A New Dawn for Chlamydia Research
These important and challenging pathogens are finally yielding to modern analytic techniques

Dan Rockey and Raphael Valdivia

The earliest reports of what were likely chlamydial infections can be found in ancient texts, where diseases of the eye are documented. Early efforts to study the etiology of chlamydial disease trace to Czech scientists Ludwig Halberstädt and Stanislaus von Prowacek, who examined the transmissibility of the infectious agent of trachoma as early as 1907 (historical descriptions of chlamydial research can be found at www.chlamydiae.com). More information about the pathogenic nature of chlamydial organisms started to emerge in the 1920s, when doctors began associating contact with psittacine birds from Africa among Europeans and Americans who developed fatal pneumonias. Subsequent researchers carefully documented the developmental cycle of these bacteria and portrayed what they observed by microscopy in detailed pen-and-ink drawings (Fig 1).

Early chlamydial research was both challenging and fascinating, and many of the original assumptions about the organism reflected the technological limits and biases of the era. For instance, in 1934, British microbiologists Samuel Bedson and J.W. Bland described it as a virus “with affinities to the bacteria” when they wrote:

In our opinion this virus is a micro-organism with bacterial affinities which is essentially an intracellular parasite and which in the early stages of multiplication produces forms much larger than normal. ... in our experience, when the elementary bodies gain access to a suitable cell, either in the animal or in tissue culture, they are soon and invariably replaced by large forms. These large forms apparently multiply for a short time without much change in size, but subsequently there is a progressive decrease in size as multiplication proceeds until the stage of the elementary bodies is reached again.” (S. P. Bedson and J. O. W. Bland, Br. J. Exp. Pathol. 15:243–247, 1934)

Early Recognition that Chlamydia Is an Important Pathogen

The nature of the infectious agent was the subject of much debate until the early 1960s, when it was finally recognized to be a bacterium. However, because its growth is restricted to the intracellular environment of host cells, traditional bacteriological approaches were of limited use. Similarly, until recently, it was impossible to genetically manipulate these bacteria. It is a tribute to many investigators over many decades that we have learned so much about the organism’s basic biology and pathogenesis without relying on tools that were available to their fellows studying free-living microorganisms.

Interest in Chlamydia biology is multifaceted. First and foremost, these bacteria are significant pathogens of humans and animals (Table 1). Important chlamydial diseases include trachoma, the leading cause of preventable blindness worldwide, and urogenital infections of humans, now the most common reportable infection in the United States. In animals, Chlamydia infections can lead to spontaneous abortion in livestock, mucosal diseases of koalas, and fatal respiratory illnesses in birds. Some of these infections, such as psittacosis with C. psittaci, are zoonotic, meaning they can be transmitted from animal to human. These bacteria are highly infectious, and can cause fatal pneumonias.

Second, the chlamydiae have coevolved with eukaryotic cells prior to the emergence of vertebrates, providing a unique example of pathogen
adaptation to intracellular environments. For instance, *Parachlamydia* and *Simkania* spp. colonize amoebae and other protozoa as commensals, mutualists, and pathogens.

<table>
<thead>
<tr>
<th>Chlamydial species</th>
<th>Associated diseases</th>
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<tr>
<td><em>Chlamydia psittaci</em></td>
<td>Diseases of birds; zoonotic lung infections in humans</td>
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<tr>
<td><em>C. abortus, C. pecorum</em></td>
<td>Arthritic, reproductive, and other diseases of ruminants</td>
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<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>Trachoma, lymphogranuloma venereum, other STI's</td>
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<td><em>Chlamydia pneumoniae</em></td>
<td>Human respiratory disease, clinical conditions in animals</td>
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<td><em>Chlamydia muridarum</em></td>
<td>Infects mice; important model system</td>
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<td><em>Chlamydia caviae</em></td>
<td>Infects guinea pigs; important model system</td>
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<td><em>Chlamydia suis</em></td>
<td>Secondary pathogen in pigs</td>
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<tr>
<td><em>Chlamydia felis</em></td>
<td>Conjunctival and respiratory infections in cats</td>
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Finally, chlamydiae undergo a complex and stereotypical developmental cycle that alternates between morphologically and functionally distinct forms with dedicated functions during infection. These developmental transitions raise fundamental questions about how cell determination and diversification is achieved in bacteria.

