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Advances and Obstacles in the Genetic Dissection of Chlamydial Virulence

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Abstract

Obligate intracellular pathogens in the family *Chlamydiaceae* infect taxonomically diverse eukaryotes ranging from amoebae to mammals. However, many fundamental aspects of chlamydial cell biology and pathogenesis remain poorly understood. Genetic dissection of chlamydial biology has historically been hampered by a lack of genetic tools. Exploitation of the ability of chlamydia to recombine genomic material by lateral gene transfer (LGT) ushered in a new era in chlamydia research. With methods to map mutations in place, genetic screens were able to assign functions and phenotypes to specific chlamydial genes. Development of an approach for stable transformation of chlamydia also provided a mechanism for gene delivery and platforms for disrupting chromosomal genes. Here, we explore how these and other tools have been used to test hypotheses concerning the functions of known chlamydial virulence factors and discover the functions of completely uncharacterized genes. Refinement and extension of the existing genetic tools to additional *Chlamydia* spp. will substantially advance understanding of the biology and pathogenesis of this important group of pathogens.

1 Introduction

Bacteria in the phylum *Chlamydiae* share a characteristic biphasic intracellular developmental cycle and reside within a parasitophorous vacuole (termed the inclusion) (Moulder 1985). This obligate intracellular lifestyle and protected intracellular niche limit opportunities for chlamydia to exchange genetic material with other microorganisms and

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pose barriers to their genetic manipulation in the laboratory. There are few examples of genetic exchange between chlamydiae and other organisms, and *Chlamydia* spp. generally have few mobile genetic elements and other horizontally acquired genes compared to free-living bacteria (Stephens et al. 1998; Kalman et al. 1999; Read et al. 2000; Horn et al. 2004; Collingro et al. 2011).

Genome sequencing and comparative genomics have played especially important roles in the investigation of chlamydial biology because methods for targeted genetic manipulation only became available recently. The first sequenced *C. trachomatis* genomes were highly similar, suggesting that static genomes might be another obstacle that would hinder genetic manipulation. However, subsequent comparisons of hundreds of genomes and the recapitulation of chlamydial recombination and transformation in vitro confirmed that some *Chlamydia* spp. readily exchange DNA, but usually only with close relatives (DeMars et al. 2007; Wang et al. 2011; Hadfield et al. 2017). Another barrier to genetic manipulation of members of *Chlamydia* spp. is that more than half of their pan-genome is comprised of a core set of orthologous genes that are present in all *Chlamydiae* (Collingro et al. 2011). Many of these core genes are predicted to mediate essential and conserved features of chlamydial development, such as maintenance of the chlamydial inclusion and the transitions between the extracellular infectious elementary body (EB) and intracellular replicative reticulate body (RB) forms, and may not be dispensable even in vitro.

Chlamydiae are estimated to have diverged from other eubacteria 2 billion years ago, and vertebrate pathogens in the family *Chlamydia* species diverged from environmental Chlamydia-like organisms (CLOs) 700 million years ago (Weisburg et al. 1986; Everett et al. 1999; Horn et al. 2004). Partial or full genome sequences from eight CLO families (*Parachlamydieaceae*, *Waddliaceae*, *Simkaniaceae*, *Rhabdochlamydiaceae*, *Criblamydiaceae* *Chlavichlamydiaceae*, and *Parilichlamydiaceae*) have been described, and there is evidence that as many as 200 more exist (Lagkouvardos et al. 2014; Taylor-Brown et al. 2015). CLO genomes contain numerous rearrangements and share little synteny with one another, which may reflect the broad taxonomic range of the hosts that these organisms parasitize (Collingro et al. 2011). Some members of *Criblamydiaceae*, *Chlavichlamydiaceae*, and *Parilichlamydiaceae* spp. are important fish pathogens (Draghi et al. 2004; Karlsen et al. 2008; Stride et al. 2013). Other CLOs can enter and proliferate in cultured human cells (Greub et al. 2003; Collingro et al. 2005; Casson et al. 2006). *S. nevegensis*, *P. acanthaomoebae*, *W. condrophila* and *Rabdochlamydia* spp. have been associated with disease in humans and livestock (Taylor-Brown et al. 2015). *Parachlamydia* can also elicit respiratory disease in experimental mouse and cattle challenge models, which is consistent with the hypothesis that they are mammalian pathogens (Casson et al. 2008; Lohr et al. 2015). The predicted expanded metabolic and recombination capacities of some CLOs, compared to *Chlamydia* spp., suggest that they might be excellent candidates for the development of both genetic tools and axenic culture, although no attempts to genetically modify CLOs have been described (Collingro et al. 2011; Omsland et al. 2014).

Unlike CLO genomes, *Chlamydiaceae* genomes share substantial synteny with one another (Read et al. 2000; Read et al. 2003; Collingro et al. 2011; Nunes and Gomes 2014). Eleven *Chlamydia* spp. (*C. trachomatis, C. muridarum, C. pneumoniae, C. psittaci, C. pecorum, C.*

abortus, C. felis, C. suis, C. cavie, C. gallinacea, C. avium) are recognized, and reference genomes are available for representatives of all of these except *C. avium* (Stephens et al. 1998; Kalman et al. 1999; Read et al. 2000; Read et al. 2003; Thomson et al. 2005; Azuma et al. 2006; Mojica et al. 2011; Schofl et al. 2011; Donati et al. 2014; Sachse et al. 2014; Holzer et al. 2016). Extant members of the genus *Chlamydia* separate into two well-supported clades based on their 16S rRNA gene sequences (Stephens et al. 2009; Sachse and Laroucau 2015). One clade contains the mouse pathogen *C. muridarum*, the pig pathogen *C. suis*, and various human pathogens in the species *C. trachomatis*, and the other clade contains the remaining species.

