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Genome organization and genomics in *Chlamydia*: whole genome sequencing increases understanding of chlamydial virulence, evolution, and phylogeny

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The genus *Chlamydia* contains important obligate intracellular bacterial pathogens to humans and animals, including *C. trachomatis* and *C. pneumoniae*. Since 1998, when the first *Chlamydia* genome was published, our understanding of how these microbes interact, evolved and adapted to different intracellular host environments has been transformed due to the expansion of chlamydial genomes. This review explores the current state of knowledge in *Chlamydia* genomics and how whole genome sequencing has revolutionised our understanding of *Chlamydia* virulence, evolution, and phylogeny over the past two and a half decades. This review will also highlight developments in multi-omics and other approaches that have complemented whole genome sequencing to advance knowledge of *Chlamydia* pathogenesis and future directions for chlamydial genomics.

KEYWORDS

Chlamydiaceae, *Chlamydia trachomatis*, genomics, genome content, whole genome sequence, next generation sequencing, tissue tropism, host tropism

1 Introduction

Within the last 15 years, advances in whole genome sequencing (WGS) technologies and expansions in publicly available chlamydial genomes have dramatically increased our knowledge on chlamydial evolution and phylogeny, genome structure, metabolic processes, and potential virulence factors of these organisms. Presently, WGS is considered the gold standard for molecular epidemiology, evolution, and genetic diversity, and has become an

integral part of cell biology studies in the genetically recalcitrant *Chlamydia*. The distinguishing feature of *Chlamydia* is its biphasic developmental cycle which alternates between extracellular infectious elementary bodies (EBs), which attach to and enter the host cell, and intracellular replicative reticulate bodies (RBs) residing in a membrane-enclosed inclusion. Upon RBs replication and inclusion growth, they dedifferentiate into EBs, to be released out of host cells and continue the infectious process (Elwell et al., 2016; Gitsels et al., 2019; Chiarelli et al., 2020). Under stress, *Chlamydia* RBs can also dedifferentiate into a non-replicating, persistent form, known as aberrant bodies (ABs) (Panzetta et al., 2018). The chlamydial lifecycle is central to the pathogenesis of these bacteria, as their survival depends on the complex host-pathogen interactions to establish an intracellular niche, subvert host cellular processes, acquire host-derived nutrients and evade the host immune response (Elwell et al., 2016; Panzetta et al., 2018; Gitsels et al., 2019; Chiarelli et al., 2020). Currently, the *Chlamydiaceae* consists of two genera: 1) *Chlamydia* (Figure 1), which harbours the following characterised species: *C. abortus*, *C. avium*, *C. buteonis*, *C. caviae*, *C. crocodili*, *C. felis*, *C. gallinacea*, *C. muridarum*, *C. pecorum*, *C. pneumoniae*, *C. poikilothermis*, *C. psittaci*, *C. serpentis*, *C. suis*, *C. trachomatis*, in addition to four Candidatus (Ca.) species: Ca. *Chlamydia corallus*, Ca. *Chlamydia ibidis*, Ca. *Chlamydia sanzinia* and Ca. *Chlamydia testudines* (Cheong et al., 2019; Zaręba-Marchewka et al., 2021) and, 2) the newly described *Chlamydiifrater* (Figure 1), harbouring the two recent species, *C. phoenicopteri* and *C. volucris* (Vorimore et al., 2021b). The availability of genome sequences for all novel and well-characterised species within the *Chlamydiaceae* provides an unparalleled opportunity to advance our understanding of the genetic diversity and taxonomy, genome biology, and gene content of these fascinating organisms (Figure 1). This review provides an overview of WGS methods and the most common genomic analyses, followed by a description of the chlamydial genome structure and content. It also provides details on the currently known events of genomic recombination as it relates to the threat of genetic transfer of antibiotic resistance genes, and

challenges to the identification of lymphogranuloma venereum (LGV) outbreaks. Finally, we conclude with a discussion of anticipated directions for chlamydial genomics.

2 Chlamydial genomics: A new gold standard for chlamydial studies

2.1 WGS and genomic analyses

For most bacterial species, obtaining high-yield DNA extracted from cultured organisms remains the gold standard for WGS. Due to the intracellular niche of *Chlamydiaceae*, culturing and isolation is laborious, generally yielding lower levels of chlamydial genetic material contaminated with host DNA. In the past decade, there have been advances in obtaining chlamydial genomes from contaminated and/or low-yielding DNA samples retrieved from clinical and environmental samples using non-targeted and targeted approaches. These developments have resulted in a substantial increase of WGS data being generated (Bachmann et al., 2014; Taylor-Brown et al., 2018). New targeted WGS approaches are preferred for epidemiological and diagnostic purposes, particularly if the organism has already been sequenced, due to lower costs, higher throughput, and increased specificity (Taylor-Brown et al., 2018). However, for chlamydial discovery (e.g. novel species), non-targeted approaches are mostly utilised. Other methods also include expensive deep sequencing of contaminated samples using bioinformatic filtering to determine chlamydial sequences.

For WGS studies using (high-yield) DNA extracted from cultured isolates, direct next generation sequencing (NGS) is commonly used. Due to decreasing costs and ongoing developments in NGS, hybrid approaches such as nanopore sequencing to complement short-read WGS, are used to improve and/or provide closed complete genomes for *Chlamydia* (Heijne et al., 2021; Zaręba-Marchewka et al., 2021). In contrast, WGS of *Chlamydia*-specific and/or as a part of metagenomic sequencing from clinical swabs often yield poor results. However, this

Genus/Species	Primary Hosts	Clinical Manifestation**	Median Chromosome Size (Mb)	GC (%)	CGI (Mb/Gen)	Plasmid	Plasmid Size (kbp)	Selected Reference(s)
<i>Chlamydia</i>								
<i>C. abortus</i>	Avian	Undetermined	1.17	39.9	986	+	7.55	10.3390/vetgenom1011405
<i>C. abortus</i> *	Ustedlock (Sheep, Goats)	Ustedlock: Fetal loss	1.14	39.9	982	-	-	10.1186/112844-017-3657-y
<i>C. avium</i>	Human	Human: Fetal loss	1.04	36.9	895	+	7.62	10.1128/PLoS.01509-10
<i>C. avium</i>	Avian	Undetermined	1.16	38.8	974	+	7.56	10.10043/system.2019.06.002
<i>C. buteonis</i>	Avian	Undetermined	1.17	39.2	981	+	7.97	10.1093/ncj/ajp121
<i>C. caviae</i> *	Rodents	Rodents: Conjunctivitis, urogenital infection, rhinitis	1.17	39.2	981	+	7.97	10.1093/ncj/ajp121
<i>C. caviae</i> *	Human	Human: Respiratory disease	1.17	39.2	981	+	7.97	10.1093/ncj/ajp121
<i>C. crocodili</i>	Reptilian	Undetermined	1.22	37.5	1029	+	7.73	10.1371/journal.pone.0252081
<i>C. felis</i> *	Feline	Feline: Conjunctivitis, rhinitis, respiratory infection	1.17	39.4	981	+	7.55	10.3390/vetgenom10007
<i>C. felis</i> *	Human	Human: Conjunctivitis, respiratory disease	1.17	39.4	981	+	7.55	10.3390/vetgenom10007
<i>C. gallinacea</i>	Avian	Undetermined	1.06	37.9	920	+	7.62	10.1128/genomc.00706.16
<i>C. muridarum</i>	Rodents	Pneumonia, urogenital infection	1.07	40.3	905	+	7.50	10.1128/NIJ.00250-15v
<i>C. pecorum</i>	Ustedlock (Sheep, Cattle, Pig) / Kudu	Ustedlock: Conjunctivitis, polyarthritis, abortion, encephalomyelitis Kudu: Conjunctivitis, urogenital tract infection	1.06	40.9	950	+	7.55	10.3390/vetgenom10121543 10.1128/NIJ.00454-11
<i>C. pneumoniae</i>	Human Amphibian Reptilian Marsupial	Pneumonia	1.23	40.6	1046	****	7.53	10.1186/1471-2164-11-442
<i>C. poikilothermis</i>	Reptilian	Undetermined	1.16	37.5	972	-	7.56	10.1076/141598-018-23897-z
<i>C. psittaci</i> *	Avian Ustedlock (Sheep, Cattle, Horses)	Avian: Emaciation, conjunctivitis, respiratory disease Ustedlock: Fetal loss, respiratory disease Horses: Mild to severe respiratory disease	1.17	39.1	977	+	7.53	10.3390/vetgenom100899
<i>C. serpentis</i>	Reptilian	Undetermined	1.20	37.5	992	+	7.53	10.10386/142598-018-23897-z
<i>C. suis</i> *	Porcine Human	Porcine: Conjunctivitis, diarrhoea, respiratory and reproductive disease	1.09	42.1	924	+	7.50	10.1093/ncj/ajp1042
<i>C. trachomatis</i>	Human	Ocular infection, urogenital infection	1.04	41.3	904	+	7.50	10.1103/ncj/212847.116
Ca. <i>Chlamydia corallus</i>	Reptilian	Undetermined	1.20	39.9	1005	+	7.52	10.10386/141598-017-10757-5
Ca. <i>Chlamydia ibidis</i>	Avian	Undetermined	1.15	38.3	955	?	?	10.1371/journal.pone.0074823
Ca. <i>Chlamydia sanzinia</i>	Reptilian	Undetermined	1.11	38.5	932	+	7.50	10.1186/112844-016-3055-x
Ca. <i>Chlamydia testudines</i>	Reptilian	Undetermined	~1.0	?	?	+	7.65	10.1371/journal.pone.0185407
<i>Chlamydiifrater</i>								
<i>C. phoenicopteri</i>	Avian	Undetermined	1.20	40.3	994	+	6.30	10.10145/system.2021.126200
<i>C. volucris</i>	Avian	Undetermined	1.20	40.4	991	+	6.32	10.10145/system.2021.126200

