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A Major Advance in Elucidating the Biology/Pathobiology of *Chlamydia trachomatis*

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The several chlamydial species have been recognized for decades as bacterial pathogens of major importance to humans and domestic animals. *Chlamydia trachomatis* was first identified as the etiologic agent in blinding trachoma in Africa and the Middle East and later was demonstrated to be a genital pathogen of widespread occurrence in both developed and underdeveloped areas of the world. Indeed, this organism is now considered to be the most common sexually transmitted bacterial pathogen in the United States, with more than a million new such infections reported annually to the Centers for Disease Control and Prevention (1). Significantly, primary genital infection with this organism often engenders severe sequelae, including fertility problems in women, arthritis in both genders, and others (2, 3). Current data indicate that these sequelae involve organisms in an unusual state designated persistence, in which metabolic and genetic activity are modified to yield a largely antimicrobial-refractory organism (3, 4).

The underlying damage caused by both primary and persistent chlamydial infections results from the severe inflammation they elicit, and an important advance in the approach to understanding how these pathogens engender that inflammatory process is provided in this issue by Song et al. from the Caldwell group at Rocky Mountain Laboratories (5). Studies done more than 2 decades ago demonstrated that as with most bacterial genomes, the chromosome of *C. trachomatis* specifies a proinflammatory Hsp60 protein encoded by *groEL*. One of several major surprises revealed by the complete genome sequence of the organism was that two additional paralogs of the *groEL* gene product also are encoded on the chromosome (for example, see reference 6). Further, while the *C. trachomatis* genome is relatively small, just over 900 coding sequences specified on the chromosome and a small plasmid, more than 200 of those apparent open reading frames encode proteins of unknown function, all or most of which, like the Hsp60's, are thought to contribute to virulence and/or the transition to and maintenance of the persistent infection state (for example, see reference 7). However, until recently a fundamental problem has prevented elucidation of how those unknown gene products singly or in concert function in disease induction by *C. trachomatis*.

That fundamental problem is a direct result of the biology of the organism. *C. trachomatis*, like all chlamydiae, is an obligate intracellular bacterium; that is, completion of its normal developmental cycle requires infection of and passage through eukaryotic host cells (8). Standard chemical mutagenesis of the organism has had only limited success, in part because chlamydiae cannot be grown axenically to support the normal methods of producing clonal populations of mutants. Development of a system for genetic manipulation of the organism thus has been a major goal for decades in the *Chlamydia* research community, but the primary problem with development of a transformation system for *C. trachomatis* has been that getting modifying nucleic acid constructs to the organisms required that they be inserted somehow into the metabolically inactive extracellular form of the organism, the elementary body, through its extremely durable cell wall, or into metabolically active chlamydiae within host cytoplasmic inclusions; this latter route involves the daunting task of getting any modifying construct across the eukaryotic host cell membrane, the inclusion membrane, and finally across the membrane and minimal cell wall of the metabolically active form of *Chlamydia*, the reticulate body. In either case, sorting out the transformants from nontransformants remains a problem.

Early attempts to develop a transformation system for chlamydiae demonstrated that nucleic acids could be inserted into elementary bodies by electroporation, but stable transformants were not produced (for example, see reference 9). A later method using electroporation was able to generate a small number of transformants, but that process required extremely large amounts of construct to be used in their production (10). The efficiency of this system was extremely low, and while it was ground-breaking in a sense, it was generally considered to be unusable for most routine experimental purposes. Most recently, the Clarke laboratory at the University of Southampton in the United Kingdom described a method for transformation that inserts modifying nucleic acids into elementary bodies using, surprisingly, a treatment with $CaCl_2$ (11). This system also displays relatively low efficiency, and clonal populations of transformants can be produced only by several sequential passages of the organism under antibiotic selection. Regardless, this procedure is workable on a routine basis, and it is this method for genetic manipulation of *C. trachomatis* that provides the context for the major advance described by Song et al. (5).

