembled genome (MAG), *Rhabdochlamydiaceae* MAG Bodden, originating from wastewater (Figure 1B). The novel family, formerly known as FEN1388 by GTDB and so far, only represented by MAGs, consists of two strains of the same species *Plastochlamydia dictyostelii*, and a second species *P. violaceii*. These isolates belong to the same genus but are only distantly related (Table 1, Table S3). The closest relative of this clade is the MAG JTS-AC372, which was derived from a geothermal spring in Norway (Figure 1B). The chlamydial phylogeny is only partly congruent with the 18S rRNA derived dictyostelid host phylogeny (Figure 1B, Table S4).

The size and GC content of the chlamydial genomes ranged from 1.38 to 1.66 Mb and 35.6 to 43.0%, respectively (Figure 1C, Table 1), both below the average of environmental chlamydiae (Köstlbacher et al., 2021; Taylor-Brown et al., 2015). The numbers of predicted coding sequences ranged from 1,064 to 1,300, thus, considerably less than other environmental chlamydiae (Table 1) (Dharamshi et al., 2023).The close relationship

between the strains, especially those occurring in the same or closely related host species, is reflected in the genome synteny (Figure 1C), which is interrupted by insertion sequences (IS) that are particularly prevalent in *P. dictyosteleii* (Table 1). The related strain *P. violaceii* encodes substantially fewer insertion sequences (Figure 1C, Table 1).

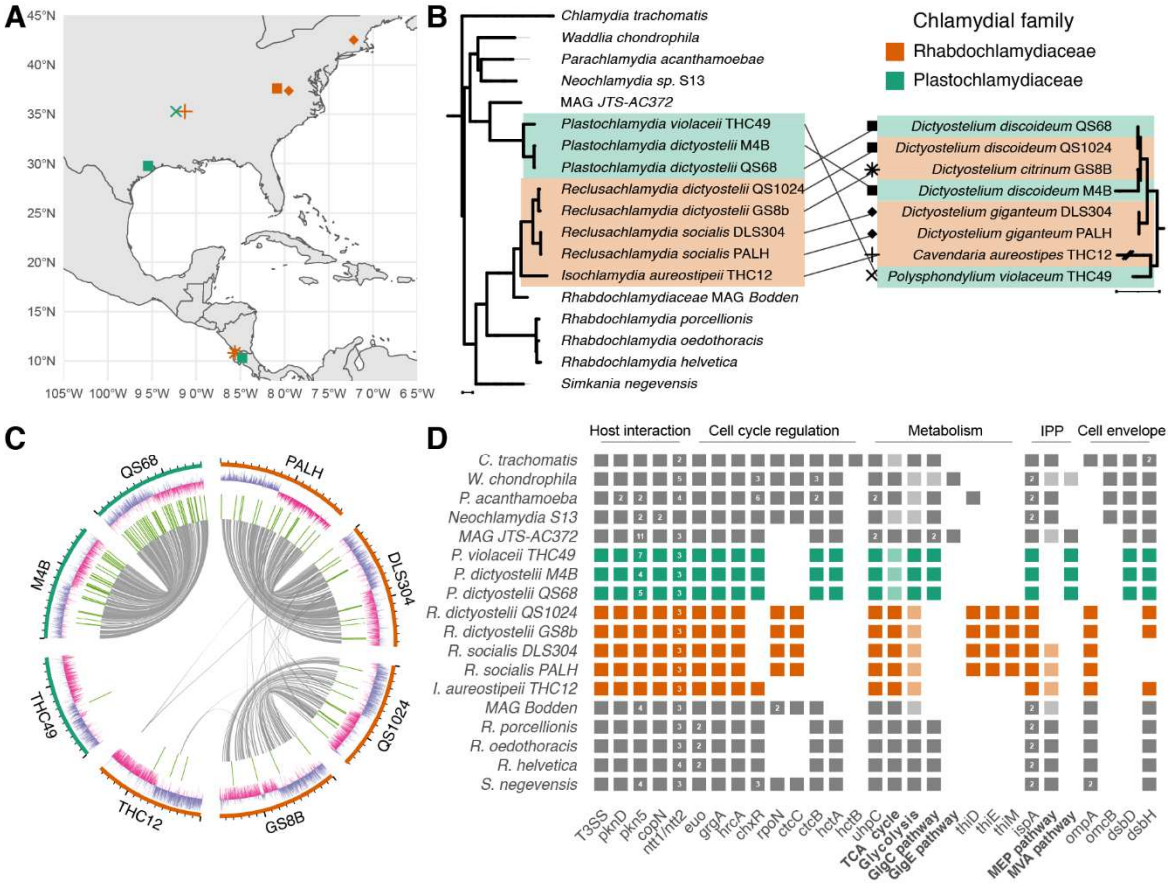

Figure 1: Chlamydial symbionts of dictyostelids belong to two different family-level clades. (A) Geographic location of the dictyostelid sampling sites Isolates from the family of the *Rhabdochlamydiaceae* or *Plastochlamydiaceae* are depicted in orange or turquoise, respectively. Isolates of *C. aureostipes*, *D. citrinum*, *D. discoideum*, *D. giganteum* and *P. violaceum* are indicated by squares, circles, triangles, diamonds and crosses respectively. Note that the positions of the isolates DLS304 and THC12 have been shifted east by one degree for better readability. (B) The symbiont phylogeny within representatives of the Chlamydiota, based on a maximum-likelihood tree generated from the alignment of 46 conserved proteins. The host phylogeny is based on the alignment of 18S rRNA gene sequences derived from the isolates and representative Eumycetozoa sequences (Table S4). The scale bar represents 0.1 substitutions per site. (C) Genomic map of the chlamydial symbionts, labelled with strain names. Coordinates in black (each tick indicates 100 kbp); affiliation of the symbiont is indicated by color of the outmost section; positive and negative GC skew in violet and magenta, respectively; position of insertion sequences in green; syntenic regions found by blast larger than 10 kbp and e-value smaller than 10^{-10} in grey. (D) Presence or absence of chlamydial genes. The order of the genomes follows the relation indicated in panel B. The presence and number of orthologs are indicated by a tile and the depicted number, respectively. Metabolic pathways are depicted as complete, incomplete or absent by filled, opaque and absent tiles, respectively.

Table 1: Genome assembly and annotation statistics of the chlamydial symbionts.

Species/Strain	Length [Mbp]	GC [%]	coding density [%]	tRNAs	rRNAs	CDSs	Hypothetical Proteins	IS elements
***P. violaceii* THC49**	1.38	35.6	89.2	36	9	1064	704	1
***P. dictyostelii* M4B**	1.54	39.4	87.3	37	9	1259	865	31
***P. dictyostelii* QS68**	1.51	39.3	86.7	37	9	1300	906	48
***R. dictyostelii* QS1024**	1.42	39.7	89.5	36	3	1121	554	10
***R. dictyostelii* GS8b**	1.59	39.2	89.5	36	3	1139	586	4
***R. socialis* DLS304**	1.66	38.6	89.5	36	3	1188	587	14
***I. aureostipeii* THC12**	1.46	43.0	90.9	36	3	1133	584	3

Genomic signatures of chlamydiae associated with dictyostelids

Low population sizes and genetic drift evokes loss of genes and cellular functions in endosymbionts, leading to increased dependency on the host (Bennett & Moran, 2015; Husnik & Keeling, 2019; McCutcheon et al., 2019). To study gene loss, we calculated orthologous groups of genes within the curated set of chlamydial and bacterial genomes (Table S1). A total of 858 (80.6%) and 780 (69.6%) orthologous groups are shared among the *Plastochlamydiaceae* and the *Reclusachlamydiaceae*, respectively. Common chlamydial virulence factors are conserved in the genomes of both *Rhabdochlamydiaceae* and *Plastochlamydiaceae*, e.g., genes vital for a functioning T3SS, the serine/threonine kinase *pknD,* the pseudokinase *pkn5* and the sensor protein *copN* (Figure 1D). Conversely, various conserved transcription factors and regulatory proteins controlling the chlamydial developmental cycle are absent. All *Reclusachlamydia* genomes lack the transcription factor *chxR* that regulates the expression of mid- and late-cycle genes (Yang et al., 2017). Likewise, only parts of the two-component signal transduction system *AtoS/AtoC* activating the σ54 transcription factor (*RpoN*) are encoded in the dictyostelid-associated genomes. The histone-like protein *HctA* is not encoded in the genus *Reclusachlamydia* and *I. dictyosteleii* (Figure 1D). Orthologs of the nucleotide transporters encoded by *ntt1/ntt2* are present in various copy numbers in all dictyostelids-associated strains (Figure 1D). A complete glycolysis pathway is encoded by *Plastochlamydia*, in contrast to *Reclusachlamydia*. Conversely, all *Reclusachlamydia* species encode a complete TCA cycle, which is largely eroded in all *Plastochlamydia* species (Figure 1D). Chlamydiae can synthesize the energy storage compound glycogen either via the conserved *glgC* or the alternative *glgE* pathway (Colpaert et al., 2021). The *glgC* pathway is fully encoded in all *Plastochlamydiaceae* in contrast to all *Reclusachlamydia* genomes (Figure 1D). Proteins related to the assembly of the cell envelope are species-specific

(Collingro et al., 2011), thus, the major outer membrane protein, *OmpA*, the chlamydial membrane complex forming, *OmcB*, and the disulfide bridge forming proteins *DsbD* and *DsbH* are all poorly conserved (Figure 1D).

Endosymbionts can gain genes by horizontal gene transfer within host species like amoebae (Dharamshi et al., 2023; Price et al., 2024). We found possible cases of HGT, as all dictyostelid-associated chlamydiae gained genes which are unusual for chlamydiae. The genus *Reclusachlamydia* encodes genes of the thiamine salvage pathway *thiD, thiE* and *thiM*, enabling them to recycle thiamine, a vital co-factor of many metabolic processes (Kraft & Angert, 2017). In the Chlamydiota, isoprenoids, biomolecules with a plethora of functions, are generated via the bacterial methylerythritol phosphate (MEP) pathway which has been lost in all dictyostelid-associated chlamydiae. Of note, the *Plastochlamydiaceae* recovered this function by encoding the eukaryotic mevalonate (MVA) pathway, rarely found in bacterial genomes (Rohmer et al., 2004).

Taken together, the patterns of gene loss and gain are consistent within *Reclusachlamydia* and *Plastochlamydia*, indicating the loss of conserved chlamydial regulatory genes. However, the patterns vary substantially between the two families.

Phenotypic adaptations and effect on host fitness

We were interested in whether the observed genomic differences between the two symbiont clades influence their developmental cycle and mode of transmission. Thus, we compared *Reclusachlamydia dictyostelii* QS1024 and *Plastochlamydia dictyostelii* QS68 as family representatives infecting the same host species, *D. discoideum*. For clarity, they will be referred to by their genus name in the rest of the result section. Both symbiotic *D. discoideum* strains were able to enter the social life cycle, leading to the formation of fruiting bodies and viable spores (Figure S1). Characterization via FISH provided evidence that *Reclusachlamydia* and *Plastochlamydia* infect spores at a prevalence of 93.9%, with a standard deviation (SD) of 2.0%, and 82.7% (SD = 9.1%), respectively (Figure 2D). The number of symbionts per cell was below ten and five chlamydiae per trophozoite and spore, respectively, which is far fewer than in other chlamydial species (Kostanjšek et al., 2004; Lee et al., 2018). Since chlamydiae are parasites, we were interested in the effects of *Reclusachlamydia* and *Plastochlamydia* on their hosts. Using antibiotics, we generated aposymbiotic *D. discoideum* QS1024 and QS68 cultures and compared their growth rate and spore production. The incubation of *D. discoideum* QS1024 and QS68 with excess food bacteria led to exponential cell growth over 48 hours, before the growth rate decreased over the next 24 hours and eventually decreased until the end of the incubation after 8 days (Figure 2A) (Table S5). The chlamydial symbionts did not significantly

influence the amoebae cell numbers at any time point ($p > 0.1$; t-test). Host spore production was increased by *Reclusachlamydia* and decreased by *Plastochlamydia*, however, not significantly ($p > 0.1$ and $p = 0.99$, respectively; t-test, Table S6).