FIGURE 1

Features of the *Chlamydiaceae* family including primary host range, clinical manifestations and genome characteristics.

[*]: Documented zoonotic transmission to humans
 [**]: Asymptomatic shedding observed possible in wild chlamydial species
 [***]: Plasmid only present in animal strains (-): Plasmid Absent [?]: Currently Unknown

limitation was overcome with the development of culture-independent sample preparation methods, further supplemented with targeted capture methods (Joseph et al., 2015; Taylor-Brown et al., 2018; Zaręba-Marchewka et al., 2020). One of these methods is probe-based sequence-specific capture using biotinylated 120mer RNA probes called baits (Christiansen et al., 2014). This approach was successfully performed on WGS of *C. trachomatis* DNA from clinical samples (Christiansen et al., 2014; Hadfield et al., 2017; Seth-Smith et al., 2021), livestock and koala *C. pecorum* DNA from mucosal animal samples (Bachmann et al., 2015), DNA from archival *C. pneumoniae* strains (Roulis et al., 2015a; Roulis et al., 2015b), as well as *C. psittaci* clinical isolates (White et al., 2022) and DNA samples from a range of hosts (Branley et al., 2016). The recent development of third generation long-read sequencing technologies such as Oxford Nanopore and PacBio has already been used to provide high-quality complete *Chlamydia* genomes, resolving complex repetitive and recombination regions (Livingstone et al., 2021; Zaręba-Marchewka et al., 2021). These technologies also contain the additional potential for understanding chlamydial biology which is yet to be fully understood. For instance, Nanopore sequencing can directly detect DNA and RNA methylation in bacteria such as 6mA through changes in voltage signals when modified bases pass through the nanopore (Tourancheau et al., 2021), while PacBio Hi-C sequencing can be used to reveal the 3D genome structure and chromatin interactions (Lamy-Besnier et al., 2021). These novel technologies can be used to explore epigenetic modifications and 3D genome structure in different *Chlamydia* species, strains and/or stages of infection.

With the decreasing costs of deep sequencing, improvements in host DNA depletion techniques and advances in bioinformatic algorithms, shotgun metagenome sequencing which involves direct, untargeted sequencing of all microbial DNA in samples without the need for culture is becoming increasingly common. Shotgun metagenome sequencing provides new opportunities to study both *Chlamydia* and the microbiome; the microbiome has been shown to impact *Chlamydia* colonisation (Edwards et al., 2019). The successful recovery of metagenome-assembled genomes (MAGs) from shotgun metagenome data can also be used to discover new genera or species, as illustrated by the discovery of novel chlamydial lineages in anoxic marine sediments (Dharamshi et al., 2020).

Following WGS, analysis of *Chlamydia* genomes mainly follows a straightforward approach including *de novo* assembly into contigs and mapping approaches. Assembled sequences are subjected to genome annotation and estimation of genomic variability using single nucleotide polymorphisms (SNPs) and comparative approaches. Finally, a variety of phylogenomic analyses are performed to establish phylogenetic relationships among analysed strains and/or species (Joseph et al., 2015; Wolff et al., 2015; Hadfield et al., 2017; Sigalova et al., 2019; Hölzer et al., 2020; White et al., 2021; Zaręba-Marchewka et al., 2021; White et al., 2022). The reads and/or assembled genomes are deposited in publicly available databases so that they can be re-used across studies, providing a valuable resource for the chlamydial research community.

In chlamydial genomic studies, *in silico* analyses elucidating virulence factors and/or gene functions to explain phenotypes are also common. However, genes encoding for hypothetical or unknown proteins account for a significant proportion (39 – 45%) of most chlamydial genomes. Such hypothetical protein genes are conserved and considered “chlamydial-specific hypothetical protein genes”, with homologues commonly found among chlamydial species (Sigalova et al., 2019; Hölzer et al., 2020). Despite major advances in the genetic manipulation of chlamydiae, the function of many chlamydial virulence, tissue tropic-, and metabolic factors remain unknown (Bastidas and Valdivia, 2016). WGS and comparative genomics are efficient ways to characterise the genome content for strains of interest, further enabling detailed investigation of specific genotypes and delineating factors for host specificity, pathogenicity and tissue tropisms. One method for determining gene function is querying predicted Open Reading Frame (ORF) or coding DNA sequences (CDS) and assigning gene functions against a reference protein database (e.g. UniProtKB or Pfam-A database). Further gene and protein sequence homology or conserved domain analyses are commonly performed in chlamydial WGS annotation studies utilising publicly available or custom databases (Voigt et al., 2012; Read et al., 2013; Borges and Gomes, 2015; Seth-Smith et al., 2017a; Borges et al., 2019; Sigalova et al., 2019; Hölzer et al., 2020; Heijne et al., 2021; White et al., 2021). Recently, Pillonel and colleagues developed “open-access ChlamDB”, a comparative genomics database containing 277 genomes covering the entire *Chlamydiae* phylum as well as their closest relatives belonging to the *Planctomycetes-Verrucomicrobiae-Chlamydiae* (PVC) superphylum. Open-access ChlamDB provides various tools for comparing, analysing and retrieving *Chlamydiae*-specific genomic data (Pillonel et al., 2020). Such *in silico* analyses enable predictions of certain phenotypic or pathogenicity traits of chlamydial organisms. However, these require experimental validation, with limitations depending on the computational methods and resources used (Zhao et al., 2020). For genes encoding hypothetical proteins, comparative genomics to reveal conserved or absent genes in different lineages or tropic strains can provide valuable insight into their potential function and help identify genes for further investigation. Finally, using WGS in phylogenetic analyses can provide fine-detailed phylogeny, epidemiological networks and evolutionary pathways of *Chlamydia* species by estimating the chlamydial molecular clock. Without WGS techniques, this level of resolution would not be achievable, and certain evolutionary important lineages could be missed.

3 Chlamydial genome organisation and content: variability within similarity and synteny

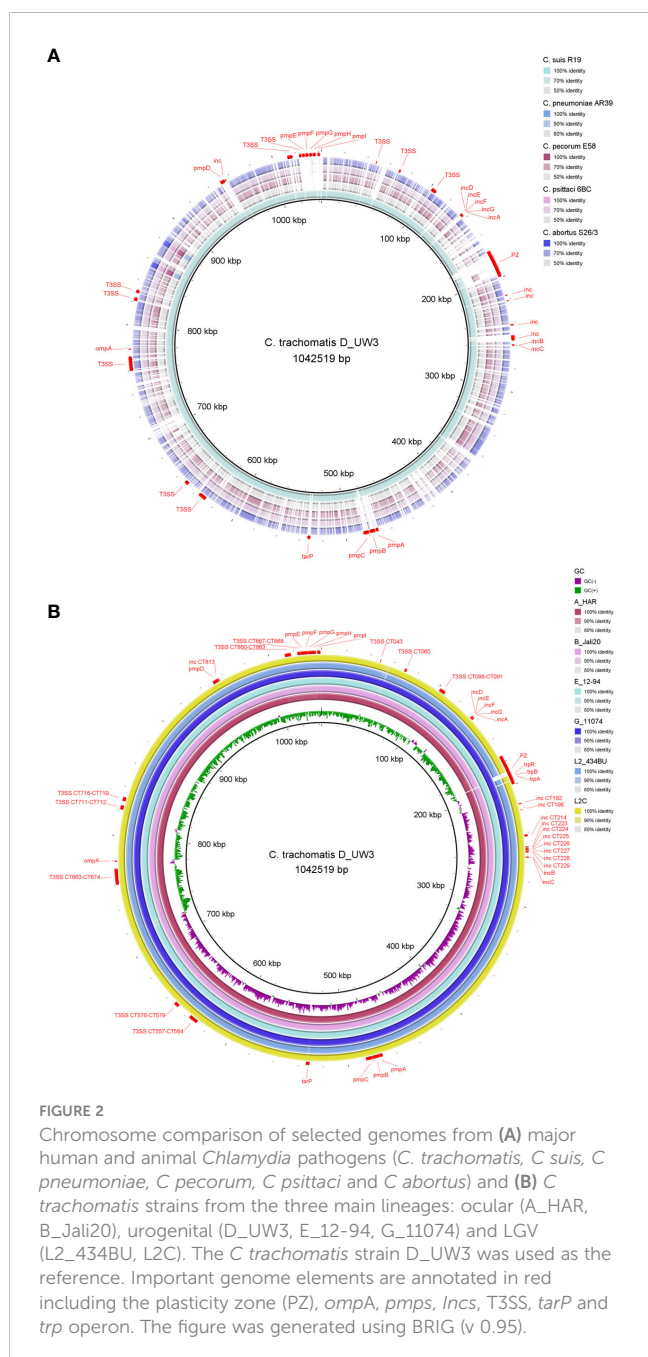
Genomes of *Chlamydiaceae* are compact, remarkably conserved and syntenic. Similar to most other obligate intracellular bacteria,

