Molecular Genetic Analysis of *Chlamydia* Species

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Abstract

Species of *Chlamydia* are the etiologic agent of endemic blinding trachoma, the leading cause of bacterial sexually transmitted diseases, significant respiratory pathogens, and a zoonotic threat. Their dependence on an intracellular growth niche and their peculiar developmental cycle are major challenges to elucidating their biology and virulence traits. The last decade has seen tremendous advances in our ability to perform a molecular genetic analysis of *Chlamydia* species. Major achievements include the generation of large collections of mutant strains, now available for forward- and reverse-genetic applications, and the introduction of a system for plasmid-based transformation enabling complementation of mutations; expression of foreign, modified, or reporter genes; and even targeted gene disruptions. This review summarizes the current status of the molecular genetic toolbox for *Chlamydia* species and highlights new insights into their biology and new challenges in the nascent field of *Chlamydia* genetics.

Contents

INTRODUCTION

Chlamydiae are a remarkably diverse phylum of obligate intracellular bacteria that encompass symbionts of unicellular eukaryotes and parasites of invertebrates along with significant pathogens of humans and livestock (53). The species with the greatest impact on human health is *Chlamydia trachomatis*, which causes primarily ocular infections (serotypes A–C) and sexually transmitted diseases (serotypes D–K, L1–L3). Ocular infections can lead to trachoma, a major cause of preventable blindness in developing areas of the world (130, 145). Urogenital infections with *C. trachomatis* are a global health burden and may result in lymphogranuloma venereum, adverse pregnancy outcomes, and/or infertility (88, 144). The second-most significant chlamydial pathogen, *Chlamydia pneumoniae*, is mainly considered an agent of respiratory disease; yet, it may also be associated with atherosclerosis and neurological diseases (18). Other species of the genus *Chlamydia*, such as *C. psittaci*, are known to infect primarily animals but can cause severe zoonotic infections in humans (75). The ecologic and pathogenic roles of chlamydial species outside the genus *Chlamydia* are not well understood.

Development of new means to treat and prevent *Chlamydia* diseases requires a better understanding of the biology of the pathogen and its strategies to invade and replicate within human host cells, to evade host immune responses, and to induce pathology. The lack of tools for molecular genetic manipulation of chlamydiae has been a great impediment for investigators who had to rely on surrogate systems and inferences based on similarities between chlamydial proteins and virulence factors previously characterized in other pathogenic bacteria. This *Chlamydia* research landscape has radically changed in the last couple of years, with a greatly expanded molecular genetic toolbox becoming available for research on *C. trachomatis*. This paves the way for exciting discoveries and critical reassessment of established concepts. In this review we summarize major factors that contribute(d) to the genetic recalcitrance of *Chlamydia* spp., currently established genetic tools and experimental approaches, and recent applications and their impact on our understanding of chlamydial biology and virulence. We conclude with a brief survey of persisting challenges and future directions.

LIFE CYCLE OF A GENETICALLY RECALCITRANT PATHOGEN

The sequence of the first *C. trachomatis* genome (strain D/UW-3/CX) was published in 1998 (123). Numerous additional strains and related species were sequenced thereafter, which provided

new insights into the coding potential of these pathogens and interstrain and interspecies genetic variability (5, 20, 37, 65, 85, 109, 110, 132, 133, 135). Genomic analysis revealed a remarkable reduction of metabolic capacities in *Chlamydia* spp. and the presence of proteins with similarities to virulence factors in other bacteria, yet it also underscored the large number of *Chlamydia*-specific genes coding for proteins of completely unknown function (5, 20, 37, 65, 85, 109, 110, 132, 133, 135). Unfortunately, the functional analysis of these proteins has been limited by the lack of tools for molecular genetic manipulation. A major barrier to the development and application of genetic tools is the peculiar life cycle of the pathogen, in particular its dependence on a host cell for replication and development. For instance, although *C. trachomatis* can maintain some degree of metabolic activity under host cell–free conditions (99), a medium allowing axenic replication of chlamydiae has yet to be developed. An ability to cultivate bacteria in liquid broth or on agar plates can greatly facilitate application of molecular genetic techniques, as is well exemplified by the case of *Coxiella burnetii*, a bacterium that until recently had been considered an obligate intracellular pathogen (97, 98).

Chlamydia spp. alternate between two morphologically, biochemically, and functionally distinct developmental stages: the infectious elementary body (EB) and the replicative reticulate body (RB) (1). EBs invade host cells and differentiate into RBs that subsequently replicate within the confines of a membrane-bound compartment (termed inclusion). Eventually, RBs differentiate back into EBs that are released by host cell lysis or by extrusion of intact inclusions to infect neighboring cells (1, 57) (**Figure 1**). During the course of infection, *Chlamydia* spp. actively remodel their growth niche by means of effector proteins that are secreted into the host cell cytosol or are inserted into the inclusion membrane (inclusion membrane proteins) (13). Genetic manipulation
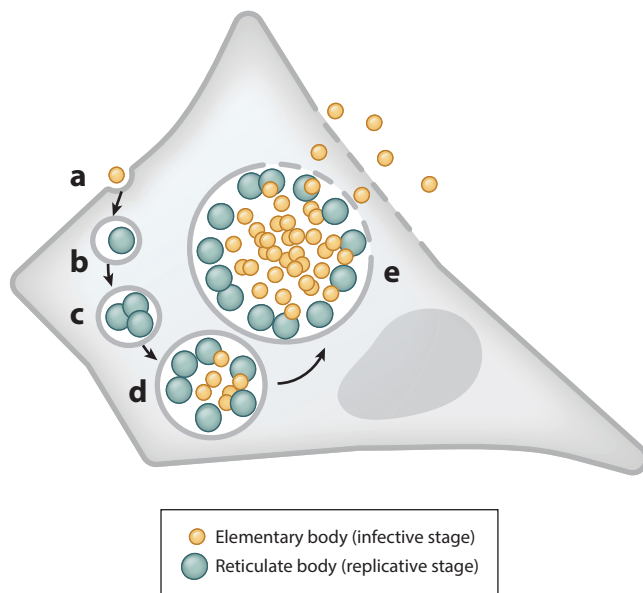