Our view of *Chlamydia* development, and how infection occurs, has evolved as new technologies became available: cell culture, contemporary microscopy (Fig. 2), biochemical and immunological tests, surrogate genetic systems, and, more recently, proteomics and genomics. Remarkably, the basics of the morphological changes underlying chlamydial development remain similar to those outlined in 1949 by Emilio Weiss (Fig. 1). However, our molecular understanding of these events has expanded considerably. When the nonreplicating form of chlamydia, called an elementary body (EB), comes in contact with epithelial cells of the mammalian genital tract, conjunctivae, or respiratory tract, a subset of proteins is translocated into the host cell to mediate EB invasion. Simultaneously, a developmental pro-
Contemporary microscopic techniques and the *Chlamydia* developmental cycle. Where visible, a complete nucleus (blue in each panel) is approximately 11–13 μm in diameter. The reticulate bodies in B are approximately 2 μm in diameter. (A) *C. trachomatis* elementary bodies (green) attached to the surface of epithelial cells. The actin cytoskeleton is highlighted (red). (B) Transmission electron micrographs of an inclusion filled with replicative reticulate bodies. (C) Fluorescence in situ hybridization-based images showing an inclusion in a cell infected with both *C. trachomatis* (green) and *C. suis* (red). Inclusions formed by wild-type strains of these species fuse during development. (D) Immunofluorescence microscopy of a mammalian cell infected with *C. trachomatis*, and labeled with antibody to the major outer membrane protein (MOMP) (red) and IncA (green). The antibody to IncA decorates the inclusion membrane as well as fiber-like structures that extend away from the inclusion into the cytoplasm of the infected cell. (Fig 2A courtesy of J. D. Dunn, Duke University; Fig 2B courtesy of J. Burnett, Oregon State University; Fig 4D courtesy of Robert Suchland, University of Washington.)
gram is activated that leads the internalized EB to change to the replicative form, called the reticulate body (RB). Within cells, chlamydiae replicate within a membrane-bound vacuole, called an inclusion. The inclusion membrane is modified via bacteria-encoded integral membrane proteins termed Inc proteins. Inc proteins interact with host cytosolic factors to establish a “chlamydia factory” that acquires lipids and nutrients from other host organelles while excluding interactions with degradative compartments. Throughout the infectious process, chlamydiae secrete proteins into the cytosol of the host cell to manipulate innate immune responses and other molecular signaling events important for bacterial survival and proliferation.

**Chlamydia Biology before and after Genomics**

Early observations of *Chlamydia*-host interactions and the identification of the bacterial proteins underlying these interactions were made when researchers could not readily manipulate the *Chlamydia* genome. Some of the experimental approaches that they did pursue were remarkable. For instance, to identify the chlamydial proteosome-activity-like factor (CPAF), an important virulence factor, investigators painstakingly used biochemical methods to isolate the protease activity from large volumes of infected cells.

Other noteworthy studies depended on antisera from patients or from animals immunized with live *Chlamydia* cells to detect potential secreted factors from among *Chlamydia* protein expression libraries. Later, investigators brought in proteomic approaches and surrogate heterologous gene expression systems to examine key aspects of chlamydial biology. As more *Chlamydia* genome sequences became available, bioinformatic and functional genomic approaches were developed to identify additional putative *Chlamydia* virulence factors.

In other microbial systems, one could easily test the role of such factors by introducing recombinant DNA to disrupt the genes encoding them and determine their role in virulence. Such approaches could not be performed in chlamydiae. Without molecular tools to generate genetic diversity in this system, investigators developed alternative approaches to directly test and evaluate gene function. For instance, the role of the inclusion membrane protein IncA was deduced by an analysis of clinical isolates in a large repository, compiled by the late Walt Stamm at the University of Washington in Seattle. This repository, which provides a broad survey of chlamydial variants in human patients, allowed for correlations to be drawn between mutations in the gene *incA* and strains displaying a fragmented inclusion morphology. Such IncA-deficient strains are associated with patients having milder symptoms.

A role for IncA in inclusion fragmentation is consistent with observations by Ted Hackstadt and colleagues at the National Institutes of Health (NIH) in Bethesda, Md., who independently arrived at the same conclusion by injecting neutralizing anti-IncA antibodies into infected cells. Nucleotide sequence analysis of more than 50 similar and, in some cases, nearly isogenic strains provides strong evidence for an association of the lack of IncA with fragmented inclusions. However, the gold standard test—a simple genetic transformation and complementation of an *incA*-negative strain with a plasmid encoding IncA—is lacking.

More recently, investigators used chemical genetic approaches to identify small molecules that interfere with *Chlamydia* virulence factors. These efforts include using inhibitors of Type III secretion, protein kinases, chlamydial protease-like activity factor (CPAF) function, and lipopolysaccharide (LPS) biosynthesis. Yet other compounds have potent activity against *Chlamydia*, but their targets remain to be characterized.

**Dawning of Chlamydia Molecular Genetics**

Through dogged efforts during the past five years, scientists made tremendous progress developing tractable genetic approaches to study *Chlamydia*. This progress reflects the availability and implementation of new technologies along with some old-fashioned microbiology.