chlamydial genomes have a significantly reduced, circularised chromosome at approximately 1-1.2 Mbp with 900 - 1500 CDSs (Figures 1, 2) (Collingro et al., 2011; Voigt et al., 2012; Joseph et al., 2015; Pillonel et al., 2018; Sigalova et al., 2019). Almost all *Chlamydiaceae* species contain a highly conserved chlamydial plasmid of approximately 6.3 - 8 kbp, apart from livestock *C. abortus* strains, human strains of *C. pneumoniae*, and rare plasmid-free strains (Zhong, 2017; Szabo et al., 2020). The reduced genome for *Chlamydiaceae* species is a result of genome streamlining rather than degradation and is thought to be due to their transition to the intracellular lifestyle and co-evolution within their eukaryotic hosts (Moran, 2002; Collingro et al., 2011; Nunes and Gomes, 2014; Dharamshi et al., 2023). Within the same chlamydial species, the genomes are highly syntenic and similar, with a sequence homology

of approximately 90% (Figure 2). Most of the differences are attributed to SNPs which have been postulated to play a role in virulence, host, and tissue tropism (Nunes et al., 2013; Read et al., 2013; Bachmann et al., 2014; Bachmann et al., 2015; Wolff et al., 2015).

Almost all *Chlamydia* spp. genomes possess several common well-characterised metabolic, species-specific, and virulence genes or genomic regions. Recent pan-genomic analyses of 227 *Chlamydia* genomes demonstrated the degree of chlamydial similarity, with over 81% of genes universally (~700 universal genes) or partially conserved (~967 periphery genes), and only 19% of genes (~380 genes) unique to singular genomes. According to homology analyses, the periphery genes within species accounted for differences in organic molecule transport and metabolism, while unique genes contributed to differences in intracellular trafficking, secretion, and vesicular transport (Sigalova et al., 2019). Due to their host-dependency and reduced genome size, *Chlamydiaceae* lack genes encoding essential metabolic enzymes, requiring them to siphon amino acids, nucleotides, and cofactors from the infected host cell. This symbiotic relationship results in phenotypic variations involving tissue and host tropisms among different *Chlamydiaceae* species. The estimated number of pseudogenes in each genome is low possibly due to genome streamlining and a strong selection pressure to remove non-essential genes (Sigalova et al., 2019). The estimated number of pseudogenes ranges from 5 - 8 in *C. pecorum*, 9 - 12 in *C. trachomatis*, *C. muridarum*, and *C. suis* to 21 in *C. pneumoniae* and 28 - 29 in *C. abortus* and *C. psittaci*, with premature stop codons commonly occurring in genes encoding hypothetical proteins, and virulence-associated genes (*pm*s, *Incs*, and cytotoxin) (Voigt et al., 2012; Bachmann et al., 2014; Borges and Gomes, 2015; Sigalova et al., 2019; White et al., 2021).

Despite a high level of genome conservation, *Chlamydiaceae* possess several distinct polymorphic regions (Figure 2), with the highest variation occurring within the plasticity zone (PZ) (Figure 3). Other highly variable elements include genes encoding polymorphic membrane (*pm*s) and inclusion membrane proteins (*Incs*), Type III secretion system (T3SS), and other metabolic pathways, such as tryptophan biosynthesis (Bachmann et al., 2014; Sigalova et al., 2019; Hölzer et al., 2020) (Figure 2). Furthermore, *Chlamydiaceae* have been shown to possess strong recombination potential, particularly within virulence-associated genes, including the major outer membrane protein (*ompA*), T3SS effector translocated actin-recruiting protein (*tarP*), *pmp* genes and the PZ (Harris et al., 2012; Read et al., 2013; Joseph et al., 2015; Borges et al., 2019; White et al., 2021).



3.1 Tissue-tropic and virulence genes revealed by WGS

3.1.1 The plasticity zone

An evolutionary conserved but highly variable genomic region, the PZ is a genomic region associated with bacterial virulence protein genes and is responsible for distinct niche characteristics specific to different *Chlamydia* species (Rajaram et al., 2015;

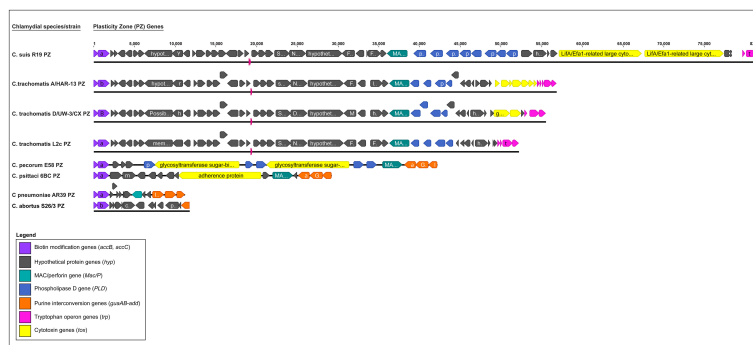


FIGURE 3

Comparison of the plasticity zone (PZ) from selected major human and animal *Chlamydia* species (*C. suis*, *C. trachomatis*, *C. pecorum*, *C. psittaci*, *C. pneumoniae* and *C. abortus*). The PZ elements are depicted in different colours, where biotin modification genes (*accB*, *accC*) are in purple, hypothetical protein (*hyp*) genes are in grey, MAC/perforin (*MAC/P*) in teal, Phospholipase D (*PLD*) in blue, purine interconversion genes (*guaAB*, *add*) in orange, Tryptophan operon genes (*trp*) in pink, and chlamydial cytotoxin (*tox*) gene(s) in yellow.

Dimond et al., 2021). Among the pathogenically diverse *Chlamydia* spp., the PZ ranges from ~5 kb for *C. avium* (five genes), ~20 – 40 kb for *C. abortus*, *C. psittaci* and *C. pecorum*, (~15 to 20 genes), to ~55 kb and ~81 kb for *C. trachomatis* and *C. muridarum* (> 45 genes) (Figure 3) (Hölzer et al., 2020; Dimond et al., 2021; Zaręba-Marchewka et al., 2021). This region commonly includes homologs to biotin modification genes *accB* and *accC*, MAC/perforin (*MacP*), a variable number of phospholipase D-like genes (*PLD*), cytotoxin (*tox*) homologs, purine interconversion genes *guaA* and *guaB*, and a different subset of species-specific hypothetical proteins. Additionally, a near-full tryptophan (*Trp*) operon can be found in the PZ regions of *C. caviae* and *C. felis*, while in *C. trachomatis* and *C. suis*, the PZ regions only contain partial *Trp* operons (Bachmann et al., 2014; Rajaram et al., 2015; Zaręba-Marchewka et al., 2021).