Little genetic distance can separate *Chlamydia* spp. and strains that infect different hosts and cause distinct diseases. For example, 99% of *C. muridarum* genes have high similarity orthologues in *C. trachomatis* serovar D (Read et al. 2000). Genomes of *C. trachomatis* strains that cause blinding trachoma (serovars A-C, trachoma), mucosa-restricted sexually transmitted infections (serovars D-K, chlamydia), and the invasive sexually transmitted disease lymphogranuloma venereum (Lymphogranuloma venereum (LGV) serovars L1-L3) share more than 99% nucleotide sequence identity (Stephens et al. 1998; Carlson et al. 2005; Thomson et al. 2008). This phenotypic diversity in the context of little genetic distance led to the proposal that host and tissue tropism of *Chlamydia* spp. is determined by a small group of niche-specific genes (Read et al. 2003). These genes are attractive targets for manipulation because their variable presence in different isolates that can be cultivated in the same conditions suggests that they are dispensable (Kari et al. 2011). In this review, we describe recent advances in the development of tools for genetic manipulation of pathogenic *Chlamydia*, the uses and limitations of these tools, and how these might be improved for dissecting mechanisms of chlamydial cell biology and pathogenesis.

2 Chlamydial Genomes Are Malleable

Statements that chlamydiae are genetically “intractable” were fixtures in the literature until recently, but evidence that this was not so emerged earlier. For example, a study in 1983 showed that temperature-sensitive (TS) *C. abortus* mutants could be isolated from infected cell cultures treated with the alkylating mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) (Rodolakis 1983). One of these TS mutants was a potent attenuated vaccine strain that afforded strong protection against the virulent parent organism in sheep (Rodolakis and Souriau 1983, 1986). Sequencing of this mutant 30 years later confirmed that it contained multiple mutations consistent with the effects of NTG (Burall et al. 2009).

Antibiotic resistance studies provided additional evidence that chlamydial genomes are malleable. Treharne observed that *C. trachomatis* became rifampin resistant when passed in increasing concentrations of this antibiotic (Treharne et al. 1989). Another study identified a fluoroquinolone-resistant chlamydial *gyrA* allele by sequencing candidate resistance alleles in drug-resistant mutants isolated by serial passage (Dessus-Babus et al. 1998). Endogenous chlamydial resistance alleles for other antibiotics were identified using similar strategies (Dreses-Werringloer et al. 2003; McCoy et al. 2003; Misiurina et al. 2004; Binet and Maurelli 2007; Demars et al. 2007). The ease with which *Chlamydia* spp. developed resistance to antibiotics in vitro initially seemed inconsistent with the paucity of antibiotic-

resistant *C. trachomatis* clinical isolates. Subsequent studies showed that laboratory selected endogenous resistance alleles have fitness costs in the absence of antibiotic explained this paradox (Binet and Maurelli 2005, 2009; Binet et al. 2010). Chlamydial metabolism has also been investigated using mutants derived by passage in toxic precursors. For example, hydroxyurea, thioguanine, trimethoprim, and sulfisoxazole-resistant *C. trachomatis* mutants provided insights into the mechanisms of chlamydial nucleotide metabolism (Tipples and McClarty 1991; Qin and McClarty 1992; Wylie et al. 1996).

Other groups have harnessed spontaneous mutants to study chlamydial pathogenesis. To test if detrimental mutations accumulate in cell culture, a *C. trachomatis* reference isolate was passaged in a murine genital tract (GT) model, and the genomes of the resulting progeny were compared to the reference isolate (Sturdevant et al. 2010). Decreased virulence of the starting reference strain compared to progeny shed late from the murine GT mapped to a frameshift mutation in *ct135*. Analogous mutations accumulated in a *C. muridarum ct135* orthologue (*tc0412*), and cell culture propagation selects against intact *ct135* (Ramsey et al. 2009; Borges et al. 2013). Another study compared the virulence of a *C. muridarum* parent isolate and its cell culture-adapted progeny in mice. Two alleles, *tc0237* and *tc0668*, that mediate *C. muridarum* attachment and pathogenicity were identified (Chen et al. 2015a; Conrad et al. 2015).

Studies of rare, naturally plasmid-negative *Chlamydia* isolates and later of parent and chemically cured strain pairs demonstrated that some key chlamydial virulence determinants are dispensable in vitro (Peterson et al. 1990; Farencena et al. 1997; Stothard et al. 1998; O'Connell and Nicks 2006). Most natural *Chlamydia* isolates, except *C. pneumoniae*, contain a highly conserved ~7.5 kb plasmid that encodes 8 open reading frames (ORFs) and at least two small noncoding RNAs (sRNAs) (Palmer and Falkow 1986; Thomas et al. 1997; Pickett et al. 2005; Albrecht et al. 2010; Zhong 2017). The ubiquity of plasmids in *C. trachomatis* reference and clinical isolates suggested that these plasmids play key roles in chlamydial biology and pathogenesis. Plasmid-free *C. trachomatis* (pCT^{-}) isolates grow with near-normal kinetics in vitro, but do not accumulate glycogen and have reduced infectivity (Matsumoto et al. 1998; Russell et al. 2011). However, plasmid-free *C. muridarum* (pCM^{-}) is highly attenuated, unable to elicit pathology, and unable to compete with wild type *C. muridarum* in the murine GT (O'Connell et al. 2007; Russell et al. 2011). Studies of pCM^{-} and wild-type strain pairs have now shown that the plasmid plays a central role in *C. muridarum* GT virulence, dissemination from the murine upper GT to the gastrointestinal (GI) tract, and colonization of the murine GI tract (Carlson et al. 2008; Lei et al. 2014; Chen et al. 2015b; Shao et al. 2017; Zhong 2017). Collectively, these studies defined plasmid-associated phenotypes and laid the groundwork for contemporary studies of plasmid ORFs using transformation and plasmid genetic engineering (discussed below).