Figure 1

The developmental cycle of *Chlamydia* spp. Chlamydiae alternate between two distinct developmental stages, the infectious elementary body (EB) and the replicative reticulate body (RB). After invasion of a host cell (*a*), the EB resides within a membrane-enclosed compartment (termed "inclusion") and differentiates to form a noninfectious RB (*b*) that will replicate (*c*) and eventually form new infectious EBs (*d*) that are released by host cell lysis (*e*) or extrusion (not shown). The infection cycle can be of variable length depending on the chlamydial species, yet is usually completed within about 40–48 hours in the case of *C. trachomatis* L2.

of *Chlamydia* spp. may target either the EB or the RB stage. RBs are very labile in cell-free environments and moreover considered to be noninfectious (129). However, inside the host cell, RBs are not easily accessible to exogenous DNA, which would need to pass four membranes (host plasma membrane, inclusion membrane, and bacterial outer and inner membranes) to reach the bacterial cytosol. While it may seem more feasible to target the EB stage for DNA transformation, this environmentally stable form has a very rigid cross-linked cell wall (50), a highly condensed nucleoid (79), and reduced metabolic activity (100). Thus, EBs may be naturally less likely to take up and integrate foreign DNA. Adding to the challenge, many chlamydial genes are differentially expressed throughout the different phases of infection (3, 12, 56, 90), most likely through the differential engagement of transcription factors, sigma factors, and changes in DNA topology (26, 71, 91, 112, 113, 149). While these regulatory systems may be exploited in the future for more precise manipulations, it is important to consider that expression of foreign or modified genes in *Chlamydia* may fail to produce the anticipated phenotype or may even disrupt chlamydial growth if it is not in tune with chlamydial development.

NATURAL COMPETENCE

Despite these technical challenges, the discovery that *C. trachomatis* has a natural capacity for DNA exchange was encouraging. First evidence for recombination among chlamydial strains came from studies describing mosaic alleles of the *ompA* gene (encoding the major antigen used for *C. trachomatis* serotyping) (17, 43, 51, 72, 81, 82, 148). Later, analysis of additional genetic loci, multilocus sequencing typing, and eventually whole-genome sequencing approaches have confirmed the occurrence of recombination events within and between serotypes of *C. trachomatis* at positions throughout the chlamydial chromosome (8, 32, 45–47, 49, 59, 62–64, 102, 120). In 2007 DeMars et al. (34) established a system to study lateral gene transfer among *C. trachomatis* in vitro. After coinfection of cells with two strains carrying different antibiotic resistances, double-drug-resistant strains could be isolated about 10^4 times more frequently than generated by spontaneous mutations (34). Subsequent studies demonstrated in vitro recombination between different serotypes of *C. trachomatis* (33, 58) and transfer of a tetracycline resistance gene from the swine pathogen *C. suis* to *C. trachomatis* (127).

The mechanism of this natural DNA exchange is unknown. While many *Chlamydia* strains naturally harbor a 7.5-kb plasmid, an essential role of the *Chlamydia* plasmid in DNA transfer is unlikely, given its nonintegrative and nonconjugative nature (111). Moreover, whereas bacteriophages have been discovered in some *Chlamydia* species (118), an essential role of phages for recombination can also be ruled out, because the great majority of *Chlamydia* isolates are devoid of phages. However, the discovery that *Chlamydia* spp. encode a homolog of the DNA-uptake protein ComEC raises the possibility that chlamydiae are naturally competent (7). Thus, DNA released from lysing RBs may be taken up by other RBs within the same host cell and may be integrated into their genome via homologous recombination.

THE GENETIC TOOLBOX FOR *CHLAMYDIA* SPECIES

Efforts to develop a system for genetic analysis in *Chlamydia* spp. have focused primarily on *C. trachomatis* and its relative *C. muridarum*, a natural mouse pathogen (31). The current molecular genetic toolbox includes methods that follow two distinct strategies: (*a*) approaches that begin with random mutagenesis followed by screening for mutants that either display a specific phenotype of interest (forward genetics) or carry a specific genetic alteration (reverse genetics), and (*b*) approaches that aim for targeted modification of the chlamydial chromosome or plasmid (reverse genetics) (**Figure 2**).