Of these PZ elements, only *accB* and *accC* are common to all *Chlamydia* and are considered the 5' boundary of the PZ, while other PZ genes vary between the species. Notably, chlamydial *tox* genes have significant homology to the large clostridial toxins (LCTs) and are present in almost all *Chlamydia* spp., however it may vary in copy number. *C. muridarum* has three *tox* copies, *C. pecorum* and *C. suis* two copies, while *C. trachomatis*, *C. caviae*, *C. crocodile*, *C. felis*, *C. gallinacea*, *C. psittaci*, *C. buteonis*, and avian *C. abortus* strains have one *tox* gene. A cytotoxin gene is absent from the PZs of the human pathogen *C. pneumoniae* and the ovine pathogen *C. abortus* (Figure 3) (Bachmann et al., 2014; Heijne et al., 2021; Zaręba-Marchewka et al., 2021). Significantly, *tox* gene polymorphisms have been useful in defining *C. trachomatis* disease phenotypes where the *tox* ORFs can be intact in urogenitotropic strains, truncated in oculotropic strains, or absent altogether in the LGV invasive strains (Figures 2B, 3) (Carlson et al., 2004; Nunes et al., 2013; Bachmann et al., 2014). Some PZ ORFs (e.g. species-specific hypothetical proteins) have no homologs or have been classified into families based on the nucleotide and/or protein sequence homology.

3.1.2 Type 3 secretion system genes

All *Chlamydia* spp. possess conserved genes encoding for structural, effector and chaperone proteins of the evolutionary

conserved T3SS, essential for the translocation of effector proteins directly into host cells. The secreted T3SS effectors are recognised as virulence factors in *Chlamydiae* (Peters et al., 2007; Bachmann et al., 2014; Mueller et al., 2014; Mojica et al., 2015). While for some species, like *C. trachomatis* and *C. psittaci*, T3SS effectors have been experimentally confirmed and/or functionally characterised (Mojica et al., 2015; da Cunha et al., 2017; Marschall et al., 2020). For other less characterised chlamydial species WGS analyses is used to identify and decipher their roles (Hölzer et al., 2020; Heijne et al., 2021; White et al., 2021).

In *Chlamydia* spp. genomes, T3SS genes consist of 20 – 25 genes, encoding for highly conserved T3SS apparatus scaffolding (e.g. chlamydial *CdsC*, *CdsJ*, and *CdsD* homologs), inner membrane components (e.g. *Cds R/S/T/U/V* and *Cds L/N/Q*), extracellular needles (e.g. *CdsF*, *CopN*), chaperons (e.g. *Ssc1-3*, *CdsE* and *CdsG*), and a variable number of genetically diverse effectors (e.g. *tarP*, *SINC*), and is usually found in four clusters across the chromosome (Betts-Hampikian and Fields, 2010; Borges and Gomes, 2015; Wolff et al., 2015; Bugalhão and Mota, 2019; Hölzer et al., 2020; Heijne et al., 2021; White et al., 2021).

In *Chlamydia* spp. genomes, 5 - 8% of the CDS are estimated to be effectors (Valdivia, 2008; Bugalhão and Mota, 2019; Andersen et al., 2021). Of the T3SS effectors, *TarP* is an extensively studied and functionally characterised T3SS effector, responsible for remodelling the actin cytoskeleton and facilitating the entry of *Chlamydia* into the host cell (Peters et al., 2007; Bugalhão and Mota, 2019; Andersen et al., 2021). Orthologs of *tarP* are present in all *Chlamydia* spp. genomes but show extensive sequence variation. Variations in the *tarP* gene sequences are hypothesised to contribute to virulence and variations to host tissue tropisms, as shown in *C. psittaci* and *C. trachomatis*. This is thought to be due to distinct N-terminal tyrosine-repeat units and C-terminal binding domains in *C. trachomatis* *TarP* (Borges and Gomes, 2015; Wolff et al., 2015). Another conserved T3SS secreted protein known as *SINC* (secreted inner nuclear membrane-associated *Chlamydia* protein) is a functionally characterised nuclear membrane targeting protein (Mojica et al., 2015; Marschall et al., 2020), which has been identified in *C. psittaci*, *C. abortus*, *C. pecorum*, *C.*

caviae, *C. felis*, *C. gallinacea*, and *C. avium* genomes (Hölzer et al., 2020). The ability of SINC to manipulate infected and non-infected host cell nuclear envelope function has been hypothesised to contribute to the high virulence of *C. psittaci* (Mojica et al., 2015).

3.1.3 Inc genes

Inc proteins are virulence factors unique to the *Chlamydiae* phylum (Heinz et al., 2010). They are typified by one or more bilobed hydrophobic domains. These hydrophobic domains consist of two transmembrane helices separated by a hydrophilic loop, allowing Inc proteins to be inserted into an inclusion membrane (Dehoux et al., 2011). Inc proteins are translocated by the T3SS with the N-terminal containing the secretion signal. Inc proteins contribute to the physical structure of the inclusion membrane and perform diverse functions, including subverting vesicular trafficking for nutrient acquisition and avoiding lysosomal degradation (Elwell et al., 2016; Bugalhão and Mota, 2019).

Inc genes constitute a significant portion of the *Chlamydia* genome, accounting for 6% to 14% of the total coding capacity in the respective species. Comparative genomics revealed a significant variation in the number of predicted inc genes in different *Chlamydia* species, with 65 - 70 genes in *C. trachomatis*, *C. muridarum* and *C. suis*, 102 - 110 in *C. psittaci* and *C. abortus* and 140 - 147 in *C. pneumoniae* (Sigalova et al., 2019). Only a subset of Incs are conserved between species with ~23 Incs shared between *C. trachomatis*, *C. muridarum*, *C. felis*, *C. caviae* and *C. pneumoniae* (Lutter et al., 2012; Dimond et al., 2021). Of the Incs that are shared between species, sequence conservation is low among *Chlamydia* (Dehoux et al., 2011). Incs conserved across species may point to common protein functions and host interactions important in *Chlamydia* species, while non-conserved Inc proteins are hypothesised to contribute to host species differences. For instance, Inc genes differences account for approximately one-third of the genetic variations between *C. trachomatis* and *C. pneumoniae*. The increased number of Inc genes in *C. pneumoniae* may allow it to adapt to a broader host range (Dehoux et al., 2011).

In addition to species differences, Inc proteins may be responsible for differences in tissue tropisms and disease severity between *C. trachomatis* LGV and trachoma biovars. Analysis of *C. trachomatis* genomes showed that Inc genes were subject to positive selection with four times more non-synonymous SNPs compared to synonymous SNPs (Almeida et al., 2012; Borges and Gomes, 2015). Amino acid changes in Inc proteins were primarily specific to LGV strains and could phylogenetically separate LGV from ocular and urogenital strains (Almeida et al., 2012; Borges et al., 2012; Lutter et al., 2012). These amino acid changes occurred in regions predicted to be exposed to the host cytoplasm and thus may be involved in host-pathogen interactions and/or immune recognition. Also, LGV strains were found to have LGV-specific expression patterns from three Inc genes (CT058, CT192 and CT214). These expression differences correlated with specific LGV-mutations in the CT192 and CT214 promoters and within the CT059-CT058 transcript. These Inc sequence and expression differences may be associated with LGV strain tissue tropism to macrophages, possibly by inhibiting phagolysosomal fusion (Almeida et al., 2012).

3.1.4 Pmp genes

Pmps are *Chlamydiaceae* unique, membrane-bound, type V surface-exposed autotransporters, associated with many chlamydia-host cell functions, including cell adhesion and virulence. All Pmps contain N-terminal Sec-dependent leader sequences (passenger domain) with multiple short repetitive motifs (GGA(I, L, V) and FxxN), a middle region and a C-terminal autotransporter β -domain. This autotransporter β -domain is responsible for translocating the protein to the bacterial surface (Mölleken et al., 2010; Nunes et al., 2015; Vasilevsky et al., 2016). The variable chlamydial pmp genes are grouped into several pmp gene families, where some are more evolutionary conserved in sequence and gene number (such as *pmpA*, *pmpB*, *pmpD* and *pmpH* families), while others are more varied (such as *pmpG* and *pmpE* families). The subtype *pmpG* is the most expanded in gene copy numbers. Among chlamydial species, the number of pmp genes ranges between seven and nine genes in *C. avium*, and *C. trachomatis*, to 18 and 21 genes in *C. psittaci* and *C. pneumoniae*, accounting for approximately 14% of the coding capacity of their genomes (Vasilevsky et al., 2016; Sigalova et al., 2019; Hölzer et al., 2020). Furthermore, bioinformatic analyses have shown that the subtype *pmpG* may undergo phase variation, by exhibiting differences in the lengths of in-frame poly(G) tracts. This may lead to random, high-frequency, on/off reversible switching of gene expression, involved in host adaptation and immune evasion (Nunes et al., 2015; Sigalova et al., 2019).