3 Moving Genes Between and into Chlamydial Genomes with Recombination and Lateral Gene Transfer

Major outer membrane protein (MOMP) gene (*ompA*) sequencing began to replace *C. trachomatis* serotyping in the early 1990s. The resulting sequences revealed that many

un-typeable clinical *C. trachomatis* isolates contained chimeric *ompA* alleles (Dean et al. 1992; Fitch et al. 1993; Lampe et al. 1993; Brunham et al. 1994; Hayes et al. 1994). Hayes understood the significance of these chimeras and proposed, "efforts to manipulate the chlamydial genome in vitro by recombination should be intensified" (Hayes et al. 1994). Sequencing of the polymorphic membrane protein (*pmp*) genes later revealed identical *pmpC* sequences associated with different *ompA* genotypes in *C. trachomatis* clinical isolates, showing that recombination between *Chlamydia* isolates could extend beyond *ompA* (Gomes et al. 2004).

Recapitulation of lateral gene transfer (LGT) in vitro confirmed that chlamydiae can exchange chromosomal DNA and led to the development LGT-based mutation mapping (discussed below) (Demars et al. 2007; DeMars and Weinfurter 2008). Demars and colleagues first demonstrated that doubly antibiotic-resistant isolates arose from co-infections with various combinations of antibiotic-resistant *C. trachomatis* L1 parents 1000- to 10,000-fold more frequently than did spontaneous double mutants from single infections with either parent alone (Demars et al. 2007). Recombination between endogenous ofloxacin resistant *C. trachomatis* serovar L1 and rifampicin resistant *C. trachomatis* serovar D parents showed that LGT could be used to exchange DNA between dissimilar parents and that large genomic regions (123–790 kb) could be exchanged (DeMars and Weinfurter 2008). Exchange of a *C. suis* tetracycline resistance island between *C. suis, C. muridarum*, and *C. trachomatis* in another study showed that LGT between different chlamydial species and heterologous regions of chlamydial genomes was possible (Suchland et al. 2009). LGT does not appear to require that the donor and recipient reside in the same vacuole for *C. trachomatis* because wild-type and non-fusogenic *incA*$^{-}$ parents produced LGT recombinants at similar frequencies (Jeffrey et al. 2013). If LGT can occur between other non-fusogenic *Chlamydia* spp. is unknown. So far, analyses of genomes from dozens of LGT recombinants has not yet identified any clear recombination hotspots associated with this process (Jeffrey et al. 2010, 2013; Brothwell et al. 2016; Muramatsu et al. 2016). The mechanisms of LGT also remain mysterious, but many *Chlamydia* spp. encode low identity orthologues of the *B. subtilis* competence genes *comEC* and *mec*, so it is plausible that DNA exchange between competent RBs mediates LGT (Stephens et al. 1998; Demars et al. 2007; DeMars and Weinfurter 2008).

Two LGT-based mutation-mapping approaches have been described, positive selection LGT (LGT) and counterselection LGT (csLGT). In LGT, host cells are co-infected with a parent that has a phenotype of interest and is resistant to an antibiotic (e.g., spectinomycin, rifampicin, trimethoprim) and a second parent that does not have the phenotype but is resistant to a second antibiotic (Nguyen and Valdivia 2012). Comparison of the genomes of the resulting doubly antibiotic-resistant progeny that have either maintained or lost the phenotype of interest can identify which allele is responsible for the phenotype (Nguyen and Valdivia 2013). Importantly, reciprocal LGT of these progenies with a third antibiotic-resistant parent can permit resistance allele recycling and serial mapping attempts (Nguyen and Valdivia 2012). In csLGT, healthy recombinant progeny is generated from parents that both have conditional or nonconditional growth defects (Brothwell et al. 2016; Muramatsu et al. 2016). For example, an LGT cross of two TS parent strains performed at the non-permissive temperature will yield temperature-resistant progeny (Brothwell et al. 2016).

Similar to LGT, comparing parent and recombinant progeny genomes can then identify the alleles linked to the phenotypes of the parents. The advantages of csLGT include avoidance of the fitness costs associated with some endogenous antibiotic resistance alleles and generation of progeny-parent pairs that differ only in the detrimental allele. Unlike LGT, enrichment of recombinants by csLGT is dependent upon the strength of the counter-selectable phenotypes of the parents.

Binet and Maurelli showed that homologous dsDNA sequences can also be directly introduced into *C. psittaci* using electroporation and recombined into the chlamydial chromosome (Binet and Maurelli 2009a, b). This clever study took advantage of the single copy rRNA operon in *C. psittaci* and distinct SNPs in the 16S rRNA gene that confer resistance to kasugamycin and spectinomycin. The engineered resistance alleles contained a silent mutation that destroyed a restriction site to monitor allelic exchange verses spontaneous antibiotic resistance and flanking homologous sequences that targeted the allele to the chromosome. This promising approach has not been replicated in other *Chlamydia* spp. or used to manipulate other genes.

4 Transformation of Chlamydia with Plasmid Shuttle Vectors

The potential of the chlamydial plasmid for genetic engineering was appreciated upon its discovery, but was not realized until almost 35 years later when *C. trachomatis* L2 was stably transformed with a vector constructed from its corresponding plasmid (pL2) (Wang et al. 2011). Electroporation of *C. trachomatis* EBs with a vector constructed from pCT and an *E. coli* plasmid (pBGS9) in which the pBGS9 chloramphenicol acetyltransferase (*cat*) gene was controlled by a *Chlamydia* promoter provided the first evidence that transformation was possible (Tam et al. 1994). Chloramphenicol resistant organisms and RBs containing the vector detected in the transformed cultures could survive up to four passages in chloramphenicol but were eventually lost. In hindsight, the shuttle vector may have been unstable because the *E. coli* plasmid elements disrupted a pCT maintenance gene (Song et al. 2013). Nonetheless, careful descriptions of positive and negative results in this study provided a roadmap for future attempts.