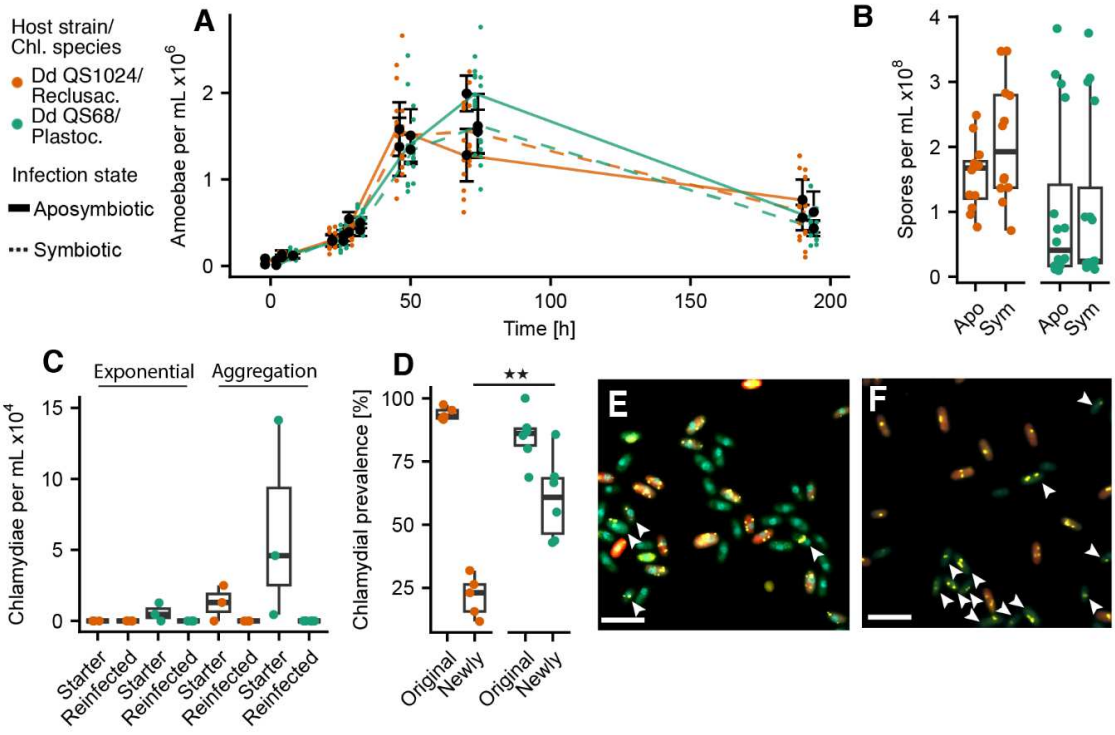

Figure 2: Interactions of *Reclusachlamydia* and *Plastochlamydia* with their respective host species. (A) Growth of symbiotic and aposymbiotic *D. discoideum* trophozoites supplemented with food bacteria and sampled after 6, 24, 30, 48, 72 and 192 hours. Neither *D. discoideum* strains growth rate was significantly affected by the symbionts ($p > 0.1$; t-test) (B) Total spore production of symbiotic and aposymbiotic *D. discoideum* populations, counted with a hemocytometer, not significantly affected by chlamydiae ($p > 0.1$; t-test) (C) Chlamydiae in the supernatant of exponentially growing and aggregating starter cultures, respectively, were used to reinfect aposymbiotic *D. discoideum* trophozoites but no chlamydiae were detected after 5 days of incubation. Chlamydial numbers were measured via dPCR. (D) Transmission of chlamydiae from symbiotic to aposymbiotic host cells during fruiting body formation quantified via FISH for *Reclusachlamydia* (E) and *Plastochlamydia* (F) spores. Originally symbiotic and newly infected dictyostelids are depicted for both isolates. Despite the lower original prevalence, the prevalence of spores newly infected with Plastochlamydia was significantly higher than for *Reclusachlamydia* ($p < 0.01$; t-test). In the fluorescence images, originally symbiotic and aposymbiotc chlamydiae are depicted in red and green, respectively, chlamydiae in yellow, and nuclei in cyan. Chlamydiae-infected, originally aposymbiotic amoebae are marked with white arrowheads. Scale bars, 10µm. In each plot, data derived from *R. dictyosteleii* and *Plastochlamydia* is depicted in orange or turquoise, respectively. Each colored dot represents a replicate; black dots indicate the mean. Boxes and error bars depict the interquartile range and the 95% confidence interval, respectively.

Chlamydial transmission depends on extracellular particles, the EBs. We used digital PCR (dPCR) to test for the presence of *Reclusachlamydia* and *Plastochlamydia* in the supernatant of symbiotic *D. discoideum* cultures during exponential growth and aggregation (Figure 2C). Aggregated cultures released more chlamydiae than exponentially growing cultures, and *Plastochlamydia* exceeded *Reclusachlamydia* numbers, however, not significantly ($p > 0.2$; t-test; Table S7). To assess the infectious potential of extracellular chlamydiae, we subsequently applied the supernatant to aposymbiotic populations of the original host, but we did not observe reinfection (Figure

2C, Table S8). To test transmission efficiency, we mixed aposymbiotic and symbiotic host cells (1:1) and let them undergo the social life cycle. After development, we tested the prevalence of chlamydial symbionts in originally aposymbiotic host cells using FISH. The ratio of newly infected amoebae was significantly higher in cultures infected with *Plastochlamydia* (60.5 %; SD = 16.6 %) than *Reclusachlamydia* (21.7 %; SD = 8.1 %) ($p < 0.01$; t-test) (Figure 2D).

Despite the genomic differences between the two chlamydial species, they showed converging phenotypes, i.e., no significant effects on host fitness and few or no extracellular particles. Nevertheless, both symbionts spread during the host's social life cycle, resulting in stable symbioses.

Chlamydial developmental cycle during the host life cycle

The phenotypic similarities to *R. socialis*, especially the apparent absence of extracellular chlamydiae from the host supernatant, raised the question of whether and when the symbionts undergo differentiation from RB to EB (Helmlinger et al., 2025). To assess this and to investigate the influence of host life stages on chlamydial developmental forms, we cryofixed symbiotic *D. discoideum* strains during exponential growth, aggregation and at the spore stage and performed transmission electron microscopy (Figure 3).

The two chlamydial symbionts varied in size and staining properties. The symbionts resembled rippled spheres with diameters from 400 to 700 nm and 500 nm to 1.2 µm for *Reclusachlamydia* and *Plastochlamydia*, respectively. The cell envelope of *Plastochlamydia* was intensively stained and easily distinguishable from host membranes. Most *Plastochlamydia* cells were surrounded by small vesicles, with a maximal diameter of 50 nm, similar to but smaller than vesicles secreted by *C. trachomatis* associated with virulence factors (Frohlich et al., 2012; Giles et al., 2006). The electron-dense staining of their membrane suggests they are symbiont-derived vesicles (Figure 3J-L). Like *Reclusachlamydia*, *Plastochlamydia* seems to reside directly in the host cytosol, however, temporarily it is located inside a vacuole (Figure 3J). In both cases, *Plastochlamydia* predominantly appears in aggregates or clusters of at least two cells in contrast to *Reclusachlamydia*, occurring as individual cells, apart from putative cases of ongoing cell division (Figure 3D). Both species did not display morphologically distinct developmental stages as seen in most (environmental) chlamydiae. During aggregation, some *D. discoideum* trophozoites formed conjunctions near *Reclusachlamydia* cells at which they appeared to exchange cytosol, also known as anastomosis (Huffman et al., 1962) (Figure 3BE).

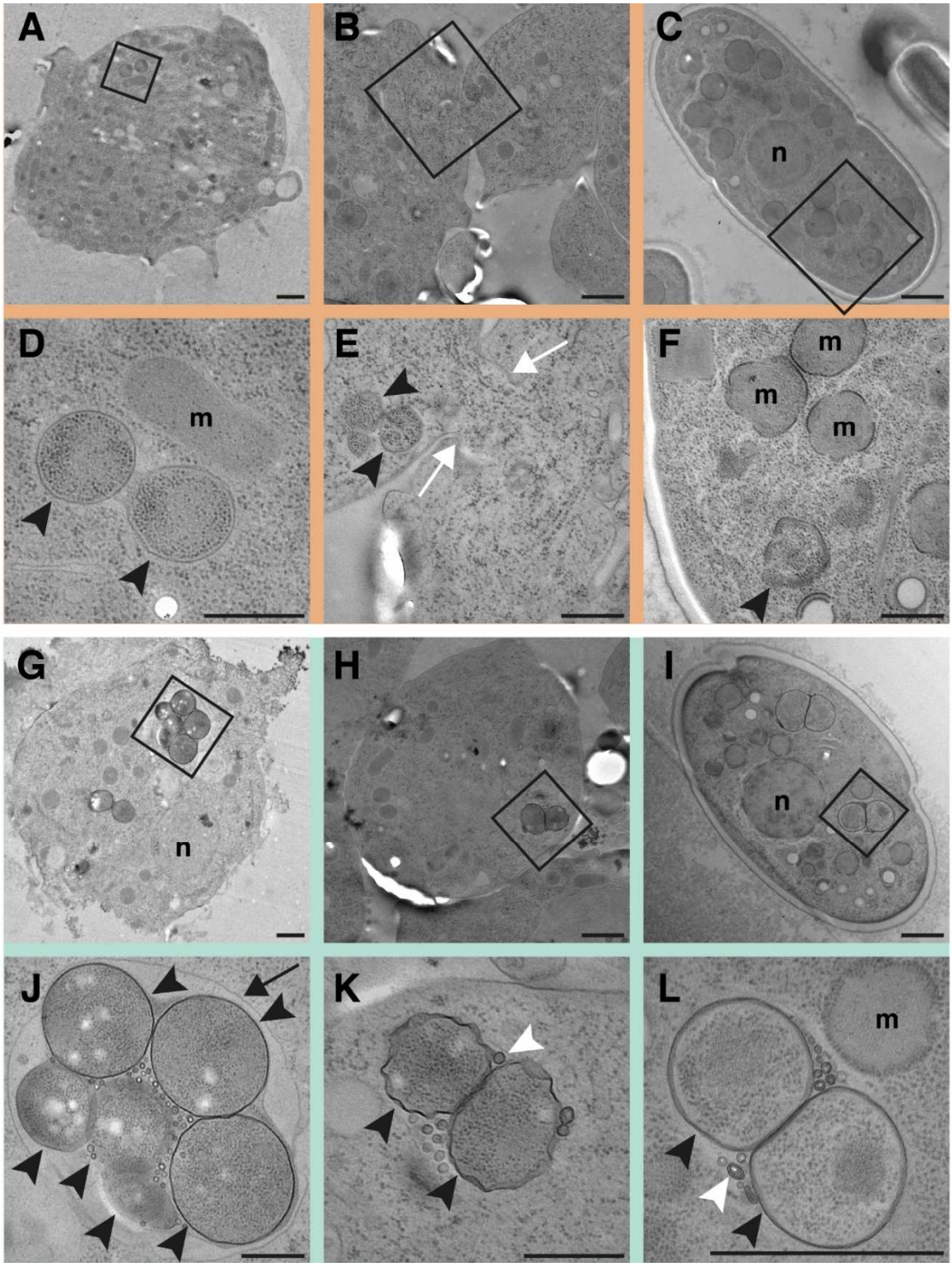

Figure 3: Morphology of *R. dictyosteleii* (A-F) and *Plastochlamydia* (G-L) in *D. discoideum* exponentially growing cells (left column), aggregating cells (middle colum) and spores (right column). In each overview (A-C and G-I) a square indicates the area shown in detail in the image below (D-F and J-L). Scale bars, 1µm and 500nm in overview or detail images, respectively; black arrowheads, chlamydiae; white arrowheads, chlamydial vesicles; black arrows, inclusion membrane; white arrows, amoebae junction; n, amoeba nucleus; m, mitochondria.

The impact of the dictyostelid social life cycle on the chlamydial transcriptome

The biphasic developmental cycle is well-reflected in the transcriptome dynamics of both chlamydial pathogens and chlamydial symbionts of amoebae (Albrecht et al., 2010, 2011; König et al., 2017; Wurihan et al., 2024). Chlamydiaceae upregulate uptake- and infection-related genes in EBs and genes involved in replication in RBs (Albrecht et al., 2010, 2011). Gene expression data from *P. amoebophila* show a stepwise activation of T3SS-related genes and a shift from carbohydrate- to amino acid-dependent metabolism during EB-to-RB transformation (König et al., 2017). The absence of extracellular forms and uniform morphology across host life stages in dictyostelid-associated chlamydiae raises the question of whether chlamydial gene expression follows these canonical chlamydial transcriptional dynamics. Alternatively, the chlamydial transcription is influenced by the substantial shifts in the physiology and morphology of *D. discoideum* during the host social life cycle. To address this, we extracted and sequenced RNA from *Reclusachlamydia* and *Plastochlamydia* symbionts derived from exponentially growing *D. discoideum* trophozoites and from spores. To test whether infection events during host aggregation (Figure 2D) are reflected in the chlamydial transcriptome, we also sequenced RNA derived from aggregating *D. discoideum* trophozoites, in either completely symbiotic culture or predominantly (80%) aposymbiotic culture.