3.1.5 ompA gene

The *ompA* gene encodes for the chlamydial major outer membrane protein (MOMP), a ~40kDa surface-exposed porin protein containing four variable domains (VD I to VD IV), contributing to approximately 60% of the chlamydial outer membrane mass (Sun et al., 2007; Confer and Ayalew, 2013; Wen et al., 2016). Due to its antigenic properties, MOMP has been an attractive vaccine candidate against human and veterinary *Chlamydia* infection (Tifrea et al., 2020; Olsen et al., 2021; Quigley and Timms, 2021). The chlamydial *ompA* is a highly polymorphic gene specific to the genus *Chlamydia*, with approximately 30% of the sequence attributed to nucleotide polymorphisms and/or recombination (Nunes et al., 2009; Harris et al., 2012; Hadfield et al., 2017). Despite its highly recombinogenic nature, the chlamydial *ompA* gene is still a widely utilised marker for clinical, epidemiological, phylogenetic, and public health studies.

3.2 New insights into metabolic capabilities revealed by WGS

3.2.1 Nucleotide scavenging and energy production genes

Comparative genomic studies commonly note differences in metabolic genes and/or lack of pathways to synthesise most amino acids, including biosynthesis of biotin, tryptophan, thiamine, folate, purines, and pyrimidines nucleotides within and among chlamydial species.

Pyrimidine and purine nucleotides are necessary for energy transduction and the biosynthesis of nucleic acids for most bacterial species. However, *Chlamydia* spp. lack the necessary genes to synthesise pyrimidine and purine nucleotides, instead hijacking host cellular systems for their energy needs (Saka and Valdivia, 2010). *Chlamydia* genomes contain species-specific differences for purine conversion genes, with only *C. caviae*, *C. felis*, *C. muridarum*, *C. psittaci*, and *C. pecorum* possessing a functional *guaAB-add* operon capable of producing AMP adenosine deaminase, GMP synthase and IMP dehydrogenase. Conversely, species including *C. trachomatis*, *C. gallinaceae*, *C. suis*, *C. pneumoniae*, and *C. abortus*, are lacking these conversion genes (Figure 3) (Voigt et al., 2012; Nunes and Gomes, 2014; Hölzer et al., 2020).

Additionally, all *Chlamydia* genomes contain the pyrimidine interconversion genes *pyrH*, *ndk* and *pyrG* (uridylylase kinase, nucleoside diphosphate kinase, and CTP synthase, respectively). These allow for the conversion of uridine monophosphate (UMP) to cytidine triphosphate (CTP). With the exception of *C. trachomatis* and *C. muridarum*, chlamydial genomes also contain the *pyrE* (orotate phosphoribosyltransferase) gene that encodes for uridine monophosphate synthetase (Saka and Valdivia, 2010; Voigt et al., 2012; Nunes and Gomes, 2014; Sigalova et al., 2019). Finally, only *C. pneumoniae* has a uridine kinase (*udk*) gene, which converts uridine or cytidine into UMP or CMP, respectively (Voigt et al., 2012; Nunes and Gomes, 2014).

Chlamydia utilises host-cell adenosine triphosphate (ATP), guanosine triphosphate (GTP), thymidine triphosphate (TTP) and uridine triphosphate (UTP) for survival (McClarty and Qin, 1993). Chlamydial species also contain a CTP synthetase gene (*Npt1*), which converts UTP to CTP and provides an alternative pathway for *Chlamydia* to obtain ATP (Saka and Valdivia, 2010; Sigalova et al., 2019). However, all other known mechanisms are lacking. Alternatively, WGS analyses have revealed that *Chlamydia* species contain genes for a complete glycolysis pathway (except for hexokinase, utilising hexose phosphate transporter to uptake host glucose-6-phosphate instead) and sodium-driven oxidative phosphorylation to generate its own ATP for energy or nucleic acid synthesis) and a hexose phosphate transporter, allowing *Chlamydia* to use glucose-6-phosphate as an energy source (Liang et al., 2018; Sigalova et al., 2019; Ende and Derré, 2020). However, this process would still depend on ADP availability from nucleotide uptake or interconversion.

3.2.2 Tryptophan operon

Trp is an essential amino acid used by bacterial species in protein biosynthesis. This pathway has been identified in chlamydial species, and is of particular interest. Genomic analyses of *Chlamydia* spp. have identified partial/complete gene loss in the *trp* operon resulting in the selective abilities of species to synthesise Trp. In human host cells, the pro-inflammatory cytokine, interferon-gamma (IFN- γ) stimulates the indoleamine-2,3-dioxygenase (IDO) enzyme to catabolise host tryptophan to kynurenine, essentially starving the invading organism. Due to this, IFN- γ treatment can be used for infection control (Bachmann et al., 2014; Somboonna et al., 2019).

A (near) complete and functional *trp* operon, which includes *trpR-trpDCFBA* and the complementary genes *kynU* and *prsA*, has been described in *C. caviae*, *C. felis*, *C. pecorum*. The *trpR* gene mediates tryptophan-dependent transcriptional repression, while the rest of the operon genes encode for anthranilate phosphoribosyltransferase (*trpD*), indole-3-glycerol phosphate synthase (*trpC*), phosphoribosylanthranilate isomerase (*trpF*), tryptophan synthase beta chain (*trpB*) and tryptophan synthase alpha chain (*trpA*). The complementary *kynU* and *prsA* encode for kynureninase and phosphoribosylpyrophosphate. However, *C. suis* and *C. trachomatis* have only partial *trpR-trpBA* genes, while the rest of the species lack all *trp* operon genes (e.g. *C. abortus*, *C. gallinaceae*, *C. pneumoniae* and *C. psittaci*) (Nunes et al., 2013; Bachmann et al., 2014; Hölzer et al., 2020). Interestingly, *C. pecorum* was reported to contain a *trp* operon outside the PZ, while *C. caviae* and *C. felis* have a near-full *trp* operon in the PZ. Similarly, *C. trachomatis* and *C. suis* have been reported to contain only partial *trp* operons located in their PZs (Bachmann et al., 2014; Hölzer et al., 2020). Using an example of *C. trachomatis*, where *trp* genes are organised as an operon with *trpR* separated by a 348-base pair (bp) intergenic region from *trpBA*, a recent study demonstrated that these could be independently transcribed and not necessarily expressed as a monocistronic transcript. Pokorzynski and colleagues showed that iron starvation can switch on the Trp salvage pathway, mediated by the iron-dependent repressor YtgR, which binds to an operator sequence within the *trpR-trpBA* intergenic region to repress transcription initiation from the alternative promoter for *trpBA* (Pokorzynski et al., 2019; Pokorzynski et al., 2020).

Furthermore, in ocular strains of *C. trachomatis*, the *trp* operon has a loss of function due to a single nucleotide deletion and frameshift in *trpA* gene encoding a truncated tryptophan synthase alpha (TrpA) subunit. Conversely, the *trp* operon genes of *C. trachomatis* urogenital strains (D – K) are intact and functional, having the ability to produce tryptophan from indole, further allowing them to recover from IFN- γ exposure and resume their infectious cycle and this is supported by experimental studies (Fehlner-Gardiner et al., 2002; Ziklo et al., 2016; Somboonna et al., 2019; Bommanna et al., 2021). For the chlamydial species, such as *C. caviae* and *C. pecorum*, which have the complementary genes, *kynU* and *prsA*, it has been shown that these species can synthesise tryptophan when limited in the presence of IFN γ , likely due to the presence of these genes (Wood et al., 2004; Islam et al., 2018). Overall, the biological significance of the identified *trp* polymorphisms remains uncharacterised. However, for some, one could infer with confidence, such as acquisition of *kynU*, which mediates anthranilate synthesis from kynurenine, instead of the canonical chorismate-to-anthranilate pathway facilitated by *trpE*. The intergenic region of the *C. trachomatis* *trp* operon has regulatory functions that potentially optimises *trp* operon expression in specific biological contexts, including iron starvation and mucosal epithelial tropism.

3.2.3 Biotin operon

Biotin is a critical cofactor involved in many central cell metabolism pathways (Fisher et al., 2012). Analysis of chlamydial genomes identified differences across species for biotin acquisition,

where *C. psittaci*, *C. abortus*, *C. felis*, *C. pecorum* and *C. pneumoniae* have an intact biotin operon (*bioBFDA*) needed for *de novo* biotin synthesis. Other species, such as *C. trachomatis*, *C. caviae* and *C. muridarum* lack a functional biotin operon and thus depend on biotin salvaging (Fisher et al., 2012; Voigt et al., 2012; Nunes and Gomes, 2014; Sigalova et al., 2019). Interestingly, some species such as *C. psittaci* can synthesise biotin *de novo* and also sequester host biotin (Voigt et al., 2012).