An approach developed by the Clarke group is the basis for most contemporary transformation protocols (Wang et al. 2011). Their initial vector consisted of a portion of pBR325 encoding β-lactamase (*bla*) and pBR *oriC* ligated into the pL2 plasmid (pBR325::L2). The shuttle vector and EBs were incubated in $CaCl_2$-Tris buffer, and McCoy cells infected with this mixture yielded stable penicillin-resistant transformants. Transformation of the glycogen-deficient pCT$^-$ *C. trachomatis* isolate 25667R with pBR325::L2 restored glycogen accumulation. This confirmed that plasmid loss–not background mutations—caused the glycogen deficiency of pCT$^-$ strains and was the first example of genetic complementation in *Chlamydia*. Finally, a similar vector containing a green fluorescent protein (GFP) gene under a *Neisseria* promoter was introduced into the backbone of the new Swedish variant plasmid (pSW2), which contains a deletion in CDS1 and a duplication in CDS3 (Seth-Smith et al. 2009). The transformants stably expressed GFP. This demonstrated that pCT-derived vectors can express foreign transgenes and that some regions of chlamydial plasmids are dispensable for their in vitro maintenance.

5 Applications of the Plasmid as a Shuttle Vector

The ability to deliver and express transgenes facilitated the development of many new approaches for investigating chlamydial biology. For example, Agaisse and Derré developed vectors that express GFP, mCherry, and cyan fluorescent protein genes under the control of chlamydial promoters (Agaisse and Derre 2013). This permitted real-time visualization of developing inclusions and their interactions with host cell organelles. Introduction of a tetracycline promoter and operator sequences upstream of a multiple cloning site in pASK permitted anhydrotetracycline-regulated expression of transgenes in *C. trachomatis* (Wickstrum et al. 2013). Further tuning of a tetracycline-regulated vector permitted comprehensive interrogation of the secretion and localization of known and putative inclusion membrane proteins (Incs) (Bauler and Hackstadt 2014; Weber et al. 2015). New chlamydial expression and shuttle vectors and are evolving rapidly, and we refer readers to a more extensive review of this topic (Bastidas and Valdivia 2016).

Despite remarkable progress and recent demonstration of transformation in additional *Chlamydia* spp., chlamydial transformation still has many limitations (Ding et al. 2013, Song et al. 2014). Inability to transform specific constructs, achieve expression of specific products from vectors and/or complement chromosomal defects is common (Nelson DE, unpublished) (Weber et al. 2015, 2017). We and others have observed subtle—and sometimes not so subtle—growth defects in transformants (Nelson and Zhong, unpublished; Wang et al. 2011). Possible explanations for these vector effects could include inappropriate timing of gene expression during the developmental cycle and/or improper levels of gene expression from multi-copy vectors (Wickstrum et al. 2013). Recently identified roles of the plasmid in the regulation of chlamydial virulence, chromosomal gene expression, and lytic exit suggest multiple reasons why manipulation of the chlamydial plasmid could elicit unanticipated phenotypes (discussed below). Considering these limitations, it is essential that vector controls be included in complementation experiments when feasible. Alternately, complementation results can sometimes be validated using phenotypic complementation and/or ectopic expression of the chlamydial gene in host cells (Clifton et al. 2004; Nelson et al. 2007).

6 Reverse Genetic Dissection of Chlamydia Plasmids

Development of transformation permitted reverse genetic dissection of the functions of chlamydial plasmids. The inability to transform non-LGV strains with pBR325::L2 or pGFP::SW2 suggested that plasmid or chromosomal determinants dictated plasmid tropism. *C. trachomatis* serovar *A* and *C. muridarum* could only be stably transformed with shuttle vectors constructed from their corresponding plasmids, indicating that plasmid sequences mediate stable transformation (Song et al. 2014). Forcing recombination between *C. muridarum* and LGV plasmids in another study revealed that CDS2 is a key determinant of plasmid tropism (Wang et al. 2014). Inactivation of specific plasmids ORFs revealed that Pgp1, 2 and 6 proteins and *pgp8*, but not Pgp8, are also essential for plasmid maintenance and identified additional *cis*-acting plasmid regulatory regions (Gong et al. 2013; Song et al. 2013; Liu et al. 2014; Zhong 2017). Pgp4 is dispensable for plasmid maintenance, but regulates multiple chromosomal genes including *glgA* (Carlson et al. 2008; Song et

al. 2013). Loss of *pgp4* is also sufficient to elicit glycogen deficiency (Song et al. 2013). Pgp4 also plays an essential and type III secretion-dependent role in the lytic exit of *C. trachomatis* (Yang et al. 2015). Collectively, these studies revealed that chlamydial plasmids play central roles in multiple aspects of chlamydial metabolism and pathogenesis.

7 Genetic Manipulation of Chlamydial Chromosomes

Four approaches for genetic analysis of chlamydial chromosomal gene functions have been reported (Table 1): Targeting Induced Local Lesions in Genomes (TILLING), TILLING by sequencing, TargeTron®, and Fluorescence-Reported Allelic Exchange Mutagenesis (FRAEM) (Kari et al. 2011; Johnson and Fisher 2013; Kokes et al. 2015; Mueller et al. 2016). Chemical mutagens are used to generate libraries of random mutants in the TILLING approaches, whereas TargeTron and FRAEM permit targeted mutagenesis (Fig. 1).