Mutagenesis-Based Approaches

Generation of *Chlamydia* mutant libraries. Random genetic alterations can be introduced into microbial genomes by various methods, such as insertional transpositions, UV irradiation, and exposure to DNA-damaging chemicals. Whereas transposon mutagenesis has been successfully applied to other obligate intracellular bacteria (10, 41, 105), so far only chemical mutagenesis has been applied to *Chlamydia* spp. to generate mutant libraries (67, 70, 89, 107). Mutagenesis was performed by exposure of infected cells to either of the DNA alkylating compounds ethyl methanesulfonate (EMS) or *N*-ethyl-*N*-nitrosourea (ENU), followed by plaque isolation of clonal strains. Variable concentrations of mutagen have been applied to achieve either a maximum of one mutation per genome—optimized for reverse genetic approaches that maximize the chance of isolating mutants that are isogenic to the parental strain at all other loci (67)—or multiple mutations per genome—optimized for forward genetic approaches to reduce the number of mutants that need to be screened (70, 89). The genetic lesions caused by EMS (or ENU) are mostly point mutations (116), which are unlikely to have significant polar effects on the expression of surrounding genes. Nevertheless, they provide a broad spectrum of modifications, including disruption of genes, but also generation of hypo- or hypermorphic alleles. The latter is of particularly great value for analyzing essential genes.

Finding mutants with specific phenotypes. Mutant collections are an excellent resource for forward genetic screens. Screening assays may be as simple as searching for mutants with altered plaque morphologies (89) or as complex as a recently described high content imaging-based screen for mutants that fail to assemble F-actin at the inclusion (70). Whole-genome sequencing of recovered mutants may aid in the identification of the causative mutation, though additional confirmation by complementation and/or by genetic linkage analysis may be required. The latter approach is based on monitoring the linkage between phenotypes and point mutations among recombinant strains generated by coinfecting host cells with the mutant strain and a strain that is wild type at all positions that are altered in the mutant (89). Linkage analysis may be particularly useful in cases in which the phenotype is due to altered gene expression or protein function, rather than a complete loss of gene function. However, the analysis relies on an ability to select for recombinants. Nguyen & Valdivia (89) and later Kokes et al. (70) solved this constraint by performing mutagenesis in a rifampin-resistant strain, enabling subsequent crosses with spectinomycin- or trimethoprim-resistant variants. In this manner, granular plaque morphologies could be linked to mutations in *glgB* (1,4-α-glucan branching enzyme) and *gspE* (ATPase required for type II secretion) that resulted in intrainclusion accumulation of insoluble glycogen (89). Similarly, loss of the ability to recruit actin could be linked to a nonsense mutation in *inaC* (inclusion membrane protein for actin assembly) (70).

Finding mutants with specific genetic alterations. Mutant libraries also enable a targeted characterization of specific genes, if mutants can be recovered. This strategy was used to study several *C. trachomatis* virulence factors, such as TrpB (tryptophan synthase β chain) (67), PmpD (polymorphic membrane protein D) (68), CPAF (*Chlamydia* proteasome-like activity factor) (16, 119, 147), TepP (translocated early phosphoprotein) (25), and HtrA (78), as well as the *C. muridarum* cytotoxins (107). Two different approaches have been applied to find specific mutants in a large collection of strains: (*a*) CEL1 nuclease-based detection of mutations (67, 95, 107), and (*b*) whole-genome sequencing (70). In the first approach the gene for which one wants to identify mutant alleles is amplified by PCR from pools of mutant strains. PCR products are denatured, slowly reannealed to enable heteroduplex formation, and digested with the nuclease CEL1 that

only cleaves at sites of mismatches. The approach based on whole-genome sequencing, while more costly, gives a comprehensive overview of every single mutation that is present in the library. It thus eliminates the need for repeated gene-specific screening of the entire collection. Moreover, with the ability to recover mutants with lesions in certain genes, Kokes et al. (70) gained additional
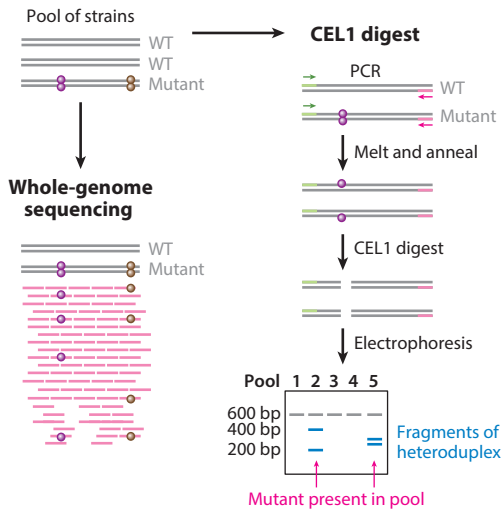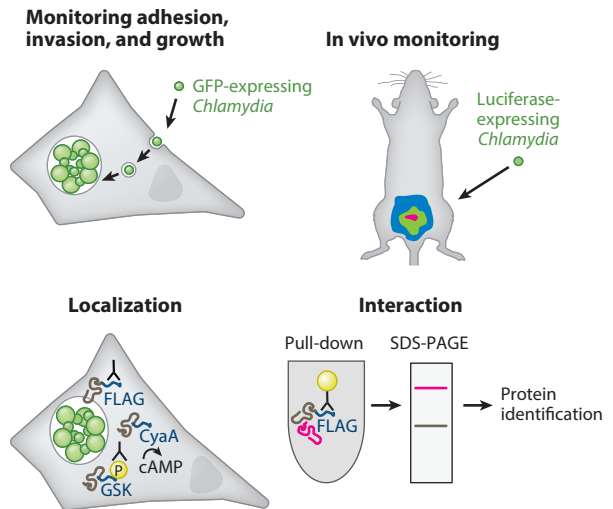