RNA sequencing yielded an average library size of 88.6 million reads per sample (SD = 12.8 million) of which an average of 7.3 million reads (SD = 4.2 million), corresponding to 8.3 % (SD = 4.9 %), mapped to the respective chlamydial genomes (Table S9). In both chlamydial species, gene expression profiles grouped by host life cycle stage (Figure 4A-C). We identified 528 (47.1% of all genes) and 857 (65.9%) genes, which were differentially expressed (DE), (false discovery rate [FDR] adjusted $p < 0.05$, log2-fold change ≥ 1), during the host life cycle in *Reclusachlamydia* and *Plastochlamydia*, respectively. The remaining genes were constitutively expressed under the tested conditions. In both species, the comparison between samples derived from exponentially growing host cells and from spores yielded the highest number of DE genes. However, the number of DE genes between aggregating host cells and spores was markedly lower in *Reclusachlamydia* than in *Plastochlamydia* (Figure 4BC). The presence of aposymbiotic host cells had no detectable effect on chlamydial gene expression profiles (permutation test; FDR adjusted $p < 0.05$) (Figure 4BC). Thus, we excluded the samples with aposymbiotic hosts from downstream analysis.

To further explore transcriptional dynamics in *Reclusachlamydia* and *Plastochlamydia*, we identified six hierarchical clusters of co-regulated DE genes per species based on their expression dynamics across the host life cycle (Figure 4DE, Table S10). We then tested each cluster for enrichment of COG categories and KEGG pathways (Table 2, Table S11) (FDR adjusted $p < 0.05$). A common pattern in both species was the enrichment of energy production related genes in clusters upregulated during the host trophozoite stages (Table 2), which is typically seen for metabolically active RBs (Albrecht et al., 2011; Stelzner et al., 2023). In *Reclusachlamydia*, host aggregation evokes upregulation of ABC transporters, which channel host nutrients across chlamydiae membranes (Bachmann et al., 2014). The enrichment of genes involved in translation among constitutively expressed genes, implies ongoing chlamydial replication in all life stages of *D. discoideum*. Notably, a majority (57%) of the constitutively expressed genes of COG category L are transposases. For comparison of co-regulated gene clusters with the canonical developmental cycle we assessed the overlap with EB- and RB-transcript sets derived from *C. pneumoniae* (Albrecht et al., 2011). Across both species, only gene cluster, *Plastochlamydia*-II, which includes genes predominantly upregulated during host exponential growth and aggregation, significantly overlaps with genes expressed in *C. pneumoniae* RBs (FDR adjusted $p < 0.01$; permutation test) (Figure 4FG, Table S12). Similarly, though not significant, trends were observed for the overlap of *Reclusachlamydia*-II with the *C. pneumoniae* RB gene set (FDR adjusted $p = 0.22$; permutation test) and for *Plastochlamydia*-VI with the *C. pneumobiae* EB gene set (FDR adjusted $p = 0.14$) (Table S12). These results indicate pronounced shifts in chlamydial gene expression across the host social life cycle, which, however, only partly resemble the canonical chlamydial developmental cycle.

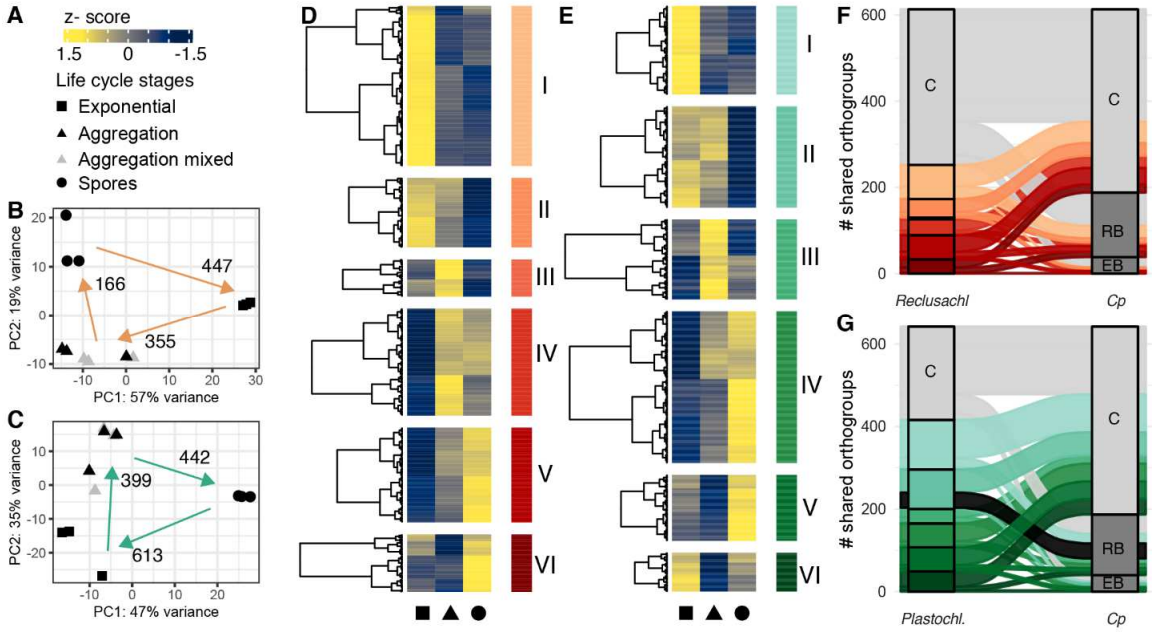

Figure 4: Gene expression of the chlamydial symbionts during the social life cycle of *D. discoideum.* Schematic of the life cycle stages, exponential growth, aggregation, aggregation of mixed (aposymbiotic:symbiotic) trophozoites, and spore stage, of which RNA was extracted (**A**). Principal component (PC) analysis plot for RNA-Seq gene expression data at the three life cycle stages shown in panel A, for *Reclusachlamydia* (**B**) and *Plastochlamydia* (**C**). The number of differentially expressed chlamydial genes between dictyostelid life stages is depicted next to the arrows connecting the groups. Clusters of co-regulated genes during the host social life cycle in *Reclusachlamydia* (**D**) and *Plastochlamydia* (**E**), indicated by colored bars and Roman letters. Overlap between genes upregulated in *C. pneumoniae* developmental cycle stages with co-regulated gene clusters of *Reclusachlamydia* (**F**) and *Plastochlamydia* (**G**); flows are colored by the source cluster, and significantly enriched overlaps are highlighted in black. Letters in temporal gene clusters of *C. pneumoniae* refer to genes expressed constitutively (C), upregulated in reticulate bodies (RB) and upregulated in elementary bodies (EB).

Table 2: COG categories and KEGG categories significantly enriched (FDR < 0.05) in temporal clusters.

Species	Cluster	COG category	KEGG pathway
Reclusachlamydia	I	[C] Energy production and conversion	Citrate cycle (TCA cycle) Oxidative phosphorylation
	II	[J] Translation, ribosomal structure and biogenesis	Ribosome
	IV	-	ABC transporters
	Const	[J] Translation, ribosomal structure and biogenesis	
Plastochlamydia	I	[J] Translation, ribosomal structure and biogenesis	Protein export Fatty acid metabolism Ribosome
	II	[G] Carbohydrate transport and metabolism [C] Energy production and conversion [T] Signal transduction mechanisms	Glycolysis/ Gluconeogenesis Oxidative phosphorylation Starch and sucrose metabolism Pentose phosphate pathway
	Const	[L] Replication, recombination and repair	-

Discussion

Wild dictyostelids are frequently associated with environmental chlamydiae of two distantly related families (Haselkorn et al., 2021; Helmlinger et al., 2025; Holland et al., 2025; Liu et al., 2024). Both families have convergently adapted to their hosts by losing vital features of the conserved chlamydial developmental cycle. The symbionts stably infect all host life stages (Figure S1) and are rarely detected extracellularly (Figure 2C), suggesting increased importance of vertical over horizontal transmission. The genomes of dictyostelid-associated chlamydiae are reduced and lack genes vital for development and extracellular survival (Figure 1D), a common pattern of advanced symbiont adaptation (McCutcheon et al., 2019; McCutcheon & Moran, 2012). The loss of regulatory genes could also result from host regulation of development (Maurya et al., 2025; McCutcheon & Moran, 2012). In line with adapted development we observe reduced morphological variation (Figure 3), and the chlamydial gene expression dynamics across the host life cycle showed little resemblance with the canonical chlamydial developmental cycle (Figure 4FG). However, many of these adaptations are less advanced in *Plastochlamydia*. It is detectable extracellularly, albeit at low abundance, and infects aposymbiotic hosts more efficiently than *Reclusachlamydia* (Figure 2CD). Its temporal gene expression partly mirrors gene expression of *C. pneumoniae* (Figure 4G) and all Plastochlamydiaceae encode a complete glycogen synthesis pathway, which may support limited extracellular survival (Figure 1D) (Colpaert et al., 2021). The high abundance of transposases in the genome of *Plastochlamydia* and their activity seen at the transcriptional level indicate ongoing genome reduction, described for endosymbionts which recently switched from horizontal to vertical transmission (De Albuquerque et al., 2020; McCutcheon & Moran, 2012; Wu et al., 2015). Taken together, these features suggest advanced but ongoing adaptation of *Plastochlamydia* to its host social life cycle.

Infection events despite the lack of extracellular particles suggests transmission by host cell-to-cell contact (Helmlinger et al., 2025). Whether transmission is actively induced by chlamydiae or occurs passively by transient exchange of cytosol between host cells remains unclear. Dictyostelids can exchange cytosol by anastomosis and cell nibbling described in *D. discoideum* and *Dictyostelium caveatum*, respectively (Huffman et al., 1962; Nakada-Tsukui & Nozaki, 2021). However, chlamydial cells next to transient cell contacts of *D. discoideum* cells during aggregation (Figure 3E) and the small effect of aposymbiotic dictyostelids on chlamydial gene expression (Figure 4B-C) suggest passive transmission. In either case, reduced capacity to exit host cells increases host dependency, which leads to attenuated virulence (Yamamura, 1993). Consistent with this

notion, the chlamydiae had no significant effects on the tested host fitness parameters (Figure 2). Nevertheless, endosymbiont maintenance entails costs to the host, which may be offset by benefits. Some environmental chlamydiae protect their hosts against pathogens, e.g., *Legionella* and giant viruses (Arthofer et al., 2022; König et al., 2019; Maita et al., 2018). Many symbionts benefit their hosts by providing nutrients and expand the ecological niche of the host (García-Lozano et al., 2024; Wilson & Duncan, 2015). The thiamine and terpenoids synthesis pathway encoded by members of the genera *Reclusachlamydia* and *Plastochlamydia*, respectively, could serve this function (Figure 1D).

Despite convergence in developmental features, transmission mode and effect on host fitness, genotypic and morphological characteristics of the chlamydial families diverge greatly. In contrast to *Reclusachlamydia*, *Plastochlamydia* is transiently found in vacuoles, exhibits strongly stained membranes and is consistently surrounded by small extracellular vesicles (Figure 3). These vesicles may relate to host interaction or manipulation (Gitsels et al., 2019). Both families lost conserved regulatory genes, however, with low variation within families but pronounced differences between families (Figure 1D). These patterns align with experimental evolution studies, in which redundancy and robustness of developmental regulatory networks leads to similar phenotypes despite differential gene loss (Arendt & Reznick, 2008; McColgan & DiFrisco, 2024; Stern, 2013).

Taken together, we showed that chlamydial symbionts of dictyostelids affiliated with evolutionary distinct lineages converged by losing conserved developmental characteristics during adaptation to their facultatively multicellular hosts. These symbioses can help reveal how host behavior influences chlamydial development and evolution.