Two methods to identify naturally occurring or mutagen-induced mutations in a gene of interest have been described: TILLING and TILLING by sequencing. TILLING was adapted from plant genetics for application in *Chlamydia* in 2011 (McCallum et al. 2000; Kari et al. 2011). Mutants that contained less than one mutation per genome on average were generated by ethyl methanesulfonate (EMS) mutagenesis and were expanded in pools in HeLa cells. The tryptophan synthase operon (*trpBA*) was PCR amplified from genomic DNA from each of these pools. Cleavage of the PCR products with a mismatch-specific endonuclease (CEL-I) then identified pools that contained both wild-type and mutant *trpBA* amplicons. Multiple *trpBA* mutants were identified, including an isogenic *trpB* null (*trpB*) mutant. Unlike the library parent, growth of the isogenic *trpB*$^{-}$ mutant was not rescued from tryptophan starvation by indole, which is consistent with the predicted function of TrpB (Fehlner-Gardiner et al. 2002; Caldwell et al. 2003; Carlson et al. 2005). A similar approach was used to isolate a near-isogenic *C. trachomatis pmpD*$^{-}$ mutant (Kari et al. 2014). Although the *pmpD*$^{-}$ mutant behaved similarly to its parent in the murine GT, it was highly attenuated in a macaque trachoma model, again demonstrating that key chlamydial virulence factors can be dispensable in vitro.

Other TILLING studies have used higher mutagen doses and different *Chlamydia* species. TILLING of a highly mutagenized *C. trachomatis* serovar D library identified three *chxR*$^{-}$ mutants and five ChxR regulated genes (Yang et al. 2017). The *chxR*$^{-}$ mutants were attenuated in mice, suggesting that ChxR is a conserved chlamydial virulence factor. TILLING also identified a collection of *C. muridarum* plasticity zone (PZ) ORF nonsense mutants in another study (Rajaram et al. 2015). These PZ mutants were similarly virulent to their parent in a murine GT model, but some were profoundly attenuated in a mouse GI tract model (Nelson, unpublished).

Kokes et al. coupled chemical mutagenesis with genome sequencing to produce a mutant library for simultaneous forward (discussed below) and reverse genetic analyses using a strategy that we refer to as “TILLING by sequencing” (Kokes et al. 2015). A rifampicin resistant *C. trachomatis* L2 isolate was mutagenized with either EMS or *N*-ethylnitrosourea (ENU) and the resulting mutants were plaque-cloned. Sequencing of the mutants in pools identified over 8,000 different mutations, including nonsense mutations in 84 *C. trachomatis*

ORFs. Detection of nonsense mutations in genes that play roles in carbon metabolism, DNA damage repair, and virulence revealed that a variety of cellular processes are dispensable for chlamydial survival in vitro. Use of a rifampicin-resistant parent in this study also facilitated downstream LGT mapping efforts used to link phenotypes to specific mutations.

Major limitations of TILLING by sequencing compared to TILLING include the higher costs of library construction, genome sequencing, analysis, and the general requirement for more sophisticated technology. However, since TILLING by sequencing libraries are clonal, multiple isolates with distinct mutant alleles of a gene of interest can be identified by inspection of the corresponding genome sequences (Kokes et al. 2015). These libraries are also amenable to phenotypic screening. For example, Snavely et al. identified chlamydial protease-like activity factor (CPAF) null mutants by screening for isolates that failed to stain with the anti-CPAF antibody (Snavely et al. 2014). Theoretically, any *Chlamydia* spp. that can be propagated in vitro, mutagenized, and clonally isolated is amenable to TILLING. The most important limitation of TILLING is mapping phenotypes to genotypes. These approaches invariably suffer from the trade-off between the overall size of the mutant library and difficulty of mapping mutations of interest in the library isolates. Fewer heavily mutagenized isolates need to be screened to identify a mutant with a given phenotype of interest, but linking phenotypes to genotypes become more difficult as numbers of background mutations increase.

The Fisher group successfully adapted an insertional mutagenesis approach (TargeTron®, Sigma Inc.) to target type II introns to specific locations on the chlamydial chromosome (Johnson and Fisher 2013). A type II intron carrying a *bla* cassette and *incA* targeting sequences was transformed into *C. trachomatis* L2 using a suicide vector. Integrants (*incA::bla*) were selected with ampicillin and confirmed by PCR and Southern blot. Similar to naturally occurring *incA* mutant strains, inclusions of the *incA::bla* mutant were non-fusogenic (Suchland et al. 2000; Johnson and Fisher 2013). Subsequently, a spectinomycin resistant intron was developed to create an *incA::aadA rsbV1::bla* double mutant (Lowden et al. 2015). TargeTron has now been used to disrupt multiple genes including the chaperones *groEL2* and *groEL3, cpoS, ct813/inaC*, and various *inc* genes in *C. trachomatis* L2 (Illingworth et al. 2017; Sixt et al. 2017; Weber et al. 2017, Wesolowski et al. 2017). The applicability of the TargeTron approach is limited by the presence of optimal intron targeting sequences within the gene of interest (Hooppaw and Fisher 2016; Key and Fisher 2017). Although insertional mutagenesis approaches can disrupt specific genes, they can also induce polar effects and are less suitable than chemical mutagenesis for generation of partial loss of function alleles.

FRAEM insertional mutagenesis utilizes DNA homology to target genomic regions of interest and introduce a selection cassette and dual reporter genes to facilitate identification of recombinants (Mueller et al. 2016). The FRAEM vector is a conditional suicide vector that places the plasmid maintenance gene (*pgp6*) under control of tetracycline-regulated operator sequence. This permits expansion of shuttle vector transformants within the population to increase the possibility of recombination between homologous sequences on the chromosome and vector, while still maintaining the ability to cure the FRAEM vector. The selection cassette contains *bla* for penicillin selection of recombinants, and GFP

to monitor transformation and integration of the selection cassette into the chromosome. Finally, visual screening of single and double crossover (allelic exchange) events is facilitated by a mCherry gene located outside of the selection cassette and flanking chromosomal homology regions. *C. trachomatis* L2 *trpA* was deleted and replaced with the shuttle-vector selection cassette in a proof of principle experiment. The limitations of FRAEM include its relative complexity, the risk of deletion of unknown *cis*-acting chromosomal sequences, and polar effects (Mueller et al. 2017). Nonetheless, FRAEM is clearly a powerful tool for targeted manipulation of chlamydial genomes.