a Chemical mutagenesis
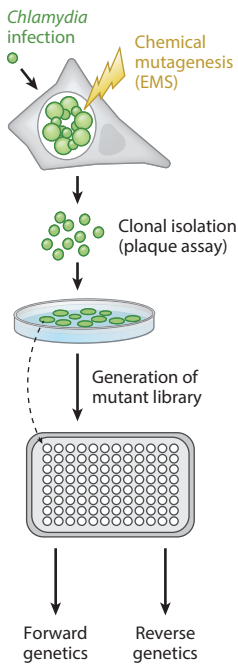
b Linking phenotype to genotype
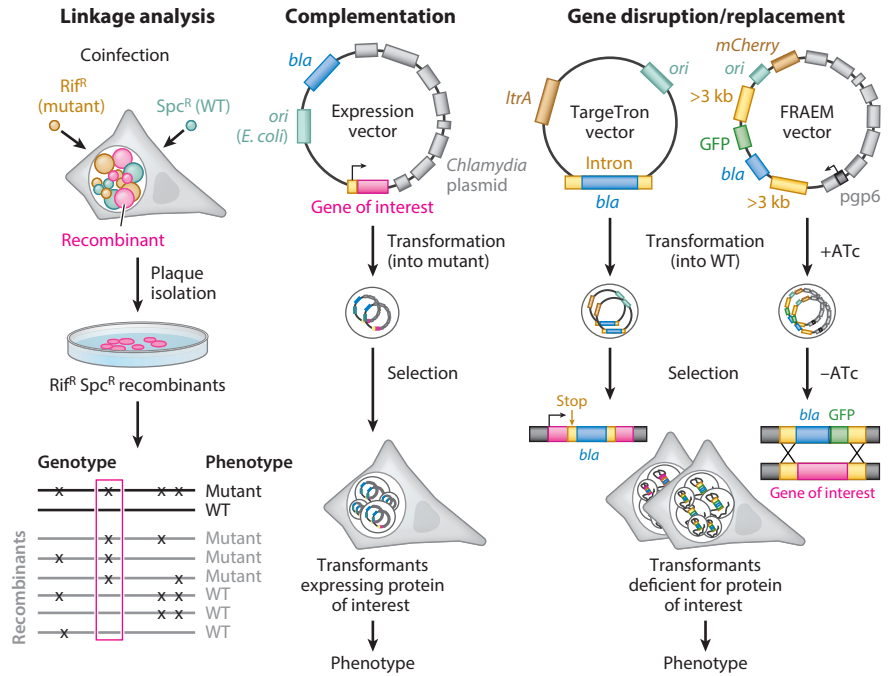
c Finding strains with specific mutations

d Application of reporters and tags

insights into the biology of *Chlamydia* spp. by identifying certain biological processes (e.g., glycogen metabolism and DNA repair mechanisms) as being expendable for bacterial viability in cell culture. Although screening approaches can initially be carried out with pools of mutants, they ultimately rely on sequencing of individual strains to determine the complete set of mutations present.

Targeted Genetic Manipulation

Generation of shuttle vectors. Targeted genetic modification of *Chlamydia* spp. is currently almost exclusively based on the introduction of plasmids. Shuttle vectors were constructed to include the natural *Chlamydia* plasmid fused to the backbone of an *Escherichia coli* cloning vector to introduce elements required for plasmid replication in *E. coli* and selection markers conferring antibiotic resistance (2, 36, 66, 73, 121, 122, 128, 140, 143, 146). A single study reported the use of a *Saccharomyces cerevisiae–Chlamydia* shuttle vector constructed to avoid introduction of an antibiotic resistance gene into *Chlamydia* (44). An overview of currently available shuttle vectors for *Chlamydia* spp. is given in **Supplemental Table 1** (follow the **Supplemental Materials link** from the Annual Reviews home page at **http://www.annualreviews.org**).

▶ Supplemental Material

Transformation methods. In 1994, Tam et al. (128) reported the first successful, though transient, transformation of *Chlamydia* spp. In this study, they introduced plasmid DNA into *C. trachomatis* EBs by electroporation. About 15 years later, Binet & Maurelli (14) used a similar approach to transform *C. psittaci* EBs—the first example of successful allelic exchange in the genus *Chlamydia*. Although electroporation conditions were similar in both studies, the authors reported variable effects of electroporation on EB viability that may be explained by differences in the buffers used (14). Interestingly, despite these encouraging early successes, electroporation has not emerged as the method of choice for current transformation protocols in *Chlamydia* spp.