3.3 Chlamydial plasmid

There is increasing evidence that the chlamydial plasmid is a key virulence factor and determinant of pathogenicity. Almost all *Chlamydia* spp., except *C. abortus* and human *C. pneumoniae* strains, contain a small (~7.5 kbp), highly conserved, non-conjugative or integrative chlamydial virulence plasmid (Pawlikowska-Warych et al., 2015; Szabo et al., 2020). Within some of the plasmid-bearing species, naturally occurring plasmidless strains can be common, as observed in *C. pecorum* (Jelocnik et al., 2015), or rare, as seen in *C. trachomatis* (Sigar et al., 2014; Jones et al., 2020).

The chlamydial plasmids contain eight CDSs, named plasmid glycoproteins 1 to 8 (pGP 1-8), and four tandem repeat sequences, with each iteration comprising of 22 base pairs near the origin of replication (Pawlikowska-Warych et al., 2015; Zhong, 2017; Szabo et al., 2020). Briefly, CDSs 1 (pGP7) and 2 (pGP8) encode a putative integrase, involved in plasmid replication, CDS3 (pGP1) is homologous to a helicase, while CDSs 4 (pGP2) is an unknown chlamydia-specific protein. CDS5 (pGP3) encodes a secreted virulence 28 kDa protein essential for establishing persistent infection (Yang et al., 2020) and CDS 6 (pGP4) encodes a chlamydia-specific transcriptional regulator for multiple genes located within both the plasmid and chromosome including pGP3 and *glgA*, a glycogen synthase gene (Song et al., 2013). A recent study also demonstrated that in *C. trachomatis* pGP3 or pGP4 are both required for infectivity (Turman et al., 2023). Finally, CDSs 7 and 8 (pGP5 and 6) are denoted as partitioning plasmid proteins (Rockey, 2011; Pawlikowska-Warych et al., 2015; Zhong, 2017; Jones et al., 2020).

Sequence and phylogenetic analyses indicate that chlamydial plasmids are evolutionarily conserved and syntenic between species, but within species, chlamydial plasmids contain unique sequence variations, further supporting the notion that chlamydial plasmids have co-evolved with the chromosome (Seth-Smith et al., 2009; Jelocnik et al., 2015; Szabo et al., 2020). In *C. trachomatis* diagnostics, the chlamydial plasmid CDSs are useful targets for diagnostic testing due to their stability, and high copy number, with up to ten copies per genome (Seth-Smith et al., 2009). However, the emergence of a urogenital mutant strain from Sweden containing a 377 bp deletion in plasmid gene CDS1 led to this plasmid locus being abolished as a diagnostic target due to false negative test results (Seth-Smith et al., 2009). This variant was characterised using WGS, and has since been occasionally reported in other countries (Piñeiro et al., 2014; Dahlberg et al., 2018; Escobedo-Guerra et al., 2019).

3.4 Other genomic elements

3.4.1 Antibiotic resistance genes

There are limited reports of antibiotic-resistant genes (ARGs) in the genomes (including both chromosome and chlamydial plasmid) of *Chlamydia* spp. However, due to observed treatment failures, WGS studies continue to monitor for chromosomal genes and mutations associated with *Chlamydia* spp. resistance to antibiotics (Borel et al., 2016; Benamri et al., 2021).

The only ARG identified in *Chlamydia* spp. is the tetracycline resistance gene *tetA(C)* found in tetracycline-resistant *C. suis* strains, the only chlamydial species to have naturally obtained a resistance gene (Borel et al., 2016; Seth-Smith et al., 2017a). The *tetA(C)* gene encodes a tetracycline efflux pump which has been integrated as a genomic island (Tet-island) into the *C. suis* invasion-like gene (*inv*) on the chromosome. This integration most likely occurred during a transposition event directed by the transposase-encoding insertion sequence IScs605 (Joseph et al., 2016; Marti et al., 2022). The Tet-island is currently the only evidence for recent acquisition of foreign DNA from other bacterial species in *Chlamydia* as it shares high nucleotide identity with a pRAS3-type plasmid from the fish pathogen *Aeromonas salmonicida ssp. salmonicida* (Marti et al., 2022).

4 Recombination and horizontal gene transfer in chlamydial genomes

Due to the biphasic and intracellular life cycle of *Chlamydia* species, chlamydiae do not commonly replicate in environments occupied by other microorganisms. Most bacterial species gain foreign DNA, including antibiotic-resistance genes through environmental interactions with other bacterial species. Despite an intracellular niche, chlamydial species have developed an increased ability to undergo genomic recombination (Harris et al., 2012; Read et al., 2013). Although *Chlamydia* genomic recombination had been identified prior to 2004, Gomes et al. demonstrated that *C. trachomatis* recombination was frequent. Using phylogenetic analysis of 19 reference strains and 10 clinical isolates, two genomic regions were found to be hotspots for interstrain recombination (Gomes et al., 2007). Genetic recombination hotspots for other *Chlamydia* species have also been identified in *C. suis* (Marti et al., 2017; Seth-Smith et al., 2017a), *C. psittaci* (Read et al., 2013; Branley et al., 2016), *C. pecorum* (White et al., 2021), and even in small regions in the otherwise monomorphic *C. abortus* (Seth-Smith et al., 2017b). Genomic regions with evidence of recombination include the genes *IncA*, *pmpps*, *tarp* and the PZ (Gomes et al., 2004; Harris et al., 2012; Joseph et al., 2012; Borges and Gomes, 2015; Seth-Smith et al., 2021).

The most convincing evidence indicating the ability of chlamydial species to undergo genetic recombination (horizontal gene transfer (HGT)) was reported in co-culture studies (Marti et al., 2022). Co-culture of *C. suis* carrying the *tetC* gene with *C. trachomatis*, *C. muridarum*, and *C. caviae* confirmed genomic integration of the *tetC* island in both *C. trachomatis* and *C. muridarum* (but not *C. caviae*) (Suchland et al., 2009). Similarly, chlamydial interspecies HGT was analysed using crosses of

tetracycline (Tc)-resistant *C. trachomatis* L2/434 and chloramphenicol (Cam)-resistant *C. muridarum* VR-123. WGS of the recombinant clones identified examples of duplications, mosaic recombination endpoints, and recombined sequences that were not linked to the selection marker (Suchland et al., 2019). A more recent study co-cultured tetracycline-resistant *C. suis* with rifamycin group-resistant *C. suis* resulting in an overall *in vitro* recombination efficiency of 28% (Marti et al., 2021). However, these observations have not been found in clinical isolates, and tetracyclines still remain part of the first-line defence antibiotics for human chlamydial infections.

5 Whole genome phylogenies resolve fine-detail relationships between *Chlamydia* species and strains

The use of WGS combined with sample-specific metadata have provided important observations on the contemporary history of current *C. trachomatis* circulating lineages within global populations (Andersson et al., 2016; Hadfield et al., 2017; Seth-Smith et al., 2021). It is well established that *C. trachomatis* is comprised of three distinct lineages, where *ompA* genotypes A, B, Ba and C are associated with trachoma, genotypes D-K associated with non-invasive UGT infections, and genotypes L1-L3 and L2b associated with invasive infections and LGV (Harris et al., 2012; Joseph et al., 2012; Andersson et al., 2016; Hadfield et al., 2017) (Figure 4A). However, phylogenomic studies show that not all strains may fit into this traditional separation, perhaps reflecting *C. trachomatis* adaptation to their respective niche and the noted recombination events. For example, whole-genome phylogenetic

analyses of ocular isolates obtained from Australian Aboriginal people with trachoma placed these isolates into two lineages that fall outside the classical trachoma lineage (A-C). Instead, these genetically distinct strains clustered within lineages that were previously occupied exclusively by UGT isolates (namely genotypes D–K). These trachoma isolates appear to be recombinants with the *C. trachomatis* UGT genome backbones, where recombination was noted in *ompA* and *pmp* loci replacing them with those *ompA* and *pmp* genotypes characteristic of ocular isolates (Andersson et al., 2016). Similarly, recent evidence has suggested that clinically relevant recombination events have occurred between two *C. trachomatis* strains (serovar D and LGV). Recent outbreaks of LGV (L2b strain) within men who have sex with men (MSM) populations have resulted in genetic recombination of the *ompA* gene from non-LGV strains (Borges et al., 2019; Borges et al., 2021). A previously undetected outbreak of LGV may have gone undiagnosed due to over-reliance on the *ompA* gene for identification. Further genomic analysis found that the *C. trachomatis* L2B strain had undergone genetic recombination, acquiring *ompA* from the non-LGV strain *C. trachomatis* D-Da (Borges et al., 2019). Furthermore, follow-up studies have indicated that this L2b/D-Da variant is spreading across Europe and, with the continued reliance on *ompA* for strain identification, the probability of further dissemination across the globe is high (Borges et al., 2021).