Materials & Methods

Maintenance of cultures

The isolation and curing of the dictyostelid strains was performed as described by Haselkorn (Haselkorn et al., 2021). For long term storage, dictyostelid spores were generated on SM/5 plates (2 g/L glucose, 2 g/L bacto peptone, 0.2 g/L yeast extract, 0.1g/L $MgSO_4$, 1.9 g/L KH2PO4, 1.0 g/L K2HPO4, 15 g/L agar), collected in Sorensen buffer (2.0 g KH2PO4, 0.29 g Na2HPO4 in MiliQ water; pH 6,0 ± 0.1) containing 25% glycogen and stored at -80°C. Working stocks of dictyostelids were generated by incubating 50 µL of dictyostelid freeze stock and 1.25 x 10^9 *K. aerogenes* on SM/5 plates incubated at 22°C. The SM/5 stock plates were exchanged every 4 weeks. To generate exponentially growing

trophozoites, spores were incubated in 25 cm^2 cell culture flasks in Sorensen buffer, supplemented with 2.5 x 10^9 *K. aerogenes*, at 22°C. Throughout this study, trophozoite concentration was assessed using the LUNA-FX7 Automated Cell Counter (Logos Biosystems, South Korea). Stocks of *K. aerogenes*, used as food bacteria, were inoculated in 100 mL LB-Medium (10 g/L tryptone, 5 g/L yeast-extract, 10 g/L NaCl) and incubated overnight at 37°C and 180 rpm. Subsequently, the bacterial cultures were centrifuged at 5,000 x g for 5 min, resuspended in PAS buffer to a final concentration of 2.5x10^{10} cells per mL and stored at 4°C.

Fluorescence in situ hybridization

Fixation was performed in suspension. Thus, the samples were centrifuged at 1000 x g for 5 min before the supernatant was replaced with 4% (w/v) paraformaldehyde in Sorensen buffer. The samples were fixed on ice for 10 min before being centrifuged again at 1000 x g for 5 min and being resuspended in Sorensen buffer and stored in the fridge.

For fluorescence *in-situ* hybridization, the samples were applied to Teflon-coated microscope slides (Marienfeld, Germany) and allowed to attach for 15 minutes. The fixed cells were covered with 10 µL of hybridization buffer (900 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 25% formamide) mixed with 1 µL of the respective probes and hybridized at 46◦C for 3 h in an atmosphere saturated with hybridization buffer. The slides were then incubated in washing buffer (20 mM Tris-HCl, 0.25 mM EDTA and 0.149 M NaCl) at 48◦C for 15 min and washed in ice-cold water for 10 s. To detect the chlamydial symbionts two Chlamydiota-specific probes each 5'-labelled with the fluorescent dye Cy3 were used simultaneously to increase fluorescence signal intensity (Chla-282, 5'- CTC AAT CCG CCT AGA CGT -3' (Pizzetti et al., 2012) and Chls-523, 5'- CCTCCGTATTACCGCAGC -3' (Poppert et al., 2002)). For the detection of amoeba the probe Euk-516 (5'- GGA GGG CAA GTC TGG T -3') 5'-labelled with Cy5 was used (Amann et al., 1990). To stain DNA, all wells were covered with 10 µL 4′,6-diamidino-2-phenylindole (DAPI) in deionized water (c = 1 µg / mL) for 3 min and subsequently washed for 7 min in 99% ethanol. Citifluor AF1 (Electron Microscopy Sciences, USA) was used to mount a coverslip. Samples depicted in Figure 2 and Figure S1 were analyzed using a Leica DMi8 Inverted Microscope equipped with a 100x oil objective and a Leica TCS SP8 X confocal laser scanning microscope equipped with a 93x glycerol objective, respectively. All images were processed with the Leica application suite X software (v3.7.6).

Growth experiments

To monitor differences in growth rates of symbiotic and aposymbiotic dictyostelid cultures, exponentially growing dictyostelid cultures were incubated at a concentration of 10^4 cells / mL in 25 cm^2 cell culture flasks (Fisher Scientific Austria GmbH, Austria) in Sorensen buffer supplemented with food bacteria at 22°C. After 6, 24, 30, 48, 72 and 192 hours the dictyostelids were detached from the bottom of the flasks with a cell scraper and trophozoite concentration was measured using the LUNA-FX7 Automated Cell Counter (Logos Biosystems, South Korea).

Horizontal symbiont transmission

To test for horizontal transmission, exponentially growing aposymbiotic and symbiotic trophozoites were stained for 30 minutes in Sorensen buffer containing CellTracker green (25µM) and deep red (2µM), respectively (Thermo Fisher Scientific Inc., USA). The stained trophozoites were washed twice with Sorensen buffer, mixed equally and a total of 10^6 trophozoites applied to nitrocellulose filters. The prevalence of chlamydiae in fully developed spores was assessed after fixation by FISH and manual counting.

Extracellular chlamydiae and reinfection

To evoke exponential growth, symbiotic and aposymbiotic trophozoites were incubated at a concentration of 10^4 cells / mL in 12-well plates (Fisher Scientific Austria GmbH, Austria) at 22°C in Sorensen buffer supplemented with food bacteria. To induce aggregation, symbiotic trophozoites were inoculated at 5 x 10^5 cells / mL without food bacteria. After 24 hours, 1 mL of the supernatant of symbiotic trophozoites was taken off, homogenized with a 25 G needle and filtered through 1.2 µm filters. Subsequently, 200 µL of each filtrate was added to the aposymbiotic trophozoites and to wells containing only food bacteria in Sorensen buffer. The remaining filtrate was frozen at -20°C. The well plates were further incubated at 22 °C for 5 days. Then trophozoites were detached using a cell scraper, homogenized with a 25 G needle, filtered through a 1.2 µm filter and frozen at -20°C. DNA was extracted from 200 µL of the aliquots using the QIAamp 96 Virus QIAcube HT Kit (Qiagen, Netherlands). The elution was used to perform digital PCR with the QIAcuity EvaGreen PCR Kit in the QiaQuity dPCR cycler following this cycling protocol: After the initial step at 95°C for 2 minutes, 40 consecutive cycles of 15s at 95°C, 15s at 61°C and 15s at 72°C, then one final step of 5min at 40°C. Imaging was performed with the QiaQuity dPCR cycler and analyzed using the QIAcuity Software Suite 3.2 (Qiagen, Netherlands).

Transmission electron microscopy

Exponentially growing, symbiotic trophozoites were incubated in single Petri dishes (Fisher Scientific Austria GmbH, Austria) in Sorensen buffer at concentrations of 10^4 (supplemented with food bacteria) and 5 x 10^5 (without food bacteria) cells per mL, to generate exponentially growing or aggregating cells, respectively. After 20 hours, the cells were detached using a cell scraper and collected by centrifugation at 100 x g for 10 minutes. In parallel, dictyostelid spores from 5 fruiting bodies were incubated on SM/5 plates covered with 2.5 x 10^9 food bacteria. After 9 days, spores of the fresh, fully developed fruiting bodies were collected in the lid and centrifuged at 100 x g for 10 minutes to produce a pellet. Cryofixation and freeze-substitution for electron microscopy of the amoebae was performed as described in (Helmlinger et al., 2025). Microscopy was performed with a Zeiss 900N transmission electron microscope (Carl Zeiss AG, Germany) in combination with ImageSP.

Genome sequencing, assembly and annotation

Symbiotic dictyostelid cultures were grown in PAS buffer (1 g/L sodium citrate, 0.4 mM $CaCl_2$, 4 mM $MgSO_4$, 2.5 mM KH_2PO_4) in twelve 175 cm^2 cell culture flasks until confluent cell layers were obtained. The supernatant was replaced with fresh PAS buffer without food bacteria, and the amoebae were detached using a cell scraper. The detached amoebae were transferred to 50 mL tubes and centrifuged at 5000 x g for 10 minutes. The resulting pellet was resuspended in PAS buffer, transferred to 2 mL Eppendorf tubes prefilled with 0.5 mL glass beads (0.25-0.5 mm diameter) and vortexed horizontally at 2700 x g for 2 min. The disrupted amoeba cells were further homogenized using a 25 Gauge needle and passed sequentially through 5 µm and 1.2 µm pore size filters to remove host cell debris and nuclei. The Wizard HMW DNA Extraction Kit (Promega, USA) was then used to extract DNA for long read sequencing according to the manufacturer's protocol.

Short and long reads were trimmed with Cutadapt (Martin, 2011) and porechop v0.2.4 (Wick et al., 2017a). Because long-read quality varied, the assembly strategy was adapted per dataset to obtain closed genomes. Long reads of *R. dictyosteleii* QS1024 and *R. socialis* DLS304 were error-corrected using Canu v2.2 (Koren et al., 2017). Hybrid assemblies combining short and long reads were generated for *R. dictyostelii* QS1024, *I. aureostipeii* THC12 and *P. violaceii* THC49 using Unicycler v0.5.0 (Wick et al., 2017b). The genome of *R. socialis* DLS304 was assembled from long reads with Flye v2.9.1 (Kolmogorov et al., 2020) and polished with short reads using Pilon v1.24 (Walker et al., 2014). For *P. dictyostelii* QS68 overlapping contigs from incomplete Flye and Unicycler

assembles were manually joined and polished with short reads using Pilon v1.24. All assemblies were annotated with bakta v1.11.4 (Schwengers et al., 2021).

The genomes of *R. dictyosteleii* GS8b and *P. dictyostelii* M4B were sequenced as described by Holland et al. (Holland et al., 2025), however, origin and orientation were adjusted to facilitate comparison between all strains.

RNA sequencing

Exponentially growing, symbiotic trophozoites were incubated at a concentration of 10^4 or 5 x 10^5 cells per mL in 75 cm^2 cell culture flask at 22°C in Sorensen buffer supplemented with or without food bacteria, respectively. In parallel, symbiotic and aposymbiotic trophozoites were mixed at a ratio of 1:5 (symbiotic:total cells) and incubated at a concentration of 5 x 10^5 cells per mL in 75cm^2 cell culture flask at 22°C in Sorensen buffer. After 24 hours of incubation, trophozoites were detached using a cell scraper, transferred to 50 mL tubes and collected at 1000 x g / 5 min. The resulting pellet was resuspended in 1 mL Sorensen buffer supplemented with 50 ng/uL Rifampicin to stall transcription and vortexed with glass beads (0.25-0.5 mm diameter) to lyse the amoebae. Subsequently, the lysate was homogenized using a 25 G syringe and filtered through 1.2 µm filter. The filtrate was centrifuged at 14,000 x g / 5 min and RNA was extracted from the resulting pellet using the RNA Reliaprep Cells kit (Promega, USA), following the manufacturers protocol. All stages were sampled as independent triplicates.

Residues of DNA were removed via ezDNase™ Enzyme (ThermoFisher, USA). A 1:1 mix of chlamydiae and dictyostelia Pan-riboPOOLs (siTOOLs; https://www.sitoolsbiotech.com/products/ribopools/pan-ribopools) was used to deplete ribosomal RNA (rRNA) from the total RNA extracts, following the manufacturer's protocol. Single-index barcoded sequencing libraries were prepared from rRNA-depleted RNA using the NEBNext® Ultra™ II Directional RNA Library Prep Kit for Illumina (NewEngland Biolabs) following the manufacturer's protocol. Sequencing libraries were then pooled and sequenced on a HiSeq6000 (Illumina) in paired-end mode (200 cycles, 2× 100 bp reads).

RNA Sequence read processing

To analyze differential expression patterns, RNA-Seq reads were quality-filtered and trimmed with Cutadapt v5.0 (-q 20,20 -m 40) (Martin, 2011) and assessed with FastQC (Andrews & others, 2010). Trimmed reads were aligned to the respective chlamydial genomes with STAR v2.7.11b (--alignIntronMax 1 --alignSJoverhangMin 999 --alignEndsType EndToEnd --outFilterMultimapNmax 20 --outSAMmultNmax 20 --

outSAMprimaryFlag AllBestScore --outSAMmapqUnique 255 --outFilterMismatchNoverReadLmax 0.08 --outFilterScoreMinOverLread 0.3 --outFilterMatchNminOverLread 0.3 --chimSegmentMin 999 --quantMode GeneCounts) (Dobin et al., 2013), and gene-level counts were generated via featureCounts v2.1.1 (-p --countReadPairs -B -C -M --fraction -s 0 -Q 0 -F SAF) (Liao et al., 2014).

Gene expression analysis

Differential gene expression analysis was performed in RStudio 2025.05.0+496 using DESeq2 v1.38.3 (Love et al., 2014). Samples derived from mixes of aposymbiotic and symbiotic amoebae were used for PCA plot generation and subsequently removed for downstream analysis. Genes with less than ten counts in more than three samples were removed before analysis. Differential expression across stages was assessed using a likelihood ratio test (LRT). Genes with Benjamini-Hochberg adjusted p-values smaller 0.05 were considered significant. For visualization, normalized counts were transformed using the regularized log transformation. Expression values were averaged across biological replicates within each stage and row-wise scaled using z-score. Subsequently, genes were clustered hierarchically using Euclidean distance and Ward's minimum variance method and visualized using ComplexHeatmap v2.14.0 (Gu et al., 2016). All clusters of co-regulated genes were analyzed for functional enrichment of COG and KEGG categories using clusterProfiler v4.6.2 (Yu, 2024). Categories with Benjamini–Hochberg adjusted p-values < 0.05 were considered significantly enriched. Genes of *C. pneumoniae* significantly upregulated in RB or EB stage (padj < 0.5; > 2x fold change) were filtered from (Albrecht et al., 2011). Overlaps between co-regulated genes of dictyostelid-associated chlamydiae and *C. pneumoniae* were based on orthogroup annotations and tested for significance using a permutation test. Overlaps with Benjamini–Hochberg adjusted p-values smaller than 0.05 were considered significant.