Wang et al. (140) described a $CaCl_2$-based method for *Chlamydia* EB transformation in 2011. This was the first report of a stable introduction of a shuttle vector into chlamydiae, which together with its ease of use has contributed to its widespread adoption. In the original protocol, EBs were incubated with plasmid DNA in $CaCl_2$ buffer for 30 min at room temperature. Subsequently McCoy cells resuspended in $CaCl_2$ buffer were added, followed by an additional incubation for

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Figure 2

The genetic toolbox for *Chlamydia* spp. (*a*) Construction of mutant libraries for forward- and reverse-genetic applications: *Chlamydia*-infected cells are exposed to a mutagenic chemical, followed by clonal isolation of the progeny. (*b*) Approaches to link phenotypes to a specific genetic alteration. (*Left*) Linkage analysis exploits the natural competence of *Chlamydia* spp. [i.e., the formation of recombinant strains during coinfection with mutant and wild-type bacteria]. Clonal recombinants are analyzed to identify mutations that are coinherited with a phenotype of interest. (*Middle*) Shuttle vectors permit complementation of mutants in *trans* with the wild-type gene, and (*right*) the use of group II intron– or homology-based gene disruption cassettes enables insertional inactivation or allelic replacement of genes in wild-type bacteria. (*c*) Approaches to screening mutant libraries for strains with mutations in specific genes: (*Left*) Whole-genome sequencing of pools of mutants gives a comprehensive overview of all mutations present in a collection of mutants. (*Right*) Monitoring of DNA heteroduplexes after the gene of interest is PCR-amplified from pools of strains. PCR products are denatured, slowly reannealed, and digested with the nuclease CEL1, which cleaves at sites of mismatches (indicative of the presence of a mutant in the pool). The smaller fragments generated by CEL1 digestion are detectable by agarose gel electrophoresis. (*d*) Expression of reporter genes in *Chlamydia* spp. facilitates analysis of infection and functional characterization of virulence factors. (*Top left*) Expression of fluorescent proteins facilitates assays for adhesion, entry, and analysis of infection in live cells. (*Top right*) Expression of luciferase enables in vivo monitoring of infection. (*Bottom left*) Expression of tagged proteins facilitates the analysis of protein localization during infection and (*bottom right*) enables analysis of protein interactions, such as via immunoprecipitation of tagged proteins from lysates of infected cells. Abbreviations: CyaA, adenylate cyclase; GSK, glycogen synthase kinase.

20 min at room temperature, seeding of cells, and, optionally, centrifugation to enhance infection (140, 141). The protocol was adapted by other groups with minor variations (e.g., 2, 9, 25, 48, 121, 146). Incubation of host cells in CaCl$_2$ buffer was later shown to be nonessential (121). Transformation efficiencies have not been calculated for either CaCl$_2$ transformation or electroporation and the effect of the incubation in CaCl$_2$ buffer on EB viability has not been assessed, making direct comparison between these transformation methods difficult.

While electroporation and CaCl$_2$ transformation facilitate DNA uptake into EBs, RBs replicating within their host cell have been targeted for transformation with dendrimer-based approaches. Polyamidoamine dendrimers are noncytotoxic, highly branched polymers that can deliver biomolecules into cells (38). Initial studies by Mishra et al. (84) indicated that dendrimers accumulate in *C. trachomatis* inclusions. Subsequently dendrimers were used to transform *C. trachomatis* with an *E. coli—C. trachomatis* shuttle vector (66). Dendrimers complexed with plasmid DNA were added to cells 16 h after infection. Plasmid replication and expression of plasmid-encoded open reading frames (ORFs) were detected within the same infection cycle (66). Plasmid-driven green fluorescent protein (GFP) expression indicated that 80% of the inclusions in the initially transfected cell culture were transformed even in the absence of antibiotic selection (66). A similar approach was applied to transform a naturally plasmid-free strain of *C. pneumoniae* with an *S. cerevisiae—C. trachomatis* shuttle vector (44). Moreover, dendrimers also enabled the delivery of antisense oligonucleotides into *C. trachomatis* RBs, which was reported to cause an efficient though short-lived downregulation of the expression of targeted genes (83). Despite the reported high transformation efficiency—which may result from a higher degree of natural competence in RBs compared to EBs—the dendrimer-based transformation method has not been broadly adopted.

Selection. Dendrimer-based transformation may be efficient enough to enable recovery of transformed clonal isolates by plaque isolation in the absence of antibiotic selection. In contrast, transformation efficiencies achieved by electroporation or CaCl$_2$ transformation are low and require antibiotic selection to enrich for transformants. Selection usually occurs through several in vitro passages with increasing drug concentrations, whereby the antibiotic is initially added 7–24 h after infection (6, 14, 36, 48, 60, 73, 86, 128, 146) or even after an initial round of infection in the absence of antibiotics (2, 9, 121, 140). However, it is also possible to select rare transformants directly in the plaque assay without prior enrichment, as reported by Binet & Maurelli (14). An optimal drug for selection should (*a*) be stable in the medium for prolonged periods of time, (*b*) be nontoxic for host cells at a concentration that effectively blocks chlamydial growth, (*c*) penetrate into the inclusion, and (*d*) produce no or only infrequent spontaneously resistant clones. The resistance marker most often used for selection of transformed *Chlamydia* spp. is the β-lactamase gene. Although β-lactam antibiotics are very efficient for selection of transformed *C. trachomatis* L2 (140), the guidelines of the U.S. National Institutes of Health (NIH) prohibit their use as selectable markers for non-LGV urogenital *C. trachomatis* (serotypes D–K) because of their therapeutic use in pregnant women infected with those strains (146). Other resistance markers have been successfully tested, including chloramphenicol acetyltransferase (128, 146), aminoglycoside 3′ adenyltransferase (76), and blasticidin S deaminase (36); the latter may be of limited use owing to the strong cytotoxicity of blasticidin toward host cells.