8 Forward Genetic Analysis of Chlamydial Genomes

Forward genetic screens have identified genes that mediate multiple aspects of chlamydial development, virulence, and immune evasion. These screens have relied on chemically mutagenized libraries because the TargeTron and FRAEM approaches are low-throughput (Table 1). In addition to the relative ease of mutagenesis, chemically mutagenized libraries contain a mixture of isolates with total and partial loss of function alleles, which permits interrogation of essential gene functions.

Nguyen and Valdivia compared the plaque morphologies of a collection of EMS mutagenized isolates derived from a rifampicin resistant *C. trachomatis* L2 parent (Nguyen and Valdivia 2012). The mutations causing altered plaque phenotypes were mapped using LGT and phenotypic linkage analysis. Three mutants that produced granular plaques had distinct missense mutations in *glgB*, a glycogen-branching enzyme. One of these missense mutations segregated with the granular plaque phenotypes when the mutant was crossed by LGT with spectinomycin and trimethoprim resistant *C. trachomatis* parents. Missense mutations in the type II secretion gene, *gspE*, were also linked to granular and small plaque phenotypes.

The same library of mutagenized *C. trachomatis* L2 isolates was screened for mutants that failed to promote F-actin assembly at the inclusion (Kokes et al. 2015). LGT with a spectinomycin parent linked this phenotype to a nonsense mutation in an uncharacterized *inc* gene, *inaC/ctl0184*. InaC expression from a plasmid complemented the *inaC* mutant, confirming the results of the LGT mapping. Another screen identified mutants that elicited increased lactose dehydrogenase (LDH) release from HeLa cells, which is an indicator of host cell death (Sixt et al. 2017). A nonsense mutation in the Inc gene *ctl0481* (renamed cpoS for chlamydia promoter of survival) was linked to the early cell death phenotype.

Two screens from the Nelson laboratory utilized an EMS-mutagenized library derived from a wild-type *C. trachomatis* L2 isolate transformed with pGFP::SW2 to avoid fitness costs associated with chromosomal antibiotic resistance alleles (Brothwell et al. 2016; Muramatsu et al. 2016). The use of pGFP::SW2 (L2-GFP) facilitated visual screens of mutant phenotypes and GFP expression served as a reporter of chlamydial metabolic activity. TS mutants that produced a reduced ratio of inclusions at 32°C versus 37°C (cold sensitive, CS) or 40°C versus. 37°C (heat sensitive, HS), compared to the library parent, were identified in the first screen (Brothwell et al. 2016). Thirty-one unique mutants with CS, TS or CS/TS phenotypes were identified and the mutations in 14 of these were mapped

by csLGT. TS alleles of genes that play roles in protein synthesis, DNA replication, fatty acid biosynthesis, and carbohydrate metabolism in other bacteria as well as conserved chlamydial genes of unknown function were identified. A second screen identified six mutants that were sensitive to interferon (IFN)-γ-mediated persistence (Sip) (Muramatsu et al. 2016). Three *sip* alleles were mapped using csLGT. One Sip mutant contained a missense mutation in *trpB*, which is consistent with the known role of *trpB* in *C. trachomatis* during indole-rescue from IFN-γ-mediated tryptophan starvation (Fehlner-Gardiner et al. 2002; Kari et al. 2011). The other two mutants had missense mutations in a predicted oxidative stress protein gene *cysJ* and a predicted small neutral amino acid transporter. These mutants suggested that the chlamydial response to IFN-γ involves more than tryptophan metabolism.

Successful genetic screens in chlamydiae hinge upon both library diversity and the ability to map specific mutations to the phenotype of interest. Multiple approaches can link phenotypes to genotypes (Fig. 1). As with TILLING, fewer isolates need to be screened to achieve saturation when a higher concentration of mutagen is employed, but parsing out the relevant mutation becomes more difficult downstream. Additionally, high mutation burdens increase the occurrence of both directly and synthetically lethal mutations. Despite the initial challenges of library construction, forward genetic screens are powerful tools for identifying the functions of chlamydial genes, which can then be validated using the reverse genetics approaches discussed above.

9 Overview and Future Perspectives

When the field of molecular microbial pathogenesis began to expand in the 1980s Stanley Falkow proposed a guide that was later deemed Koch's postulate of molecular pathogenesis (Falkow 1988). Paraphrasing these postulates: (1) the phenotype under investigation should be associated with a pathogen; (2) specific inactivation of the gene associated with the virulence phenotype should reduce pathogenicity; and (3) reversion or replacement of the mutant gene should restore pathogenicity. Falkow also stressed that genetic approaches for testing these postulates were not rigid, and that a lack of relevant in vivo models was a serious obstacle for identifying virulence factors of many genetically tractable pathogens. The latter points may be especially pertinent to the current state of chlamydial genetics where our perspective is that the excitement about the latest tools has sometimes led to incomplete consideration of their limitations as well as the best model systems in which the mutation associated phenotypes would be apparent.