Statistical analysis and visualization

All statistics were performed in RStudio 2025.05.0+496 using rstatix (Kassambara, 2023). Data visualization was performed with ggplot2 v3.5.2 (Wickham, 2016), cowplot v1.2.0 (Wilke, 2024), ComplexHeatmap v2.14.0 (Gu et al., 2016), ggalluvial v0.12.5 (Brunson, 2020), genefilter v1.80.3 , EnhancedVolcano v1.16.0 (Blighe et al., 2022), pheatmap v1.0.13 (Kolde, 2019) and clusterProfiler v4.6.2 (Yu, 2024).

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Author contributions

Conceptualization and methodology, L.H., and M.H.; investigation, L.H., T.H., S.D., N.C., T.L. and A.C.; resources and data curation, A.C.; writing – original draft, L.H.; writing – review and editing, all authors; funding acquisition, M.H. and A.C.

Declaration of interests

The authors declare no competing interests.

Supplementary material

Figure S1. Visualization of the chlamydial symbionts in dictyostelid trophozoites and spores by fluorescence in-situ hybridization, related to Figure 1.

Data S1. Formal description of chlamdyial species, related to Figure 1.

Table S1. Sampling site coordinates of all isolates, related to Figure 1.

Table S2. Genomes of chlamydiae and outgroup species used to calculate the chlamydial phylogeny and groups of orthologous genes, related to Figure 1.

Table S3. ANI and AAI values of the chlamydial symbionts, related to Figure 1.

Table S4. Genomes used to calculate an 18S rRNA based phylogeny of Eumycetozoa, related to Figure 1.

Table S5. Dictyostelid cell numbers during cell growth experiment, related to Figure 2.

Table S6. Dictyostelid spore production, related to Figure 2.

Table S7. Chlamydial genome copy numbers for reinfection, related to Figure 2.

Table S8. Chlamydial prevalence in symbiotic populations and formerly aposymbiotic spores, related to Figure 2.

Table S9. RNA reads mapped against chlamydial genomes, related to Figure 4.

Table S10. Affiliation of *Reclusachlamydia* and *Plastochlamydia* genes with respective clusters of co-regulated genes, related to Figure 4.

Table S11. Enriched COG categories and KEGG pathways per cluster of co-regulated genes, related to Figure 4.

Table S12. Overlap of clusters of co-regulated genes between *Plastochlamydia*, *Reclusachlamydia* and *C. pneumoniae* related to Figure 4.

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Towards an understanding of commensalism and tolerance in dictyostelid-chlamydia symbiosis

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Abstract

Pathogens evoke two substantially different evolutionary pathways in host species: resistance and tolerance. While resistance aims to reduce the load of parasites, tolerance aims to offset the fitness cost at a given pathogen load. Pathogens which have adapted to tolerant hosts develop increased virulence towards naïve host species. Chlamydiae present a phylum of obligate intracellular pathogens, infecting a wide host range, including humans and amoebae. Social amoebae, or dictyostelids, which undergo facultative multicellular stages are prone to chlamydial infection in the wild, however under laboratory conditions they show no detectable fitness reduction. Additionally, the chlamydial symbionts occur at high prevalence, but low symbiont loads per host cell. Because these patterns are consistent with tolerance, we hypothesized these chlamydiae induce harm on competing naïve dictyostelid strains, thereby giving a competitive advantage to their original hosts. By mixing reporter strains and chlamydiae-associated dictyostelids at different ratios, we created a competitive set-up to quantitively test this hypothesis. The analysis of reporter strain abundance after co-incubation measured by fluorescence flow cytometry, did not reveal a statistically significant effect of the symbiont on host competitiveness. We did, however, find evidence for transmission of the chlamydial symbionts to the dictyostelid reporter strain, demonstrating horizontal transmission to naïve hosts. Taken together, our results do not suggest that chlamydial virulence in naïve host strains increased due to tolerance related mechanisms, however, dictyostelid-associated chlamydiae can infect and persist in naïve dictyostelid hosts possibly facilitating their spread in the environment.

Introduction

Symbiosis and tolerance

Symbiosis, defined in the late 19th century by Anton de Bary, means the co-existence of two unlike organisms (De Bary, 1879). Depending on the net benefit of these associations for the partners symbiosis is further differentiated into parasitism, mutualism and commensalism (Van Beneden, 1876). While a mutualistic symbiosis leads to increased fitness of both partners, parasitism increases one partner's fitness but decreases the other partner's fitness. The classification of symbioses as mutualistic and parasitic are widely recognized, but the outcome of symbiotic interactions can change over time and depending on context (Bronstein, 1994; Drew et al., 2021). In contrast, commensalism,

the type of symbiosis in which one partner benefits from the association and the other is unaffected, is often overlooked (Mathis & Bronstein, 2020). Two forms can be distinguished, no-cost and balanced-cost-benefit commensalism, both resulting in an overall absence of fitness costs on one of the symbiotic partners (Mathis & Bronstein, 2020). Due to the practical difficulties to provide evidence for the absence of fitness effects, the concept of commensalism is debated (Zapalski, 2011). Conceptually, however, commensalism can be seen as a transient tipping point between mutualistic and parasitic interactions (Mathis & Bronstein, 2020). When hosts are confronted with parasites they can reduce their fitness costs by two opposing strategies, resistance or tolerance (Råberg et al., 2007). While resistance reduces the fitness cost by reducing the load of parasites, tolerance leads to reduced costs for a given parasite load (Kutzer & Armitage, 2016). Resistance includes barriers to infection (e.g. mucus, cell walls), mechanisms that clear the infection (immune response) and strategies that reduce the spread of the infection (e.g., apoptosis) (Roy & Kirchner, 2000). With decreased parasite prevalence, selection pressure for the resistance trait decreases and the trait will be successively lost. Conversely, tolerance leads to increased prevalence of the parasite and thereby increased benefits of the tolerance trait to the host. In other words, resistance traits evoke a negative feedback loop while tolerance traits evoke a positive feedback loop (Roy & Kirchner, 2000). Hence, tolerance increases parasite prevalence and parasite replication rate but reduces fitness cost of the parasite, thereby turning the parasite into an apparent commensalist (Miller et al., 2006). When an intolerant host is confronted with the evolved parasite, it can however suffer catastrophic levels of mortality (Miller et al., 2006).

Chlamydiae and dictyostelids

Chlamydiae are obligate intracellular parasites with global impacts on human health (World Health Organization, 2025). *Chlamydia trachomatis*, the major pathogen among the chlamydiae, infects eyes or genitals and – in severe cases – leads to blindness or infertility, respectively (Murray & McKay, 2021). Despite decades of research, no effective chlamydiae vaccine exists. Antibiotics can cure infections, but asymptomatic cases frequently go untreated and resistance can emerge (Poston, 2024). All chlamydiae share a biphasic developmental cycle: extracellular elementary bodies (EBs) enter host cells, convert within an inclusion to replicative reticulate bodies (RBs) that scavenge host metabolites. After redifferentiation into EBs the chlamydiae exit the host via extrusion or lysis (Stelzner et al., 2023). Besides the human and animal infecting Chlamydiaceae, there exists a variety of environmental chlamydiae, which share this conserved developmental cycle. Many of the environmental chlamydiae have been isolated from amoebae, due to

their cultivability (Collingro et al., 2020). A group of amoebae, the dictyostelids, have been frequently found infected with environmental chlamydiae in the wild (Haselkorn et al., 2021). Dictyostelids evolved a sophisticated social life cycle upon nutrient depletion. This so-called social life cycle starts with aggregation of tens of thousands singular cells, which stepwise develop to become a multicellular structure called the fruiting body. The fruiting body consists of a stalk of dead amoebae holding thousands spores aloft, which can disperse and start new dictyostelid population at suitable spots (Kessin, 2001). Since the stalk is a common good, provided by few cells to the population and prone for exploitation by cheaters, kin discrimination mechanisms evolved to restrict access to the fruiting body (Ostrowski, 2019). Besides stalks and spores, dictyostelids can differentiate into sentinel cells, removing toxins and bacteria during multicellular stages (Chen et al., 2007). Yet, some symbiotic bacteria, including *Burkholderia*, evade these immune strategies and can access the spores . Notably, infection with *Burkholderia* leads to lower spore productivity, often used as an indicator of dictyostelid fitness (Shu et al., 2018). However, *Burkholderia* also induces the carry-over of food bacteria, which can be used as food source when spores germinate in nutrient deficient locations and thereby increase survival rates (DiSalvo et al., 2015). Additionally, *Burkholderia* inhibits growth of competing *D. discoideum* strains by molecules secreted to the supernatant (Brock et al., 2013). Taken together, *Burkholderia* infections pose an example of context dependent symbiosis outcome, e.g., the counter-intuitive potential of parasites to transiently increase host fitness.

Here we aimed to understand the relation of *D. discoideum* strains with their natural chlamydial symbionts. The symbionts have a reduced potential to leave their dictyostelid host cells and to transmit to novel hosts (Chapter 2). Additionally, they are apparent commensalists (Chapter 2). Since chlamydiae thrive by scavenging nutrients from the host, they inflict at least a minimal fitness cost on their host, which could be easily overlooked in monoxenic cultures in the lab. However, in competition with uninfected dictyostelids even small decreases in fitness should lead to host extinction over evolutionary time spans. Two alternative scenarios can solve this dilemma for the dictyostelid-associated chlamydiae: either (1) the chlamydial symbionts provide a service to their hosts, thereby reducing the negative effects of nutrient scavenging or (2) the chlamydiae permanently infect novel hosts counteracting their own extinction. Therefore, we aimed to quantify the effect two well adapted dictyostelid-associated chlamydiae have on a naïve reporter strain, *D. discoideum* NC28.rfp. We confronted populations of *D. discoideum* NC28.rfp with *D. discoideum* QS68 and *D. discoideum* QS1024 either uninfected or infected with adapted chlamydial symbionts and measured the relative shifts

in reporter strain spores after co-incubation. Additionally, we monitored chlamydial transmission to the reporter strains.

Results

Effect of chlamydiae on host competitiveness

Tolerance against pathogens is a mechanism to offset negative effects on host fitness. Conversely, tolerance can indirectly favor higher pathogen virulence in naive hosts (Miller et al., 2006). We hypothesized that dictyostelids evolved tolerance against their associated chlamydiae, due to apparent absence of detectable effects on dictyostelid fitness and the high prevalence of symbionts within the host population and low amount of chlamydiae per host cell. To test this hypothesis, we let symbiotic and aposymbiotic competitor strains compete against the fluorescent reporter strain *D. discoideum* NC28.rfp by mixing vegetative cells at defined ratios (10:90, 30:70, 50:50, 70:30; reporter:competitor) and inducing fruiting body formation by nutrient depletion. Subsequently, we collected the spores and quantified the fraction of fluorescent (reporter) spores by flow cytometry. The strains used as competitors against *D. discoideum* NC28.rfp were *D. discoideum* QS1024 originally associated with *Reclusachlamydia dictyostelii* QS1024 and *D. discoideum* QS68 originally associated with *Plastochlamydia dictyostelii*.

Using fixed fluorescence gates, pure *D. discoideum* NC28.rfp cultures yielded 77.8% ± 15.0 % fluorescent spores (n = 20), indicating that about 22% of reporter spores fall below the fluorescence threshold under these conditions. Conversely, only 0.4% ± 2.4% of spores (n = 81) were classified as fluorescent among *D. discoideum* QS68 and *D. discoideum* QS1024 (symbiotic and aposymbiotic). Taken together, these results supported the use of fluorescence flow cytometry and the chosen thresholds for the distinction of reporter and competitor spores.