Similarly, phylogenomic analyses confirmed that modern human *C. pneumoniae* strains have evolved separately from animal strains (Roulis et al., 2015b), while further demonstrating a distinct Australian indigenous *C. pneumoniae* clade pre-dating European exploration of the continent (Roulis et al., 2015a). Phylogenomic analyses have demonstrated that whilst the global

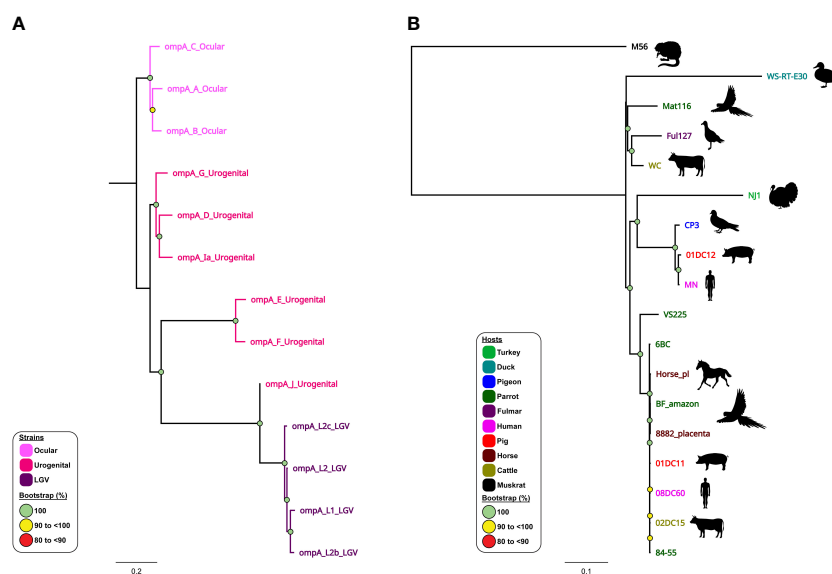


FIGURE 4

Phylogenomic relationships of *C. trachomatis* and *C. psittaci* reference strains. Midpoint-rooted maximum-likelihood (ML) core-genome phylogenetic trees constructed using Parsnp version 1.2 of (A) 13 aligned publicly available complete *C. trachomatis* genomes; and (B) 18 aligned publicly available complete *C. psittaci* genomes. For *C. trachomatis*, genome sequences are coloured according to tissue tropism/known genotypes (as depicted in the figure legend). For *C. psittaci*, genome sequences are coloured according to their respective host (as depicted in the figure legend). Corresponding host silhouettes are shown adjacent to sequence names. Bootstrap values greater than 0.8 are shown. Branch lengths represent the nucleotide substitutions per site, as indicated by the scale bar.

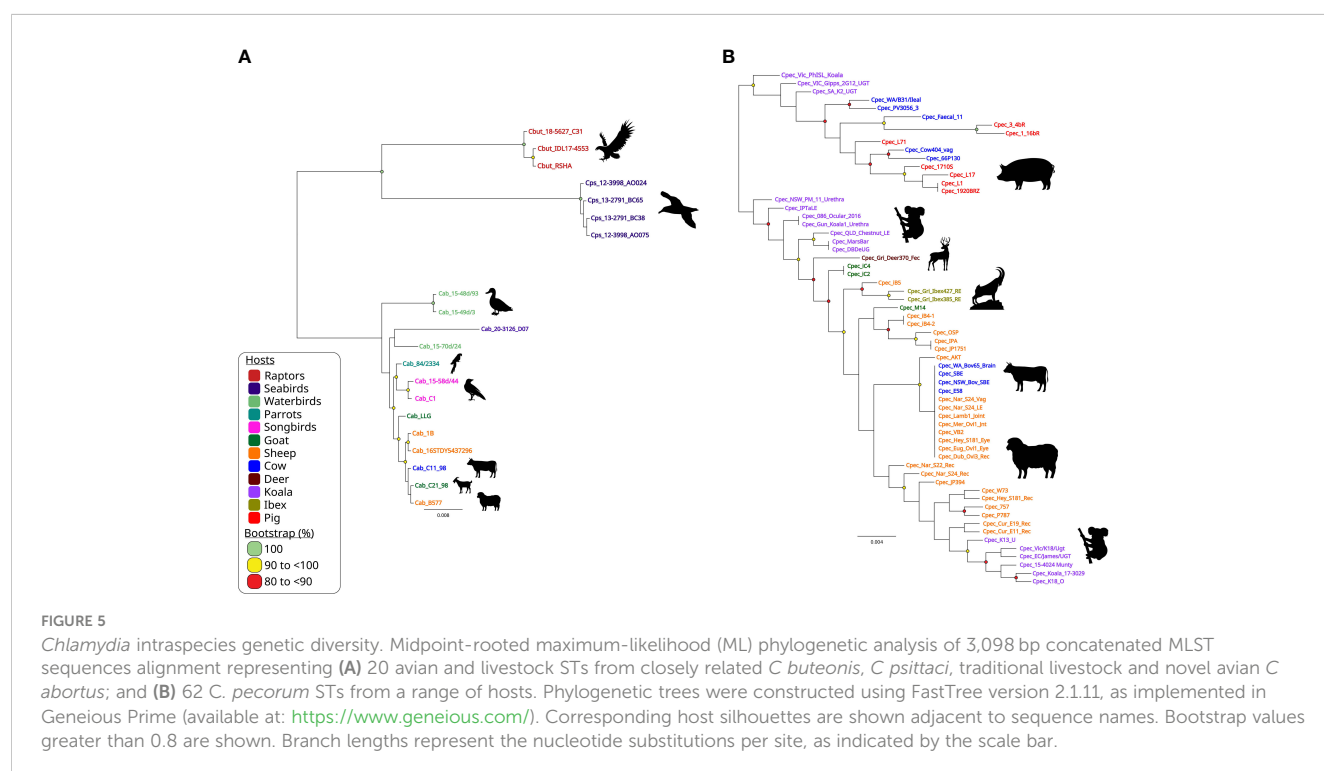
C. psittaci population is genetically diverse, there are also recently emerged, globally distributed and highly clonal lineages (such as *C. psittaci* ST24 strains) that infect humans, parrots, and, most recently, horses. Within this highly clonal ST24 lineage, the equine, human and parrot strains differ by less than 200 single nucleotide variants (SNVs), evenly distributed around the highly conserved and syntenic chromosome (Read et al., 2013; Branley et al., 2016; Jenkins et al., 2018; White et al., 2022; White et al., 2023). However, between genetically diverse *C. psittaci* lineages (such as ST24 and pigeon-associated clade) SNVs and differences in gene content were noted in distinct chromosomal regions, including *T3SS*, *ompA*, *Inc* and *pmp* genes (Voigt et al., 2012; Wolff et al., 2015; Hölzer et al., 2020; Vorimore et al., 2021a) (Figure 4B). The difference in gene content and SNVs likely plays a role in *C. psittaci* host tropism, adaptation and species-specific pathogenicity (Wolff et al., 2015; Favaroni et al., 2021). Furthermore, other phylogenomic analyses have genetically separated distinct avian-type *C. abortus* strains from the traditional ruminant *C. abortus* and/or avian *C. psittaci* strains (Longbottom et al., 2021; Zaręba-Marchewka et al., 2021) and confirmed that ruminant *C. abortus* genomic diversity is low level with no recombination detected, contrasting other related species (Seth-Smith et al., 2017b).

5.1 Complementing WGS studies: Gene-centric molecular epidemiology of chlamydial infections

WGS is considered the norm for evolutionary and epidemiological studies of many pathogens, including the human pathogen *C. trachomatis*, with the most available WGS data among

chlamydial genomes (Harris et al., 2012; Bowden et al., 2021; Seth-Smith et al., 2021). However, numerous molecular epidemiology and/or genetic diversity studies of *C. trachomatis* and veterinary species still rely on gene-centric typing methods using a single (e.g. *ompA*) or multiple conserved gene markers (e.g. multi locus sequence typing (MLST)) due to decreased costs. Genotyping using the full-length as well as fragments of the highly variable *ompA* gene is employed in genetic diversity studies for veterinary and human chlamydial infections, as it provides an initial molecular characterisation of the infecting strains (Liu et al., 2019; Rawre et al., 2019; Robbins et al., 2019).