There is no single or perfect approach for genetic manipulation of chlamydiae for all applications and choosing the best method for a given study requires consideration of the strengths and weaknesses of the existing tools. For example, forward genetic screens that employ TILLING approaches benefit from the ability of mutagens to introduce desirable point mutations that are less likely to cause *cis*- and *trans*-acting effects, but are limited by downstream mutation mapping. A report describing two *C. trachomatis* Himar transposon mutants suggests that transposon mutant libraries that could help circumvent this limitation may soon be available (Fischer et al. 2017). TargeTron and FRAEM can alter specific genes, but these approaches introduce disruptions and/or deletions that could disrupt *cis*- and *trans*-acting sequences. These limitations have been circumvented in other bacteria

using CRISPR/Cas9 gene editing since inactivation of specific targets is achieved without altering the larger genomic context, and adaptation of this approach for chlamydiae could be valuable (Luo et al. 2016; Zhang et al. 2017). Looking more long-term, adaptation of methods pioneered in *E. coli* for markerless allelic exchange and single copy gene expression from neutral loci seem like promising approaches for inactivating chlamydial genes while avoiding *cis*-and *trans*-acting effects and for expressing chlamydial proteins at physiological levels (Datsenko and Wanner 2000). Multiple tools in the chlamydial genetic toolbox remain limited by transformation efficiency, and further optimization of transformation techniques including electroporation and dendrimer-based approaches could be fruitful (Tam et al. 1994; Binet and Maurelli 2009a, b; Mishra et al. 2012; Gerard et al. 2013; Kannan et al. 2013). Probably the most significant impediment in the genetic dissection of chlamydial pathogenesis remains that these organisms need to be propagated in host cells. Genetic manipulation of other "obligate" intracellular organisms has clearly benefited from the development of anexic growth media, and promising findings that might be on the horizon for chlamydiae too (Omsland et al. 2009, 2012; Haider et al. 2010).

Finally, the overwhelming focus on the development of tools for a single model strain, *C. trachomatis* L2 434/Bu, has limited our ability to use genetic approaches to dissect crucial aspects of chlamydial pathogenesis. Despite the lack of a good in vivo model for *C. trachomatis* L2, the advent of genetic approaches has greatly enhanced our understanding of chlamydial biology. The challenge is now to extend these approaches to other *Chlamydia* spp.—especially those for which relevant animal models exist. LGV biology differs from the more common and medically important trachoma biovar strains in many ways. Trachoma biovar strains primarily infect columnar epithelial cells that protect the conjunctiva, urethra, GI, lung, cervix, and upper female reproductive tract. In contrast, LGV biovar strains infect via epithelial micro-abrasions where they enter into and proliferate in lymphatic cells. Innate immunity also rapidly clears *C. trachomatis* strains in mice (Morrison and Caldwell 2002). In contrast, *C. muridarum* can cause long-lasting infections in mice that are cleared by adaptive immunity (Barron et al. 1981; Morrison and Caldwell 2002). Existing murine *C. muridarum* pulmonary and GI infection models also mimic aspects of these infections with *C. trachomatis* STI strains in humans and the latter model was crucial to the discovery of the virulence role of chlamydial plasmids (Morrison and Caldwell 2002; Ramsey et al. 2009; Yeruva et al. 2013; Rank and Yeruva 2014). Virulence factors initially identified in the mouse GT were more important for *C. muridarum* GI colonization, showing that the tissue choice can also profoundly impact relevance of specific chlamydial virulence factors in the same animals (Shao et al. 2017). Beyond *C. muridarum, C. caviae* Guinea pig models can investigate urethral infections and sexual transmission (Rank et al. 2003; Wang et al. 2010). Primate models of trachoma and cervicitis exist that use the same isolates that cause these diseases in humans (Wolner-Hanssen et al. 1991; Kari et al. 2008). Understanding the animal and tissue-specific context in which chlamydial virulence genes are required during infection will shed light on both chlamydial biology and the limits of a given infection model. Looking forward, adapting the existing genetic tools to additional species and strains could enhance the utility of genetic approaches for dissecting mechanisms of chlamydial pathogenesis.

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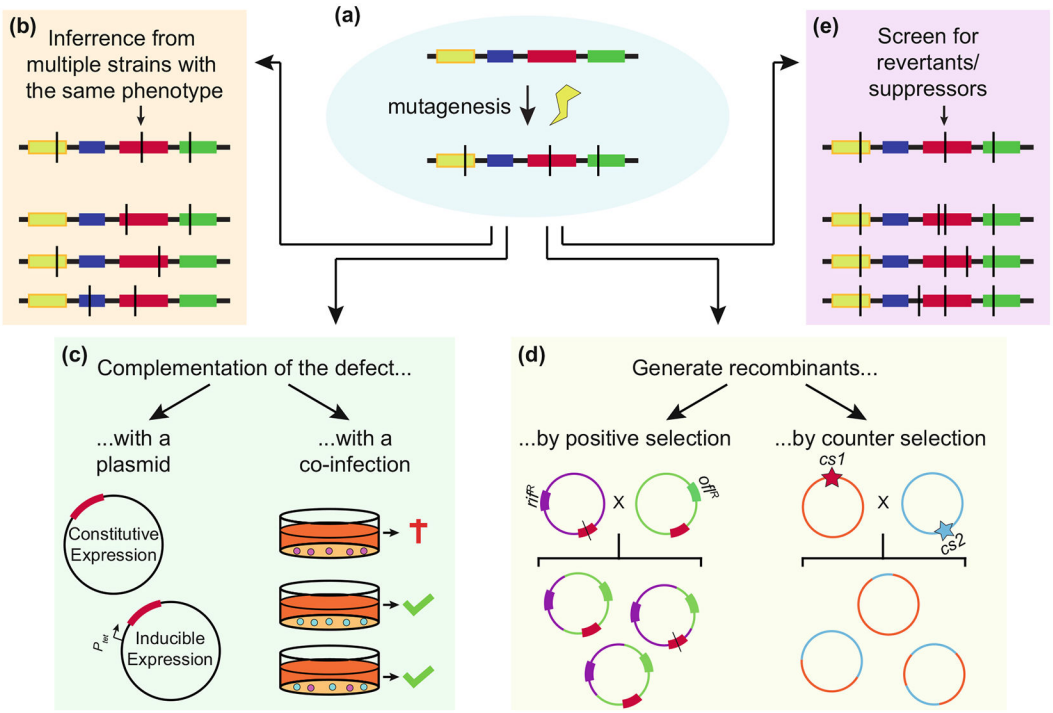

Fig. 1.