Co-culturing reporter and competitor strains reduced the fraction of fluorescent spores in the resulting fruiting bodies in proportion to the initial competitor fraction (Figure 1), as expected from the starting ratios. The influence of chlamydiae on the abundance of reporter strain differs strongly depending on the competitor identity. In three out of four initial mixes, the presence of chlamydiae in *D. discoideum* QS1024 decreased the relative abundance of reporter strains, indicating a positive effect of chlamydiae on their host (Figure 1A-D, Table S1). However, neither of these differences was significant (t-test, $p > 0.39$). Counterintuitively, chlamydiae decreased the mean abundance of their host in

developed fruiting bodies when the initial reporter strain was most abundant (Figure 1A). Notably, these trends were reversed when *D. discoideum* QS68 was competing with the reporter strain. In three out of four initial mixes, the presence of chlamydiae increased the abundance of reporter strain spores, indicating a negative effect on host fitness (Figure 1D-G). Again, none of these shifts was significant (t-test, $p > 0.09$) and the co-incubation with initially 90% *D. discoideum* QS68, the trend was reversed (Figure 1D, Table S1).

Subsequently, we aimed to see whether the chlamydiae were transmitted from chlamydiae-associated dictyostelids to *D. discoideum* NC28.rpf. Thus, we assessed the presence of chlamydiae in the spores collected from 50:50 (reporter:competitor) mixed strains (Figure 2). Chlamydiae were readily detectable using DNA stains and fluorescence microscopy. We could detect transmission of both *Reclusachlamydia* and *Plastochlamydia* in the analyzed spore mixes.

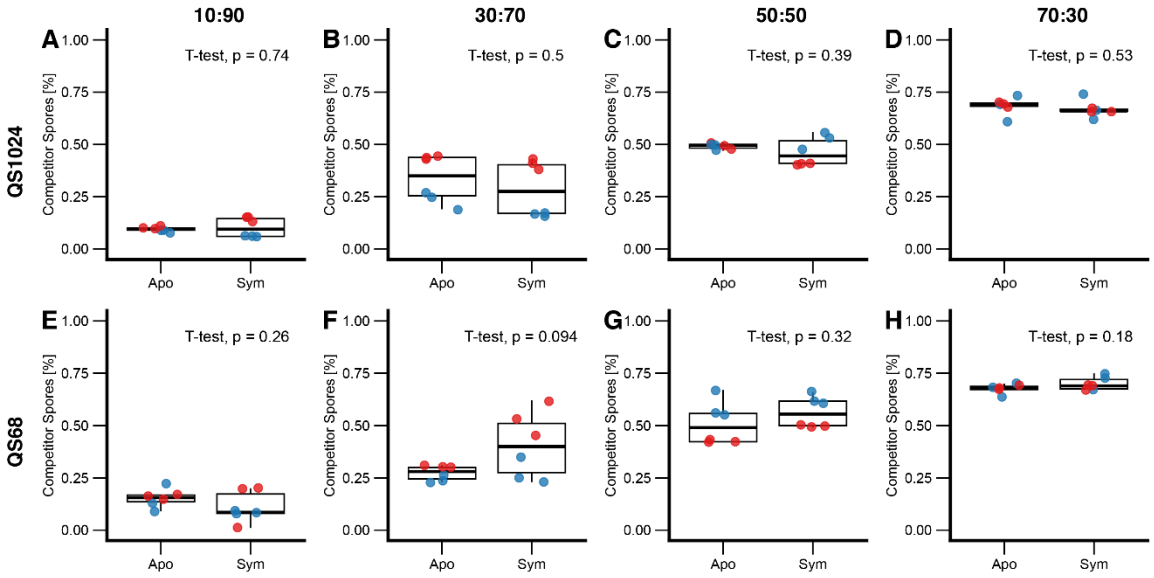

Figure 1: Effect of chlamydial infection on host strain competitiveness during fruiting body formation. Symbiotic and aposymbiotic host strains *D. discoideum* QS1024 (A-D) and *D. discoideum* QS68 (E-H) were mixed with a reporter strain *D. discoideum* NC28.rfp at defined ratios. After spore development the ratio of fluorescent spores was assessed using flow cytometry and plotted on the y-axis. Competitor host strains are indicated in bold letters on the left; initial ratios of reporter to host strain are indicated in bold numbers above the respective plots. Data derived from the first and the second biological repeat is depicted in blue and red, respectively. Each biological repeat consists of three replicates. Median and interquartile range (IQR) are indicated by the line and the box, respectively. The whiskers extend to the most extreme values within 1.5 x IQR. Insets show the results of a two-sided t-test comparing the results of aposymbiotic and symbiotic competition with the reporter strain.

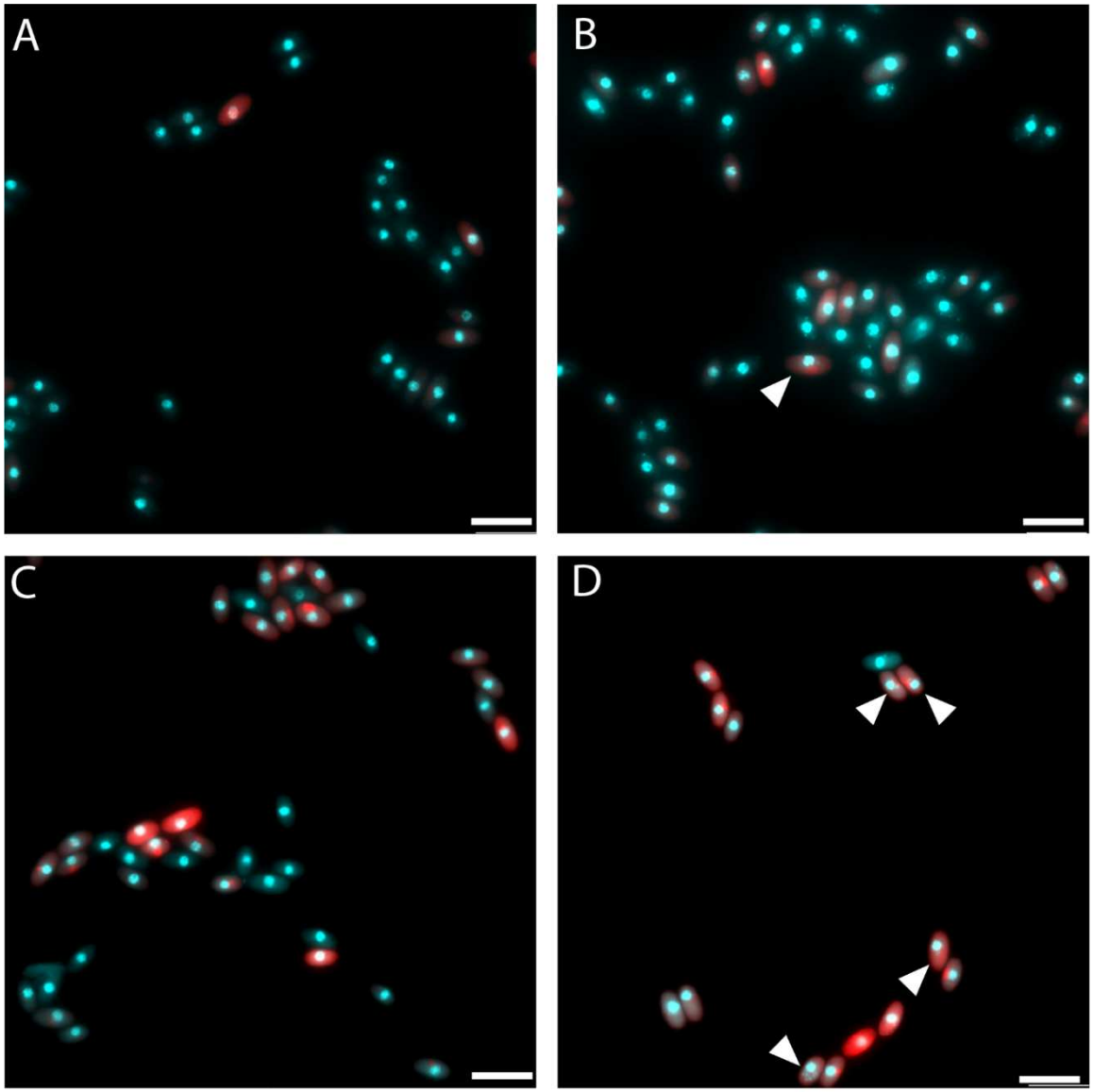

Figure 2: Transmission of chlamydiae to reporter strains during fruiting body formation. The reporter strain D. discoideum NC28.rfp was mixed with the chlamydiae-associated dictyostelids D. discoideum QS1024 aposymbiotic (A), symbiotic (B) and D. discoideum QS68 aposymbiotic (C) and symbiotic (D), respectively. After the formation of fruiting bodies the spores were checked for the presence of chlamydiae using fluorescence microscopy. White arrowheads indicate reporter strain spores infected with chlamydiae. DNA is depicted in cyan, RFP is depicted in red. Scale, 10µm.

Discussion

The chlamydial phenotype, with high prevalence and no detectable significant effects in host fitness assays led us to hypothesize that the dictyostelid hosts evolved tolerance against their chlamydial symbionts. Tolerance leads to reduced fitness costs at a given parasite load (Kutzer & Armitage, 2016). By relaxing selection on pathogen burden, tolerance can allow more virulent pathogen genotypes to persist, potentially increasing harm in naive hosts lacking tolerance (Miller et al., 2006; Roy & Kirchner, 2000). Our assays showed no significant effect of dictyostelid-associated chlamydiae on the competitiveness of their host strains, however, general trends towards increased and decreased host spore production induced by *R. dictyostelii* QS1024 and *P. dictyostelii* QS68, respectively (Figure 1). These trends are also seen in pure host cultures (Chapter 2). Without testing absolute spore production, we cannot distinguish whether the shifts we observed in these assays are related to the presence of reporter strains, i.e., a competitive advantage. All statistical tests were hampered by the high variability of reporter strain abundance between the biological repeats. Normalization methods could reduce the variation, however, we aimed to work with least steps of data manipulation to keep the results trackable and unskewed.

The presence of both chlamydial species in spores of the reporter strain (Figure 2), shows that (1) the chlamydiae can infect naive host strains and (2) they can be retained within these naïve host spores. Neither are chlamydiae cleared by immune mechanisms of the naïve hosts nor do they lyse all infected naïve host cells. This suggests a host range beyond their original host strain, that was not previously described. However, we did not assess how the symbiosis between reporter strain and chlamydiae develops beyond spore formation, i.e. whether the association is stable and increasing in prevalence over several generations or whether it breaks down due to immune mechanisms or chlamydial virulence. It is tempting to speculate how these dynamics unfold in a more natural context. Do host switches occur and at which frequency? Which role does phylogenetic host distance play in these transmission events? And lastly, if dictyostelid-associated chlamydiae can effectively spread to novel host populations why is only approximately a quarter of wild *D. discoideum* populations infected with chlamydiae (Haselkorn et al., 2021)?

Taking together, our results do not provide evidence for a strong advantage of chlamydiae-infected dictyostelids in competition with naïve dictyostelids. However, we show that transmission of chlamydiae to naive dictyostelid strains is possible.

Material and methods

Strains

Dictyostelium discoideum strain NC28.1 was originally collected in North Carolina and subsequently engineered to express red fluorescent protein (*rfp*) (Buttery et al., 2012; Francis & Eisenberg, 1993). Both origin and curing of chlamydiae-associated strains *D. discoideum* QS68 and *D. discoideum* QS1024 are described in Chapter 2 of this thesis.

Growth conditions

For long term storage, dictyostelid spores were generated on SM/5 plates (2 g/L glucose, 2 g/L bacto peptone, 0.2 g/L yeast extract, 0.1g/L $MgSO_4$, 1.9 g/L KH2PO4, 1.0 g/L K2HPO4, 15 g/L agar), collected in Sorensen buffer (2.0 g KH2PO4, 0.29 g Na2HPO4 in MiliQ water; pH 6,0 ± 0.1) containing 25% glycogen and stored at -80°C. Working stocks of dictyostelids were generated by incubating 50 µL of dictyostelid freeze stock and 1.25 x 10^9 *K. aerogenes* on SM/5 plates incubated at 22°C. The SM/5 stock plates were exchanged every 4 weeks. To generate exponentially growing trophozoites, spores were incubated in 25 cm^2 cell culture flasks in Sorensen buffer, supplemented with 2.5 x 10^9 *K. aerogenes*, at 22°C. Stocks of *K. aerogenes*, used as food bacteria, were inoculated in 100 mL LB-Medium (10 g/L tryptone, 5 g/L yeast-extract, 10 g/L NaCl) and incubated overnight at 37°C and 180 rpm. Subsequently, the bacterial cultures were centrifuged at 5,000 x g for 5 min, resuspended in PAS buffer to a final concentration of 2.5x10^{10} cells per mL and stored at 4°C.