Stability of transformed *Chlamydia* spp. *E. coli—C. trachomatis* shuttle vectors generated by Wang et al. (140) in 2011 and by other researchers thereafter are maintained by clonally isolated transformants over numerous passages and even in the absence of continued antibiotic selection (2, 143). The endogenous plasmid is lost during transformation and replaced by the shuttle vector

owing to plasmid incompatibility (2, 9, 140). Some investigators prefer to use plasmid-free strains of chlamydiae as recipients (36, 146), which may potentially enhance transformation efficiencies by avoiding plasmid competition. The copy number of plasmids introduced into *C. trachomatis* was reported to be similar to that of the original plasmid (140, 141), though studies with transformed *C. muridarum* reported an increased copy number (73, 74).

To define the function of plasmid-encoded genes, several modified shuttle vectors (both for *C. trachomatis* and for *C. muridarum*) that contain deletions of individual plasmid ORFs have been generated. These studies collectively showed that stable transformants cannot be obtained when CDS2 (*pgp8*), CDS3 (*pgp1*), CDS4 (*pgp2*), or CDS8 (*pgp6*) are deleted, leading the authors to conclude that these ORFs are likely essential for plasmid maintenance (48, 73, 121). Gene disruption by point mutations further showed that it may be the CDS2 coding sequence, not the encoded protein, that is essential, pointing to a role for the noncoding RNA encoded in this region (48). Together, these observations may explain why the first transformation of chlamydiae, reported by Tam et al. (128) in 1994, was only transient; CDS3 was likely disrupted in the shuttle vector used in this study.

Plasmid tropism. While shuttle vectors containing the *C. trachomatis* E:SW2 backbone could be transformed into *C. trachomatis* serotypes L2, D, and F (2, 36, 138, 140, 146), Song et al. reported that transformation of plasmid-free strains of *C. trachomatis* serotype A or of *C. muridarum* was only possible when the shuttle vector contained the backbone of the plasmid naturally found in those strains (122). This phenomenon was termed "plasmid tropism" (122). Interestingly, Wang et al. described a successful transformation of *C. muridarum* with the *C. trachomatis* shuttle vector pGFP::SW2 (139). Recovery of the plasmid from transformed chlamydiae revealed that pGFP::SW2 had recombined with the endogenous *C. muridarum* plasmid to form a hybrid plasmid (termed pSW2NiggCDS2) that was similar to the original shuttle vector yet contained the entire CDS2 from the natural *C. muridarum* plasmid (139). The authors thus suggested that CDS2 may be a major determinant of plasmid tropism, though the observation that pSW2NiggCDS2 was occasionally lost from *C. muridarum* indicates that other factors may contribute as well (139). Interestingly, Gérard et al. reported that a shuttle vector based on the *C. trachomatis* L2 plasmid was maintained for up to 5 passages in a human pathogenic strain of *C. pneumoniae*, suggesting that stable transformation of these naturally plasmid-free bacteria may be possible (44).

Promoters for heterologous gene expression. Early studies demonstrating expression of foreign genes in chlamydiae made use of promoters driving constitutive expression, such as standard promoters used in *E. coli* cloning vectors (140), the promoter from the *Neisseria meningitidis porA* gene (140), or the promoter of the *C. trachomatis incD* gene (2). In contrast, for reintroduction of original or modified genes into mutant strains, researchers preferentially chose to express genes under control of a promoter identical to the promoter of the endogenous gene, reasoning that this may help to maintain the gene's developmental specific regulation (25, 70). Of special note in this context is the introduction of a system for inducible gene expression in *Chlamydia* spp. that enables modulating the timing of transcription and may in some circumstances also help to avoid toxicity. The system was first described by Wickstrum and colleagues (143) and was subsequently applied by several researchers (6, 131, 142).

Stable genetic modifications of the chlamydial chromosome. Successful gene replacement in *Chlamydia* spp. by allelic exchange was reported as early as 2009 (14). In this study Binet & Maurelli constructed a plasmid harboring parts of the *C. psittaci* rRNA operon that differed from the endogenous operon at four single-nucleotide positions. Two mutations caused kasugamycin and

spectinomycin resistance, respectively, one mutation caused loss of a restriction site, and the fourth mutation was silent. After transformation in *C. psittaci*, double-resistant clones were obtained that had also coinherited the other mutations, consistent with the notion that the introduced rRNA operon had replaced the endogenous genes on the bacterial chromosome (14). Direct isolation of these recombinants in a plaque assay also enabled these investigators to optimize various experimental conditions. Recovery of recombinants was improved by the use of high amounts of circular, nonmethylated DNA during transformation, whereas the recombination frequency dropped when the length of flanking regions with strict homology to the target sequence was reduced to below 2 kb (14).