Mutation mapping in chlamydia. **a** Chemical mutagenesis strategies can result in multiple mutations (denoted by black lines). Multiple approaches can resolve which mutation(s) (shown in red) are linked to a phenotype. **b** Additional mutants with the same phenotype that have different mutant alleles of the same gene can be isolated. **c** A plasmid that expresses the wild-type allele of the gene can be used to complement the phenotype. Alternately, co-infection with wild-type chlamydia and/or ectopic expression of the gene in host cells can be used to complement phenotypes associated with secreted effectors, inclusion membrane transporters, or enzymes that detoxify the inclusion. **d** LGT can generate recombinants using endogenous antibiotic resistance or counter-selectable alleles. **e** Continued passage of a mutant in detrimental screening conditions can also generate revertants and intragenic suppressors that can identify genes linked to detrimental phenotypes (Nelson, unpublished). The original mutation is indicated by the arrow.

Table 1

Genetic analysis of chlamydial chromosomal gene functions

Method	Strain	Gene(s)	Advantage	Caveat	References
Serial passage with increasing selection Non-mutagenized organisms were serially passaged in increasing concentrations of a selection agent	*C. trachomatis* L2	Various	Mutants with one or a few mutations per genome	Mutants may not arise; isolation of mutants highly dependent on selection conditions	Tipples and McClarty (1991)
In vitro serial passage Organisms were passaged 10Xin between embryonating hen's eggs and 17 X in HeLa 229 cells. Virulence selection in mice	*C. trachomatis* D/UW-3/CX	*ct135*	Isolate phenotypic mutants with one or a few mutations per genome	Mutants may not arise; isolation of mutants highly dependent on selection conditions	Sturdevant et al. (2010)
In vitro serial passage and selection *C. muridarum* was passaged for 28 generation in vitro using unassisted and assisted attachment infection models	*C. muridarum*	*tc0237, tc0412, tc0668*	Isolate phenotypic mutants with one or a few mutations per genome	Mutants may not arise; isolation of mutants highly dependent on selection conditions	Chen et al. (2015a, b)
Chemical mutagenesis NTG mutagenesis and screening for temperature-sensitive mutants	*C. psittaci* AB7	TS genes	Identify multiple phenotypically related mutants; mutate essential genes	Multiple mutations requiring mapping to determine the causative TS allele(s)	Rodolakis (1983)
Chemical mutagenesis & antibiotic LGT EMS mutagenesis and screening for diverse plaque morphologies Antibiotic recombination to map causative alleles	*C. trachomatis* L2	Glycogen genes	Identify multiple phenotypically related mutants; map causative alleles	Labor intensive library creation, screening, and mapping; compare multiple recombinants to identify causative allele; antibiotic recombinants may have growth defects	Nguyen and Valdivia (2012)
Chemical mutagenesis & WGS EMS or ENU mutagenesis and screening for mutants that failed to promote F-actin assembly	*C. trachomatis* L2	*inaC*	Identify multiple phenotypically related mutants; rapidly map causative alleles	WGS of a large mutant library may be cost prohibitive; mutants may not share the same mutation related to phenotype of interest	Kokes et al. (2015)
Chemical mutagenesis & csLGT EMS mutagenesis of a GFP-expressing parent and screening for mutants. Counter selection recombination to map causative alleles	*C. trachomatis* L2	TS & IFN-γ persistance genes	Identify multiple phenotypically related mutants; map multiple causative alleles at the same time and isolate isogenic recombinants	Labor intensive library creation, screening, and mapping; selection of recombinants limited by strength of phenotype	Brothwell et al. (2016), Muramatsu et al. (2016)
Allelic exchange Homologous recombination of linear and circular suicide plasmids	*C. psittaci* 6BC	*16S rRNA*	Modify genes with specific mutations	Electroporation equipment; suicide vector may be maintained episomally after a number of passages	Binet and Maurelli (2009a, b)
TILLING for null mutant EMS mutagenesis and screening for null mutants within a subset of mutants found to have mismatched sequence in a region of interest	*C. trachomatis* D/UW-3/CX	*trpB*	Knock out any nonessential gene; mutate essential genes	Labor-intensive library creation, screening, and mapping; potential for multiple chromosomal mutations that need to be mapped and complemented	Kari et al. (2011)
TargeTron—gene disruption Homologous Recombination of a group II Intron on a suicide plasmid	*C. trachomatis* L2	*incA*	Disrupt any nonessential gene	Proprietary algorithm to determine appropriate targeting sites; potential lack of insertion sites in 5′ sequence of the gene of interest; transformation efficiency	Johnson and Fisher (2013)

Method	Strain	Gene(s)	Advantage	Caveat	References
FRAEM—gene knockout Homologous recombination of an insertional cassette using a conditional suicide plasmid	*C. trachomatis* L2	*trpA; ctl063-065*	Knock out any nonessential gene(s); transient suicide plasmid with fluorescent reporter	Polar effects; transformation efficiency	Mueller et al. (2016)

Abbreviations used: *NTG N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; *EMS* ethyl methanesulfonate; *ENU N*-ethylnitrosourea; *WGS* whole genome sequencing; *TILLING* targeting induced local lesions in genomes; *FRAEM* fluorescence reported allelic exchange mutagenesis; *LGT* lateral gene transfer; *cs* counterselction; *TS* temperature sensitive