Competition experiment

Symbiotic dictyostelids were cultured in Sorensen buffer supplemented with food bacteria in 75cm^2 Nunc cell culture flasks. In logarithmic growth phase, the supernatant was exchanged, all amoebae were transferred to a 50mL falcon tube and harvested by centrifugation at 1000 x g for 5min. The pellet was resuspended in 500µL Sorensen buffer before assessing trophozoite concentration using the LUNA-FX7 Automated Cell Counter (Logos Biosystems, South Korea). Subsequently, the suspensions were diluted to a trophozoite concentration of 2 x 10^7 trophozoites per mL. Fractions of these suspensions were then mixed at defined ratios – 90:10, 70:30, 50:50, 30:70 (competitor strain:reporter strain). A total of 2 x 10^6 amoebae (100µL) of these mixes and pure cultures were applied to nitrocellulose filters in a humid atmosphere within petri dishes, to induce fruiting body production.

After 7 days, the nitrocellulose filters with attached fruiting bodies were transferred to 5mL Eppendorf tubes filled with 700µL Sorensen supplemented with 0.1% SDS. The tubes and filters were vortexed thoroughly before transferring the content to fresh 1.5 mL Eppendorf tubes. Fixation of these samples was performed in suspension. Thus, the samples were centrifuged at 1000 x g for 5 min before the supernatant was replaced with 4% (w/v) paraformaldehyde in Sorensen buffer. The samples were fixed on ice for 10 min before being centrifuged again at 1000 x g for 5 min and being resuspended in Sorensen buffer and stored in the fridge.

DAPI staining

For DAPI staining, the fixed spores were applied to Teflon-coated microscope slides (Marienfeld, Germany) and allowed to attach for 15 minutes. To stain DNA, all wells were covered with 10 µL 4′,6-diamidino-2-phenylindole (DAPI) in deionized water (c = 1 µg / mL) for 3 min and subsequently washed for 7 min in 99% ethanol. Citifluor AF1 (Electron Microscopy Sciences, USA) was used to mount a coverslip. Slides were analyzed using a Leica DM4B epifluorescence microscope equipped with a 100x oil objective and the Leica application suite X software (v3.7.6).

Fluorescence activated flow cytometry

Fixed spores were analyzed at the Beckman Coulter CytoFLEX S flow cytometer configured with 4 lasers (405 nm, 488 nm, 561 nm, 638 nm) using the provided software CytExpert v2.6 (Beckman Coulter, USA). Detector gain and threshold settings were optimized on unstained and stained controls and held constant across all samples. The flow rate was set to 60 µL/min. FSC threshold was set at 5 x 10^4 to exclude debris. Acquisition stopped as soon as either 100,000 events or 100µL were monitored, latest after 110 seconds. Parameters recorded were FSC-A, FSC-H, SSC-A, SSC-H, and fluorescence at 561nm as area. Data were filtered in CytExpert v2.6 by sequential gating: FSC-A vs SSC-A to discriminate spores. A manual polygon gate was drawn on 561nm versus SSC-A to identify RFP events within the parent spore population. Gate boundaries were applied uniformly to all samples.

Statistical analysis and visualization

All statistics were performed in RStudio 2025.05.0+496 using rstatix (Kassambara, 2023b). Data visualization was performed with ggplot2 v3.5.2 (Wickham, 2016), cowplot v1.2.0 (Wilke, 2024) and ggpubr (Kassambara, 2023a).

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Synthesis – A scenario leading to stable dictyostelid-chlamydiae symbioses

Symbiosis has driven major evolutionary transitions (Smith & Szathmary, 1997), however, establishing endosymbiosis raises mutual dependencies and risks (Bennett & Moran, 2015; Keeling & McCutcheon, 2017). The majority of symbiotic relationships arise from parasitic interactions, either by host exploitation (Sørensen et al., 2019) or parasitic symbionts (Sachs et al., 2014). Over evolutionary time frames, symbionts and hosts can evolve reciprocal exploitation that increases the fitness of both partners (Herre et al., 1999). The symbioses between dictyostelids and chlamydiae show distinct signs of co-adaptation. In the synthesis of this thesis, I will develop my idea of how co-adaptation between dictyostelids and their associated chlamydiae has developed. Similar to chapter 2 and 3, *Plastochlamydia* and *Relusachlamydia* refer to *Plastochlamydia dictyostelii* QS68 and *Reclusachlamydia dictyostelii* QS1024, respectively.

Infection at first sight - Establishing the relationship

Soil-dwelling amoebae like dictyostelids thrive by phagocytosing bacteria and are thus often infected with parasites, including environmental chlamydiae (Haselkorn et al., 2021; Price et al., 2024). Several environmental chlamydiae can infect *D. discoideum* laboratory strains but they are located within typical chlamydial inclusions and cleared before the transition to spores (Horn et al., 2000; Skriwan et al., 2002). Conversely, dictyostelid-associated chlamydiae are found directly in the host cytosol, with *Plastochlamydia* transiently located in vesicles (Chapter 1, Figure 2; Chapter 2, Figure 3). This suggests that the ancestors of dictyostelid-associated chlamydiae have evolved mechanisms to escape the inclusion. This shift might has improved access to host resources and evade inclusion-targeted defenses (Flannagan et al., 2012; Pizarro-Cerdá & Cossart, 2006). With increased adaptation to the dictyostelid host, the described chlamydiae found a way to retain within the host spores (Chapter 1, Figure 1; Chapter 2, Figure S1). This retention requires the evasion of host immune mechanisms that specifically eliminate bacteria in multicellular structures (Chen et al., 2007). Only members of the genera *Burkholderia, Amoebophilus* and *Procabacter* were so far shown to evade these responses (Haselkorn et al., 2021; Shu et al., 2018). The spore poses a safe harbor for the environmental chlamydiae to evade environmental stress, thus a mechanism to sustain within, increases chlamydial fitness (Wang et al., 2023). In turn, the ability to safely sustain and transmit vertically to descendants reduces the importance of horizontal transmission via

extracellular particles (Chapter 1, Figure 4; Chapter 2, Figure 2). Nevertheless, the chlamydiae can still be transmitted horizontally but only via direct transmission using host cell-cell contacts (Chapter 1, Figure 5; Chapter 2, Figure 2). Like many symbionts, the chlamydiae evolved a mixed mode of transmission, i.e., vertical via spores and horizontal via cell-cell contact and thereby maintain remnants of independence from the host. However, horizontal transmission is purely contact dependent, therefore the possibility of infecting novel hosts is reduced. Even more so, since the dictyostelid hosts actively discriminates against distantly related species (Hirose et al., 2011; Ostrowski, 2019). The reduced potential to infect novel host species ultimately leads to the phylogenetic congruence between *Reclusachlamydia* spp. and their hosts (Chapter 2, Figure 1). Conversely, the phylogeny of *Plastochlamydiaceae* and their hosts indicate transmission to novel hosts and replacement of prior associations with *Reclusachlamydia* spp.

Active or passive horizontal transmission?

Despite the low abundance of EBs, dictyostelid-associated chlamydiae can still be transmitted during host aggregation. This is either mediated via passive or active transmission. A passive mode, i.e., transmission not mediated by chlamydial proteins, must exploit existing host behavior that enables the free passage of symbionts between host cells. One possibility how cell content can move between dictyostelid cells passively is via trogocytosis, often referred to as “cell nibbling”. This behavior was, however, only observed in *Dictyostelium caveatum*, which phagocytose *D. discoideum* and other *D. caveatum* cells (Nakada-Tsukui & Nozaki, 2021). It is therefore unlikely to facilitate chlamydial transmission. A second possible route to exchange cytosol, is via fusions frequently formed between dictyostelids (Huffman et al., 1962; Huffman & Olive, 1964) These possibly mediate the exchange of mitochondria (Bloomfield et al., 2019). I observed merging cells via light microscopy and transmission electron microscopy in vicinity to *Reclusachlamydia* (Chapter 2, Figure 3) Similar structures, called nanotubes, bridge human embryonic kidney cells and are actively used by *C. trachomatis* for transmission (Jahnke et al., 2022). A similar mechanism could lead to the observed behavior.

Active transmission, i.e., transmission via a mechanism that is encoded and expressed by the symbiont, requires adaptations of the symbiont. The intracellular pathogen *Mycobacterium tuberculosis* and *Mycobacterium marinum* can infect novel hosts by piercing through the cell membrane of adjacent host cells. This mechanism is mediated by the GTPase *RacH* and the autophagic machinery expressed by *D. discoideum* (Gerstenmaier et al., 2015; Hagedorn et al., 2009). To explain the cell-cell contact dependent transmission of *R. socialis* I speculated whether an analogous mechanism is

feasible (Chapter 1, Discussion). The described GTPases contain CAAX motifs that are prenylated by prenyl transferases, leading to attachment of the GTPases to membranes (Okuwa et al., 2001). Prenyl transferases were found in the genomes of all members of the genus *Reclusachlamydia*: *R. socialis* PALH (14 encoded), *R. socialis* DLS304 (9), *R. dictyostelii* QS1024 (2), *R. dictyostelii* GS8b (5) (Chapter 2, Annotation tables). No prenyl transferases were annotated in the *Plastochlamydiaceae*, yet, their representative infects novel hosts faster than *Reclusachlamydia*. This suggests a negligible role of prenyl transferases in transmission or simply the presence of more effective mechanisms in *Plastochlamydia*. Besides *Mycobacterium*, a variety of endosymbionts, e.g., *Burkholderia*, *Rickettsia*, *Listeria* or *Shigella*, infect adjacent cells in the absence of extracellular particles. To do so, they escape the phagosome and use the host actin cytoskeleton within the host cytoplasm to move to the cell membrane and push through the membrane into adjacent host cells (Ermolaeva et al., 2021). The dictyostelid-associated chlamydiae are mostly located in the host cytosol, however, I did not observe actin tails in electron microscopy (Chapter 1, Figure 2; Chapter 2, Figure 3), comparable to,e.g., *Rickettsia* (Van Kirk et al., 2000).

At this point, I can only speculate how dictyostelid-associated chlamydiae infect naïve host cells. Since passive transmission via cell bridges does not necessarily require the evolution of novel mechanisms but makes use of existing dictyostelid behavior, it is the more parsimonious strategy and therefore more likely. Whether the chlamydiae can use virulence factors to move towards or through these bridges could be the topic of future research.

Falling down the rabbit hole - enslaved by a social amoeba

When the chlamydial symbionts shift their main transmission mode from horizontal to vertical transmission, it is accompanied by grave changes in the endosymbiont genome (Bennett & Moran, 2015; McCutcheon et al., 2019). Most prominent is genome size reduction in endosymbionts due to gene loss (McCutcheon & Moran, 2012). The genomes of dictyostelid-associated chlamydiae, are on average smaller than the average genome of environmental chlamydiae, i.e., they show signs of genome reduction (Chapter 2, Table 1) (Köstlbacher et al., 2021). The coding density of the dictyostelid-associated chlamydiae lies between 85-90%, strikingly close to what is known from specialized insect endosymbionts (McCutcheon & Moran, 2012). Genome reduction occurs via mobile elements, chromosome rearrangements, gene inactivation, pseudogene accumulation and deletions (McCutcheon & Moran, 2012; Moran, 1996). The higher abundance of transposons in *Plastochlamydiaceae* than in *Reclusachlamydia spp.* indicates that these

genome rearrangements are still ongoing (Chapter 2, Figure 1) (McCutcheon et al., 2019). The high number of eukaryote-like domains among the genes of *R. socialis* (Manuscript 1) indicates chlamydial communication with or manipulation of the host (Schmitz-Esser et al., 2010). An important form of interaction with the host is the secretion of effectors via the conserved structural genes of the T3SS (Elizabeth A. Rucks, 2023). These genes are encoded in all dictyostelid-associated chlamydiae (Chapter 2, Figure 1). Conversely, regulatory genes of the developmental cycle are lost in *Plastochlamydia* and *Reclusachlamydia* alike, consistent with limited differentiation across the host life cycle. However, the specific gene loss deviates substantially between the two chlamydial families. The loss of regulatory genes of endosymbionts can be a result of a constant environment provided within host (McCutcheon & Moran, 2012) and of the host taking control over symbiont replication and development (Maurya et al., 2025). Alternatively, these deviations suggest the redundancy of developmental genes and the robustness of chlamydial developmental pathways (Arendt & Reznick, 2008; McColgan & DiFrisco, 2024; Stern, 2013).