Despite this success, the first targeted disruption of a gene on the *Chlamydia* chromosome, described by Johnson & Fisher (60) in 2013, was not based on the principle of allelic exchange. Instead, the authors adapted the TargeTron system (Sigma-Aldrich), which is based on a modified group II intron that can be retargeted to integrate into the chromosome at a specific site. Using a vector that contains a promoter sequence functional in *Chlamydia* spp. and a β-lactamase gene in the intron, Johnson & Fisher generated a *C. trachomatis* L2 strain harboring an insertional inactivation of the *incA* gene, thereby confirming the functionality of the system and the role of IncA in mediating inclusion fusion (60). A modified system using a marker conferring spectinomycin resistance was later used to disrupt the *rsbV1* gene in *C. trachomatis* L2 (131) and to generate an *incA rsbV1* double-knockout strain (76). Insertional gene disruption was stable during in vivo passage in the mouse in absence of antibiotics (76). A major limitation of the system is that only a restricted number of positions within a gene can be targeted for intron insertion. Intron insertions may also have polar effects on the expression of neighboring genes.

More recently, Mueller et al. (87) developed a system for fluorescence-reported allelic exchange mutagenesis (FRAEM) in *C. trachomatis*, allowing replacement of chromosomally encoded genes with a genetic cassette containing an antibiotic resistance gene and encoding a fluorescent marker. Key to this accomplishment was the generation of a conditionally replicating vector for *C. trachomatis*, which was achieved by placing the plasmid CDS8 (*pgp6*), and thus plasmid maintenance, under the control of a Tet-inducible promoter (87). Removal of the inducer, while selecting for an antibiotic resistance cassette flanked by *Chlamydia* sequences on the plasmid, enables the isolation of strains arising from rare allelic exchange events wherein the chromosomal locus is replaced by the version contained in the plasmid.

APPLICATIONS OF *CHLAMYDIA* GENETICS

Revisiting the Role of the *Chlamydia* Plasmid in Virulence

The methods described above provided the genetic tools to ultimately prove (or disprove) the contribution of a specific bacterial gene to a virulence trait according to molecular Koch's postulates (39). This was first demonstrated by studies revisiting the role of the *Chlamydia* plasmid. Plasmid-free strains of *C. trachomatis* or *C. muridarum* can occasionally be isolated (40, 80, 103, 124) or can be generated by novobiocin treatment (23, 94). Those strains differ from plasmid-bearing strains because of (*a*) a lack of intrainclusion glycogen accumulation, (*b*) reduced bacterial movement in inclusions, (*c*) altered inclusion morphology, and (*d*) reduced expression of several bacterial chromosome-encoded genes (termed plasmid-dependent genes) (21, 80, 92–94). Plasmid-free strains have been variably reported to have no, minor, or significant growth or plaque-forming defects in vitro (21, 80, 92–94, 114, 147) and are significantly attenuated in vivo in the murine urogenital infection model (21, 93). While the reintroduction of the complete chlamydial plasmid (as part of a shuttle vector) into plasmid-free strains restored normal gene expression, glycogen

accumulation, and inclusion morphology (44, 73, 140, 141), plasmids deficient for CDS6 (pgp4) failed to restore these phenotypes, which led to the identification of Pgp4 as the transcriptional activator of plasmid-dependent genes (44, 48, 121, 138). Pgp5, in contrast, was proposed to act as transcriptional suppressor of the same set of genes, at least in *C. muridarum* (73). Recently, Pgp4 was proposed to regulate *Chlamydia* spp. exit from host cells, potentially explaining defects in plaque formation observed in plasmid-free strains (147). The reintroduction of the plasmid also restored *Chlamydia* virulence, and plasmid-encoded Pgp3, Pgp4, and Pgp5 were shown to be important contributors to disease pathology in mice (55, 74, 108).

The reduced virulence of plasmid-free strains prompted several investigators to study their potential use as a live attenuated *Chlamydia* vaccine (69, 93, 96, 106). While variable success was reported, our new understanding of the role of individual plasmid-encoded genes, as well as our ability to introduce modified plasmids, may enable the generation of strains with improved ability to induce protective immunity. The identification of the *Chlamydia* plasmid as a virulence factor, however, also has implications for the design and application of *Chlamydia* shuttle vectors, as these vectors are known to replace the endogenous plasmid (2, 9, 140). Moreover, it has been reported occasionally that shuttle vectors can be maintained at increased copy numbers (73, 74) and that changes in plasmid size or sequence, such as caused by deletion of single ORFs, can affect the expression of other plasmid-encoded ORFs (73). Further studies are needed to explore whether these effects have consequences on virulence and how they can be avoided during vector design.

Validation of *Chlamydia* Virulence Factors

The need for molecular validation of the contribution of a bacterial gene to a virulence trait is best exemplified by the case of the *Chlamydia*-secreted protease CPAF (151). Based on CPAF's ability to cleave a diverse set of host cell proteins in vitro and detection of cleavage products in host cell lysates (150), CPAF has been implicated in the modulation of multiple host cell functions, including resistance to apoptosis (104), Golgi fragmentation (28), and inhibition of NFκB activation (27). The discovery that a significant amount of host protein proteolysis occurs during sample preparation (22, 61) indicated that the role of the protease during infection needed to be reassessed. By characterizing CPAF-deficient *C. trachomatis* mutants, Snavely and colleagues provided compelling evidence that CPAF is not essential for modulating the aforementioned host cell functions, though the protein still appeared to be important for chlamydial growth (119). Further analysis of these mutants by other groups confirmed that CPAF is dispensable for Golgi fragmentation (35) and suggested that CPAF may contribute to genomic instability in host cells (16) and *Chlamydia* exit by host cell lysis (147).