Most isolates of *C. trachomatis* possess a 7.5-kbp plasmid that specifies eight open reading frames for proteins plus noncoding RNAs (12, 13). While the plasmid is not required for survival of the organism, researchers have long held that one or more of the genes included on it function importantly in virulence (for example, see reference 7). *C. trachomatis* produces iodine-stainable glycogen in its cytoplasmic inclusion, and the Caldwell laboratory had demonstrated using a plasmid-less isolate that one or more functions encoded on the plasmid are instrumental in transcriptional control of the glycogen synthase gene (*glgA*), located not on

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the plasmid but on the chromosome (14). Importantly, in the report describing development of the $CaCl_2$-based transformation method, the Clarke laboratory confirmed that plasmid-based function by demonstrating glycogen production following transformation of the plasmid into that same plasmidless strain of *C. trachomatis* (11). The cardinal questions at issue, then, became the following. Which coding sequence(s) on the plasmid is responsible for governing expression of chromosomal *glgA*? Is expression of other chromosomal or plasmid genes encoding virulence or other functions also governed by that plasmid gene(s)? And what functions do the other coding sequences on the plasmid specify?

Given the newly available ability to insert the chlamydial plasmid into the plasmidless strain, the experiment of primary importance was to clone the 7.5-kbp plasmid in an antibiotic resistance-containing vector, individually mutagenize each of the eight coding sequences on the plasmid, transform them independently into the plasmidless strain of *C. trachomatis*, subject the transformants to antibiotic selection, and assess phenotypes variously. The initial experiments performed indicated that some transformations produced either no or unstable antibiotic-resistant chlamydiae, indicating that one set of the mutagenized genes at issue, comprising *pgp1*, *pgp2*, *pgp6*, and *pgp8*, all are involved in plasmid maintenance. These observations are consistent with earlier predictions based on the homologies of several of these predicted gene products with proteins in the databases known to be involved in plasmid maintenance. Interestingly and as pointed out in the report, *pgp2* encodes a product with no known homology to other proteins in the databases, and thus its precise function remains to be elucidated. Mutants for *pgp3* to *pgp5* and *pgp7* showed no phenotype relating to plasmid maintenance, and for two of these the result was somewhat surprising. *pgp5* encodes a protein with homology to ParA, which is involved in plasmid partitioning in other organisms, and *pgp7* specifies a putative integrase/recombinase. Song et al. hypothesize that the chromosome of *C. trachomatis* may encode additional copies of these genes, thereby allowing complementation of their absence from the mutagenized plasmids (5).

Most interestingly, *C. trachomatis* given the cloned plasmid with a mutation in *pgp4* displayed a phenotype virtually identical to that of the plasmidless strain itself, i.e., some abnormalities in inclusion morphology and severely attenuated expression of *glgA*, and thus no iodine-stainable glycogen production. Transcriptome analysis of these transformants given the nonfunctional *pgp4* identified more than three dozen genes whose transcription is affected significantly by its loss, including *pgp3* and many chromosomal genes encoding products of currently unknown function. Additional experiments demonstrated that it is the *pgp4* gene product that functions as the primary regulator of expression for these genes. At this point, it is unclear how many of these genes support pathogenesis or persistence directly or indirectly, but their identification clearly points researchers in the direction of elucidation of their functions.

Thus, the report by Song et al. (5) employs the new and much-needed transformation method to establish a milestone in research on *C. trachomatis*. Production of glycogen has been asserted to be a contributor to virulence (15, 16), and the *pgp4* gene product should be considered a virulence factor for this reason and because observations from another laboratory have indicated that the *pgp3* gene product is inserted into the host cytoplasm by infecting *C. trachomatis* (17). It seems highly likely that most, perhaps all, of the chromosomal genes whose expression is governed by Pgp4 will be determined to be virulence factors as well. The importance of the work by Song et al. is, in part, that the observations presented provide the first demonstration that the new transformation method for chlamydiae can be used routinely for assessment of chlamydial gene function and, in part, that these data provide the first clearly defined experimental direction concerning the question of which of the many genes encoding known or unknown chlamydial products should be addressed to dissect details of pathogenesis and persistence. What is needed next is a transformation method that allows knockdown and/or knockout of expression of specific chlamydial genes, as well as the demonstration that the method of inserting nucleic acids into *C. trachomatis* also functions equally well for other chlamydial pathogens, including *Chlamydia pneumoniae*. Such approaches, in concert with the transformation system developed by the Clarke laboratory and other genetic methods (for example, see references 16 and 18), will continue and indeed accelerate the revolution in our molecular understanding of chlamydiae and the diseases they cause.

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