I was interested in chlamydial gene expression dynamics in the absence of these otherwise conserved regulatory genes. Is symbiont physiology now more tightly coupled to host cellular state than to an intrinsic, cyclic program? RNA-Seq studies of chlamydial gene expression rely on highly artificial infection regimes to synchronize the infection and obtain clear RB or EB signals (König et al., 2017). In contrast, due to the lack of infective, extracellular particles the dictyostelid-associated chlamydiae in my experiments were extracted from running cultures only synchronized by the life stage of their host. Despite this limitation, I obtained clear up- and downregulation of clusters of chlamydial genes across our replicates depending on the host life stage (Chapter 2, Figure 4). This indicates a reaction of the chlamydiae to the changing intracellular environment, including nutrient supply and oxidative stress (Aloum et al., 2019). Conversely, it suggests that the chlamydial gene expression does not consist of mixed RB and EB states, which would dilute the respective signal. The observed upregulation of pathways related to carbon metabolism in exponentially growing host cells suggest that chlamydiae are actively replicating during this phase (Chapter 2, Table 2). However, the comparison with a member of the *Chlamydiaceae* shows that these fluctuations are only distantly resembling canonical chlamydial development. The longevity of the chlamydiae within the spores indicates that the chlamydiae are in a state of reduced activity, yet the gene expression does not resemble EB gene expression in *C. pneumoniae*. Alternatively, they might resemble the persistent, aberrant state of *Chlamydiaceae* (Brockett & Liechti, 2021; Panzetta et al., 2018).

Either way, the reduced potential to leave the host cells drastically restricts the potential host range of the dictyostelid-associated chlamydiae to the few organisms which closely interact with their dictyostelid hosts. Due to kin discrimination this pool of potential hosts is restricted even within the clade of dictyostelids (Ostrowski, 2019). Thus, the chlamydial symbionts are ultimately tied to their hosts evolutionary fate and goodwill (Husnik & Keeling, 2019). It is therefore likely that the chlamydiae presented here are on their way to extinction.

Open questions and future directions

The final section of this thesis comprises questions that remained unanswered during my work on these symbioses and possible answers to these questions. It also comprises experimental approaches that failed and how protocols can possibly be improved. It specifically concerns symbiont transmission, evolution and ecological context.

Host switches, symbiont replacement and interactions with the soil microbiome

The use of *D. discoideum* as a model organism has propelled the generation of numerous mutated cell lines, many readily available at dictybase (https://dictybase.dev/). Among many other findings, these cell lines have led to the discovery of *Mycobacterium* cell-cell transmission mechanisms (Hagedorn et al., 2009). Originally, I aimed to develop a protocol for infection of *D. discoideum* reporter strains with dictyostelid-associated chlamydiae. However, several infection assays of an axenic strain of *D. discoideum* with *Reclusachlamydia* were unsuccessful on agar plates, in nutrient medium, in buffer, with or without food bacteria. The success of this effort was hampered by low efficiency of FISH at that time and my lack of my experience in working with axenic *D. discoideum*. Since *Reclusachlamydia* and *Plastochlamydia* can infect a monoxenic *D. discoideum* reporter strain, I would still not exclude that infection with dictyostelid-associated chlamydiae is possible. Possible reasons for the unsuccessful transmission are either the reduced bacteria uptake due to the axenic lifestyle of the reporter strain or genetic divergence of the reporter strain, reducing interactions with *D. discoideum* QS1024. Since *Plastochlamydia* is more infectious (Chapter 2, Figure 2), future efforts should focus to use this genus for infection of axenic *D. discoideum* strains. Alternatively, using existing protocols for mutation of the original host strains could be tried. More generally, the question arises whether the dictyostelid-associated chlamydiae can infect novel dictyostelid strains, reporter strains and natural isolates alike. Are the chlamydiae trapped in their rabbit hole within their specific host strain or did they retain the potential to infect

novel dictyostelid hosts? How closely related must a host be in order to be infected? What happens when two naturally infected host strains compete? Is one host strain outcompeted? Is a chlamydial strain outcompeted? Are the fates of the partners tied to each other? How important is community context? *Burkholdiera* induces co-infection with food bacteria (DiSalvo et al., 2015) possibly due to a suppressed or overwhelmed immune system of the host (Bennett & Moran, 2015). At a later stage of my work, I observed increased chlamydial prevalence in a D. discoideum QS68 isolate contaminated with Burkholderia. Due to time constraints, I was not able to elucidate whether the *Burkholderia* symbiont was responsible for this increase in chlamydial prevalence. Thus, the microbiome of the putative host cell might also influence infection with chlamydiae in the wild.

Amoebae in the environment encounter not only diverse soil bacteria but also lethal viruses (Samba-Louaka et al., 2019). While chlamydiae are pathogenic to their amoebal hosts they can protect their hosts against giant viruses and *L. pneumophila* via yet unexplored mechanisms (Arthofer et al., 2022; König et al., 2019). I aimed to infect dictyostelids with giant viruses, of the order Tupanvirus. Despite positive reports in the literature I observed only cytopathic effects but did not establish stable infection under our conditions used (Abrahão et al., 2018). Therefore, the question of whether dictyostelid-associated chlamydiae provide their host protection against giant viruses remains unanswered. Likewise, I tried to infect dictyostelids with environmental chlamydiae, and test for symbiont protection by the dictyostelid-associated chlamydiae (Skriwan et al., 2002). Unfortunately, the infection with *Parachlamydia acanthamoebae* UV7, chosen due to its high infectivity, was unsuccessful or yielded ambiguous results.. Using the exact chlamydial species described before should enable us to test for protective symbiosis against other chlamydiae (Skriwan et al., 2002).

Parasites, commensalists or mutualists?

The influence of the symbionts on the hosts remains obscure. Our fitness tests did not result in significant effects of the symbionts (Chapter 2, Figure 2; Chapter 3, Figure 1). Thus, under the tested conditions, the symbiont fulfills the definition of a commensal (Mathis & Bronstein, 2020). That said, fitness assays in the lab will never be holistic and reflect the situation in the environment only partly. A wide range of fitness assays, e.g., spore survival and germination rates, could further determine how the symbionts affect the host. Conversely, the benefit for the dictyostelid-associated chlamydiae seems obvious: like many endosymbionts they gained shelter and nutrients. By transmitting directly via host cell-cell contact, the symbionts additionally reduce the risk of getting lost in

extracellular space. Over longer evolutionary time scales, however, this path is speculated to lead to extinction (Husnik & Keeling, 2019). Nevertheless, according to our phylogenetic analysis, *Rhabdochlamydiaceae* are stably associated with the dictyostelid hosts. However, extinction events are not visible in this tree. It is therefore conceivable that similar chlamydiae lineages have gone extinct and they go unnoticed. However, the symbiont *P. dictyostelii* M4B breaking the congruency of the *Reclusachlamydia* could indicate symbiont extinction and replacement (Manuscript 2, Figure 1). After working on this symbiosis for years, it remains enigmatic to me, whether a symbiont that does not provide benefits but only scavenges nutrients from the host, shouldn't inevitably lead to the extinction of the host and thereby itself. Shouldn't the symbiont at least transiently increase host fitness? Or can the symbiont decrease host fitness if it is infectious enough to constantly infect novel, naïve hosts? Is the transmission rate of the dictyostelid-associated chlamydiae high enough to produce constant influx into these novel dictyostelid populations? How distantly related can hosts be to be prone to infection?

To the best of my knowledge, only members of the *Burkholderia*, *Amoebophilus* and chlamydiae were described as stable endosymbionts of dictyostelids, being vertically transmitted within spores (Haselkorn et al., 2019, 2021). While Burkholderia shows context-dependent negative and positive consequences for host fitness, Amoebophilus and Chlamydiae are both devoid of an effect on host fitness, i.e., apparent commensals (Chapter 1, Figure 4; Chapter 2, Figure 2) (DiSalvo et al., 2015; Haselkorn et al., 2021).

Why are these bacteria not only successfully dividing within dictyostelids but adapted to an apparently commensalistic or mutualistic lifestyle? What distinguishes them from others like members of the genus *Mycobacterium* or *Legionella*, which kept their parasitic lifestyle in *D. discoideum*?

Resolving some of these questions will add to our understanding of chlamydial evolution, evolution of endosymbionts in the light of facultative multicellularity and the transition of pathogens to commensalistic or mutualistic entities.

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Abstract

Symbiosis has led to major transitions in evolution, including the emergence of eukaryotic cells. The relationship between intracellular symbionts and their host is delicate and shifts between states reciprocal benefit and pathogenicity. A major human pathogen is the obligate intracellular bacteria *Chlamydia trachomatis.* The bacteria regularly go through a biphasic developmental cycle consisting of a replicative intracellular stage and a transmissive extracellular stage. This trait is shared among all chlamydiae, including the numerous species infecting protists. Among those protists, the dictyostelids are especially susceptible to chlamydial infections. All dictyostelids frequently undergo a social life cycle, which includes stages of facultative multicellularity. In this thesis, my colleagues and I characterized chlamydial symbionts from eight dictyostelid isolates comprising two distantly related families, with in-depth analysis of three isolates. Across these systems, genome sequencing revealed extensive, lineage-specific gene loss, especially of regulators of the canonical developmental cycle and related genes. Consistent with these genomic signatures, I observed the loss of discrete morphological stages typical of chlamydiae, altered gene expression dynamics, and the absence of extracellular forms, detectable via digital PCR. This aligns well with the chlamydial ability to transmit vertically through the social life stage of the host, seen by transmission electron microscopy and fluorescence microscopy, and horizontally via host cell-to-cell contact. I do not detect significant effects on the host growth rate and spore production induced by the chlamydiae indicating reduced pathogenicity of the dictyostelid-associated chlamydiae. Taken together, I suggest that chlamydiae adapt to the facultative multicellularity of the host via gene loss, distinct changes in transmission mode and the loss of the conserved chlamydial developmental cycle. Thereby, this thesis adds to our understanding of chlamydial evolution and adaptation connecting it to endosymbiosis theory.

Zusammenfassung

Symbiose ist ein bedeutender Motor der Evolution, und hat unter anderem zur Entstehung eukaryotischer Zellen geführt. Die Beziehung zwischen intrazellulären Symbionten und ihren Wirten ist vielseitig und bewegt sich zwischen wechselseitigem Nutzen und Parasitismus. Einige der bedeutendsten Parasiten des Menschen sind obligat intrazelluläre Bakterien wie *Chlamydia trachomatis*. Dieser Krankheitserreger durchläuft regelmäßig einen biphasischen Entwicklungszyklus, der aus einer replizierenden intrazellulären Phase und einer übertragbaren extrazellulären Phase besteht. Dieses Merkmal vereint alle Chlamydien, auch die zahlreichen Arten, die Protisten infizieren. Unter diesen Protisten sind auch Dictyosteliden anfällig für Infektionen mit Chlamydien. Alle Dictyosteliden durchlaufen einen wiederkehrenden sozialen Lebenszyklus, der Stadien fakultativer Multizellularität beinhaltet. In dieser Arbeit charakterisierten meine KolegInnen und ich Chlamydien zweier enfernt-verwandter Familien aus acht Dictyosteliden-Isolaten, mit einer detaillierten Analyse dreier Isolate. Die komplett sequenzierten Genome dieser acht Dictyostelid-assoziierten Chlamydien zeigten einen umfangreichen, familienspezifischen Genverlust, insbesondere der Regulatoren des kanonischen Entwicklungszyklus und verwandter Gene. Im Einklang mit diesen genomischen Signaturen beobachteten wir den Verlust der spezifischen, für Chlamydien typischen morphologischen Stadien, veränderte Genexpressionsdynamiken und das Fehlen mittels digitaler PCR nachweisbarer, extrazellulärer Formen. Dies passt gut zu unseren Erkenntnissen, dass Chlamydien vertikal durch die soziale Lebensphase des Wirts übertragen werden, gezeigt durch Transmissionselektronenmikroskopie und Fluoreszenzmikroskopie, sowie horizontal über direkten Zell-Zell Kontakt zwischen Wirtszellen. Wir fanden keine signifikanten, durch die Chlamydien ausgelösten Effekte auf die Wachstumsrate und Sporenproduktion des Wirts. Das impliziert eine verringerte Pathogenität der Dictyosteliden-assoziierten Chlamydien. Zusammengefasst konnten wir zeigen, dass sich Chlamydien an die fakultative Multizellularität ihres Wirts anpassen, und zwar durch Genverlust, deutliche Veränderungen der Übertragungsmodi und den Verlust des konservierten chlamydialen Entwicklungszyklus. Damit trägt diese Arbeit zu unserem Verständnis der chlamydialen Evolution und Anpassung bei und fügt sich in die Theorien zur Evolution von Endosymbiose.

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