However, the insufficient resolution and noted recombination in the chlamydial *ompA* genes, led to use of species-specific higher-resolution genotyping methods, such as MLST (Versteeg et al., 2018a; Jelocnik et al., 2019). The chlamydial MLST is globally adopted as a rapid, universal fine-detailed molecular typing tool for both human *C. trachomatis* and veterinary chlamydial pathogens (Figure 5). As a technically easier and cheaper alternative to WGS, MLST utilises the Chlamydiales PubMLST database (<http://pubMLST.org/chlamydiales>) hosted on a platform that provides easy to use sequence and phylogenetic analyses. Furthermore, WGS studies are often supplemented with extended genotyping of samples using MLST due to high congruency with WGS phylogenetic clustering (Guo et al., 2017; Jenkins et al., 2018). Core genome and/or traditional MLST-derived phylogeny is highly congruent with WGS and/or SNP-derived phylogenies, and avoids the need to reconstruct computationally heavy phylogenies (Jolley et al., 2018; Patiño et al., 2018; Versteeg et al., 2018a; Jelocnik et al., 2019; Florida-Yapur et al., 2021). Chlamydial MLST was effectively used in uncovering the global epidemiology of *C. trachomatis* strains (Herrmann et al., 2015; Danielewski et al., 2017),



identifying clonal psittacine, horse and human strains of *C. psittaci* during an outbreak (Anstey et al., 2021) as well closely related but genetically diverse avian chlamydial strains (Figure 5A), distinguishing diverse koala *C. pecorum* strains to aid in translocation of animals (Figure 5B) (Fernandez et al., 2019) and other examples (Batteiger et al., 2014).

6 Complementing WGS studies: Multi-omics integration for a systems biology understanding of chlamydial infections

Systems biology is the large-scale study of how genes, proteins, metabolites and other regulatory elements in an organism interact together using various integrated high-throughput multi-omic techniques, including genomics, transcriptomics, proteomics and metabolomics. Due to their biphasic, intracellular lifecycle, *Chlamydia* remain difficult to genetically manipulate; multi-omic approaches to understanding *Chlamydia* biology and pathogenesis are thus essential (Figure 6).

Genomics forms the basis for systems biology and allows genes/proteins expressed during different conditions to be mapped and identified using complementary transcriptomic and proteomic methods. In *Chlamydia*, these approaches have provided insights into homologous virulence factor expression differences between *C. psittaci* and *C. abortus* which may be related to disease severity in humans (Beder and Saluz, 2018). Transcriptomics/proteomics studies have also revealed the host and pathogen genes expressed during the three phases of chlamydia infection- early, mid and late (Albrecht et al., 2010; Humphrys et al., 2013; Hayward et al., 2019), the chlamydial gene expression changes in response to stress such as iron limitation and exposure to IFN- γ (Belland et al., 2003; Brinkworth et al., 2018) and how *C. trachomatis* alters host

chromatin accessibility by inducing epigenetic modifications (Hayward et al., 2020). These studies also led to the identification of new sRNAs in *C. trachomatis* (Albrecht et al., 2010), the role of the chlamydial plasmid in infection (Porcella et al., 2015) and insights into chlamydial-induced epithelial to mesenchymal cell transition (Zadora et al., 2019). It has also provided key insights into expression differences between EBs and RBs in *C. trachomatis* including increased T3SS proteins in EBs (Østergaard et al., 2016; Skipp et al., 2016). Recently, genomics was used to reconstruct the first *Chlamydia* genome-scale metabolic model (GSMM). GSMMs are mathematical models used to computationally describe the movement of metabolites within an organism. This model was then integrated with proteomic data to elucidate metabolic differences and altered flux between EBs and RBs (Yang et al., 2019).

Finally, multi-omics methods have also been essential for vaccine development and the discovery of new drug targets against *Chlamydia*. Immunoproteomics have been used to identify new *C. trachomatis* T-cell antigens for vaccine development and to identify immunogenic proteins in *C. abortus* outer membrane complexes (Karunakaran et al., 2008; Longbottom et al., 2019). The use of RNA-seq and metabolomics identified essential host metabolites and pathways for chlamydia infection, such as glutamine and guanine nucleotide biosynthesis. Targeting of these host-pathways was shown to inhibit chlamydia infection (Rother et al., 2018; Rajeev et al., 2020).

Currently, most multi-omics approaches in *Chlamydia* involve characterising gene expression at the bulk population level from thousands to millions of *Chlamydia* and/or host cells within a given sample (Hayward et al., 2021; Pokorzynski et al., 2022). However, this approach ignores the important variations between individual cells. Future single-cell and spatial approaches such as single-cell RNA-sequencing (scRNA-seq) in bacteria (Kuchina et al., 2021) and/or host cells (Hayward et al., 2019) for interrogating host-pathogen responses to chlamydia infections may reveal meaningful differences in cell-to-cell responses to and/or from *Chlamydia* as

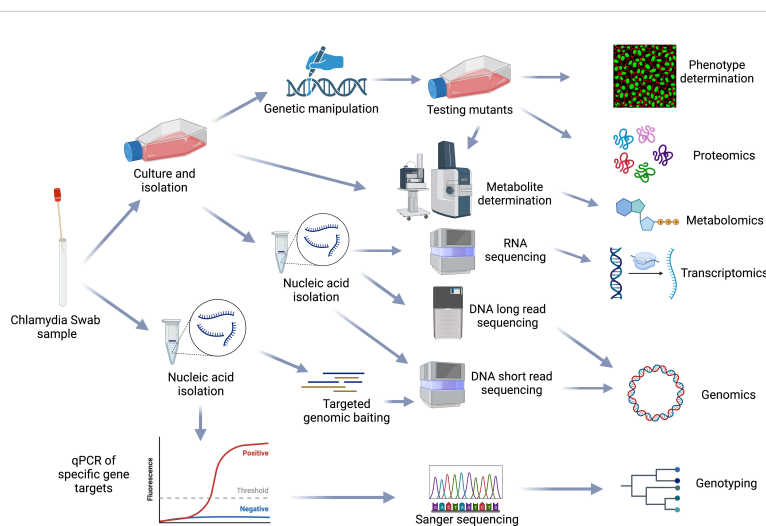


FIGURE 6

Chlamydial omics. Representation of the workflows from sample processing to multiple omics analysis methods used in the chlamydial field. Figure created using BioRender (available from <https://www.biorender.com/>).

well as identify the spatial distribution of important cell populations/lineages during infection. These existing and future omics techniques can be applied to *Chlamydia* infected tissues or advanced organotypic/organoid models, which better represent the host factors encountered (Nogueira et al., 2017; Versteeg et al., 2018b; McQueen et al., 2020; Dolat and Valdivia, 2021; Dolat et al., 2022; Edwards et al., 2022). This may provide insight into the pathogenesis *in vivo* and shed light on differences in genes or virulence factors required when infecting different host cells.

7 Future directions

The members of family *Chlamydiaceae* are an important species in the list of intracellular bacteria. They are highly adapted to their environments and depend on host cell machinery to survive and replicate. Their host range is one of the largest known, with predictions of a chlamydial species for every cell type on the planet (Collingro et al., 2020). With respect to *C. trachomatis*, it is the most common sexually transmitted infection and results in millions of cases of infertility and blindness across the globe. Improvements in WGS techniques have given rise to a deeper understanding of the chlamydial genome and the specific adaptations that have allowed specific species to thrive in different host environments. WGS has also resulted in new understandings of genetic recombination within species (but also interspecies) and has highlighted the issues around single gene sequencing to predict species evolution. In addition to enriching our understanding of *Chlamydia*, advances in genomics provides opportunities for rapid diagnosis of chlamydial infections and antimicrobial resistance detection using targeted single-gene nanopore sequencing or direct metagenome sequencing (Gu et al., 2020; Zhou et al., 2022). The advantages of using genomics for diagnosis is the ability to reveal the presence or absence of different virulence and antimicrobial-resistant genes, plasmids or important lineage/phylogenetic markers that may guide treatment and/or outbreak management.

The increasing number of chlamydial genomes sequenced and in publicly available databases poses challenges for future genome analysis as phylogenetic trees become more computationally intensive to construct. Thus, the future development of a *Chlamydia* hierarchical genome typing scheme based on core genome MLST or SNPs, such as those developed for *C. trachomatis* (Patiño et al., 2018; Versteeg et al., 2018a) will provide a standardised, universal and stable method for rapid

chlamydia genotyping and disease surveillance. These hierarchical schemes can rapidly assign sequence types to infer phylogenetic relationships, require less intensive computation, and are made up of multiple SNPs or MLST schemes with increasing resolution to provide both short and long-term epidemiological information for outbreak detection and long-term lineage tracking, respectively. Many aspects of the *Chlamydiae* are still hidden, but with more and more genome-scale data published every day, our understanding of this complex and widespread bacterial species continues to improve.

Author contributions

Conceptualisation: MJ, LL, GM. Formal analysis: LL, SP, VK, GM and MJ. Data analysis: LL, SP, VK and MJ. Writing, original draft preparation: LL and MJ. Writing, figure preparation: LL, VK and SP. Writing, review and editing: LL, SP, VK, GM and MJ. All authors have contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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