Increasingly affordable DNA sequencing technologies are a boon to those seeking to develop experimental genetic approaches to study *Chlamydia*. Importantly, the increasing ease of use of sequence analysis software has made it simpler for molecular biologist to dabble in genomic sequencing analysis. *Chlamydiae* have relatively simple, small genomes containing about 1 million base pairs, making them amenable to whole-genome sequencing approaches. To place
the technological changes facilitated by contemporary sequence analysis in perspective, the sequence of the first Chlamydia trachomatis genome (strain D/UW3) was published by Richard Stephens of the University of California, Berkeley, and his colleagues in 1998. That project required several years to complete and cost approximately $1 million. Now sequencing the chlamydial genome can take 2-3 days and costs less than $500. It is sobering to realize that average chlamydial genomes can be smaller in size than a large eukaryotic gene!

How are investigators exploiting genome sequencing technologies to better understand Chlamydia? First, in a flurry of activity, investigators determined the genome sequences of many laboratory and clinical chlamydial isolates. For example, Simon Harris of the Wellcome Trust Genome Campus in Hinxton, Cambridge, United Kingdom, and colleagues early in 2012 provided data for 38 such isolates to the research community. Altogether, approximately 60 Chlamydia trachomatis sequences, and several related chlamydial species are available. These datasets constitute a gold mine for comparative genomics studies. As more and more genome sequences come online, especially from various branches of the Chlamydiae, they will enable us to better address fundamental question about the evolution of the chlamydiae, strain-specific virulence properties, and DNA exchanges among these species.

Indeed, genome sequencing suggests that Chlamydia is rather adept at exchanging DNA in nature. Mounting evidence indicates that clinical Chlamydia strains are mosaics of the classical serologically defined groups (serovars). These lateral gene transfers (LGTs) were first recapitulated in the laboratory by Robert DeMars at the University of Wisconsin. We expanded this work, showing that chlamydiae can exchange DNA both within and among related species, and that an antibiotic resistance gene can be transferred. While antimicrobial resistance is virtually absent in human pathogenic chlamydiae, these microorganisms have the potential to acquire such resistance genes.

**Other Genetic Technologies Are Appearing on the Horizon**

Rapid genome sequence analysis is also enabling investigators to implement forward and reverse genetic strategies, in which DNA-damaging agents are used instead of transposons to generate mutations. Laszlo Kari and Harlan Caldwell at NIH and their collaborators adapted an approach used in plant genetics to screen for mutants in specific genes. The system is based on heteroduplex DNA analysis of target genes amplified from pools of ethane methyl sulfonate (EMS)-treated cells to identify clones with mismatches (mutations) in genes of interest. In proof-of-principle experiments, these investigators identified strains bearing loss-of-function mutations in a tryptophan biosynthesis gene.

We have implemented similar strategies to establish genotype-phenotype associations among EMS-generated mutants. In initial experiments, genome sequencing analysis of mutants sharing a particular phenotype—in this case, abnormal glycogen accumulation—helped establish strong correlations between insoluble glycogen precipitates and mutations in the gene encoding a glycogen branching enzyme. Causality between individual mutations and the corresponding phenotype was then established by harnessing LGT. In brief, recombinant strains generated from coinfection between wild-type and mutant Chlamydia were analyzed to determine whether a particular mutation identified by whole genome sequencing is genetically linked to a particular trait. In essence, we are performing classical genetic analysis without molecular genetic tools.

Finally, a new development in Chlamydia biology is opening this pathogen to molecular genetic analysis. Ian Clarke of the University of Southampton recently reported the stable transformation of Chlamydia with a shuttle vector consisting of an endogenous chlamydial and a ColE1 plasmid. His report follows decades of efforts in this area, and builds upon research by Priscilla Wyrick of East Tennessee State University in Johnson City and Anthony Maurelli of the Uniformed Services University of the Health Sciences in Bethesda, Md., who provided the first demonstration that recombinant DNA could be stably introduced into these organisms. Remarkably, stable plasmid was introduced via calcium chloride-based transformation, one of the oldest techniques in the book. This exciting discovery is a major breakthrough in Chlamydia research.

Progress has also very recently been made in the specific alteration of chlamydial transcription. A group at Wayne State University used a dendromere-based delivery of oligonucleotides
to interfere with the transcriptional machinery within infected cells, leading to a 90% reduction in transcription of the target gene.

*Chlamydia* research has always been constrained by technical limitations. It will be exciting to witness how research on this clinically important pathogen will blossom in the coming years and how the corresponding findings will help shape the next generation of prevention and treatment strategies.

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**Suggested Reading**


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