Plasmid-based *trans* complementation of mutant phenotypes was first applied for the analysis of the effector proteins TepP and InaC, confirming their role in recruiting specific host proteins during infection and establishing a role of InaC in mediating Golgi fragmentation (25, 70). Recruitment of host Arf GTPases and 14-3-3 proteins was enhanced during infection with a complemented InaC mutant, suggesting that gene dosage effects associated with *trans* complementation may under some circumstances also aid in deciphering protein function (70). Likewise overexpression helped Thompson and coworkers (131) to establish the role of RbsW and RbsV1 as components of a phosphoregulatory network that regulates expression of housekeeping genes and bacterial growth in *C. trachomatis*.

Studying Protein Localization and Interactions

Chlamydia-secreted effector proteins constitute an important set of virulence factors. Traditionally these proteins were identified by (*a*) in silico predictions (4, 54, 115), (*b*) their interaction with

known T3S chaperones (25, 42, 101), (*c*) their appearance in cytosolic 2D protein maps of infected cells (52, 117, 134), (*d*) their secretion in heterologous T3S systems (e.g., 30, 125, 126), and (*e*) analysis of subcellular localization by immunostaining and/or cell fractionation (e.g., 29, 77, 151). The ability to express epitope-tagged variants of candidate effector proteins eliminates the need for effector-specific antibodies and enabled the confirmation of secretion of CPAF and demonstration of secretion and inclusion membrane localization of several known and candidate inclusion membrane proteins (9, 142). Moreover, glycogen synthase kinase and adenylate cyclase tags were applied to confirm direct exposure of secreted proteins to the host cell cytosol (9). To enhance sensitivity and to enable monitoring of protein secretion in live cells, Mueller & Fields adapted a β-lactamase reporter system for application in *C. trachomatis* (86). Finally, expression of tagged bacterial proteins also facilitates the identification of interacting proteins, as has been recently applied to infer that *C. trachomatis* CT398 (CdsZ) may interact with and regulate components of T3S and transcription (6).

Application of Reporter Genes

The expression of fluorescent proteins, initially applied as a proof of principle for stable expression of foreign proteins in *Chlamydia* spp. (2, 136, 140, 143), has been developed into a tool to facilitate not only estimation of transformation success and plasmid stability (2, 36, 44, 66, 140, 146) but also assays for bacterial adhesion and invasion, monitoring of chlamydial infection during live cell microscopy, and direct flow cytometric enumeration of bacterial particles (2, 136). The technology was also proposed to have the potential to revolutionize high-throughput drug screens (136). A redox-sensitive variant of GFP was applied to monitor the oxidation state of the *Chlamydia* cytosol during the course of the developmental cycle (137). Finally, a luciferase-expressing strain of *C. muridarum* has been generated, allowing us for the first time to monitor the course of chlamydial upper genital tract infection in live mice (19).

FUTURE PERSPECTIVES

The last five years have seen tremendous advances in our ability to perform molecular genetic analyses of *Chlamydia* spp. These new techniques have not only greatly extended our ability to study the function and molecular interactions of *Chlamydia* proteins, but also for the first time enabled us to prove whether a specific bacterial gene is associated with a specific virulence trait. Indeed, we now have the capacity to remove and reintroduce specific genes into *Chlamydia* spp. to fulfill molecular Koch's postulates, a procedure that should be the gold standard for any future analysis of a *Chlamydia* virulence factor. To complement the genetic toolbox for *Chlamydia* spp. and to eliminate the risk of unpredictable virulence attenuation resulting from manipulation of the endogenous plasmid, future developments need to focus on advancing our capacity to introduce genetic modifications directly into the chlamydial chromosome. Of major interest will be systems that enable gene disruption in a non-site-restricted manner, in *cis* complementation of mutations with single gene copies to avoid the gene dosage effects associated with plasmid-based complementation, and replacement of endogenous genes and regulatory elements with modified versions to study gene function. The recent design of a suicide plasmid for *C. trachomatis* and its successful application for allelic exchange (87) is an encouraging development. While this system clearly has great potential for further refinement, alternative approaches, such as those making use of heterologous site-specific recombinases, have been successfully applied to *Coxiella burnetii* (11) and may be applicable to *Chlamydia* spp. as well. Further advancements of systems for molecular genetic manipulation in *Chlamydia* spp. may also require further improvements of transformation

efficiencies, which may be aided by a better understanding of the mechanisms of DNA uptake and recombination in *Chlamydia* spp. Moreover, an expansion of the molecular genetic toolkit to other previously untapped chlamydial strains and species, including environmental chlamydiae and those of veterinary importance, will require additional methodological adaptations and development of new tools. Finally, although this genetic system marks the beginning of a new era in *Chlamydia* research, it also introduces new challenges requiring caution: careful selection of adequate controls, establishing infrastructure for stock keeping and sharing strains, and developing efficient measures to prevent spread of antibiotic resistance or new virulence traits from the laboratory to clinically relevant strains. Investigators also need to be aware of the risk of extended passage of *Chlamydia* spp. in cell culture leading to inactivation or downregulation of virulence factors required for infections in vivo (15, 24). Appropriate selection of parental strains is therefore critical, as is accurate confirmation that only the expected modifications occur in the strains produced.

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Contents