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MICROBIOLOGY *of* CHLAMYDIA

Almen L. Barron

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Microbiology of Chlamydia

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PREFACE

Organisms classified in the genus *Chlamydia* were once considered to be unusual viruses and are now known to be obligate intracellular procaryons with a unique reproductive cycle. Older medical knowledge associated these organisms with trachoma, inclusion conjunctivitis, lymphogranuloma venereum, and psittacosis. Current medicine is concerned with their role in sexually transmitted disease, infertility in females, pneumonitis of the newborn, and possibly pneumonia in the absence of an avian reservoir. In this book we attempt to state the microbiology of *Chlamydia* as we understand it with regard to their nature as microorganisms and as pathogens. The editor is grateful to the contributors for their cooperation, patience, and commitment to excellence.

Almen L. Barron

THE EDITOR

Almen L. Barron Ph.D. is Professor and Chairman, Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock. A Canadian by birth, he received his Ph.D. at Queen's University, Kingston, Ontario in 1953. He joined the Department of Bacteriology, University of Buffalo (later State University of New York at Buffalo) in 1954 to work on the Salk vaccine field trial under the direction of Dr. David T. Karzon. He remained at Buffalo until 1974 when he assumed his present position. Research at Buffalo was mainly focused on Echoviruses and viral immunology. In 1964 he received a Fulbright Research Scholar award and worked in the laboratory of the late Professor Hans Bernkopf, Hebrew University — Hadassah Medical School, Jerusalem, Israel. It was there that his interest in *Chlamydia* was kindled. Early research was on chlamydial hemagglutinin and biological properties. Later studies involved the role of *Chlamydia* in genital tract infections using animal models, which have continued to the present. Other activities have included co-editing *Microbiology: Basic Principles and Clinical Applications* (Macmillan) with Dr. Noel R. Rose, membership on Bacteriology and Mycology Study Section, NIH, and service on the editorial boards for *Infection and Immunity* and *Proceedings of the Society for Experimental Biology and Medicine*.

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Chlamydia as Organisms

Chapter 1

CHARACTERISTICS OF CHLAMYDIAE

James W. Moulder

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I. INTRODUCTION

The chlamydiae have been assigned to the order *Chlamydiales,* which is comprised of one family, *Chlamydiaceae,* with a single genus, *Chlamydia,* and two species, *C. trachomatis* and *C. psittaci.*[1-3] The description of the genus *Chlamydia* in *Bergey's Manual of Systematic Bacteriology* follows this classification,[4] and it provides a generally satisfactory characterization of the chlamydiae infecting humans, other mammals, and birds. However, the present state of chlamydial taxonomy is unsatisfactory in at least two respects. First, it takes no account of the many reports of chlamydia-like organisms living intracellularly in invertebrate hosts, and second, being based solely on phenotypic similarities and differences, its relation to the evolutionary history of chlamydiae is uncertain.

This chapter will describe the definitive properties of the genus *Chlamydia,* how its species and biovars are distinguished one from the other, and how recent advances in chlamydial biology and bacterial phylogeny allow construction of a plausible evolutionary history of chlamydiae. Finally, it will consider ways in which the familiar *C. trachomatis* and *C. psittaci* and the as yet poorly characterized chlamydia-like inhabitants of invertebrates may all be accommodated within the order *Chlamydiales,* perhaps by the creation of new families and genera.

II. PROPERTIES OF THE GENUS *CHLAMYDIA*

A. Definition of the Genus *Chlamydia*

The genus *Chlamydia* is defined by the properties listed in Table 1. Presently, only strains of *C. psittaci* and *C. trachomatis* are known to fit this definition. With one exception, the chlamydia-like organisms of invertebrates have not been propagated in the laboratory. So, apart from morphology, their characteristics are largely unknown.

1. Obligate Intracellular Habitat

Although no chlamydia has so far been observed to grow extracellularly, either in nature or in the laboratory, serious and sustained efforts to achieve host-free multiplication have not been made. With our rapidly increasing understanding of chlamydial biology, such efforts may soon become worthwhile.

2. Developmental Cycle

Like many other intracellular parasites,[5] chlamydiae have evolved morphologically distinct infectious and reproductive forms.[6-8] Chlamydial elementary bodies never divide. Their role is to carry the infection from one cell (or one host) to another, where they reorganize into reticulate bodies which multiply by binary fission in membrane-bound intracytoplasmic vacuoles or inclusions. Reticulate bodies do not infect new host cells. Instead, they reorganize into new generations of elementary bodies to complete the developmental cycle. The most critical structural difference between elementary bodies and reticulate bodies appears to be the extent to which their outer membrane proteins are complexed by disulfide cross-linking. These proteins are extensively cross-linked in elementary bodies but not in reticulate bodies.[9-15] There is a strong temptation to ascribe the many biological differences (Table 2) between the two chlamydial cell types to this structural difference, but direct evidence for a cause and effect relationship is largely lacking.

3. Gram Negative Envelope Without Peptidoglycan

Envelopes of both elementary bodies and reticulate bodies resemble those of host-independent Gram-negative bacteria in that they are made up of an inner cytoplasmic membrane and an outer membrane,[7,8,17,18] are disrupted by polymyxin B and ethylenediaminetetra-

Table 1
CHARACTERS THAT DEFINE THE GENUS *CHLAMYDIA*

Obligate intracellular habitat.
Developmental cycle with morphologically distinct infectious and reproductive forms.
Gram negative envelope without peptidoglycan.
Genus-specific lipopolysaccharide.
Patches of hexagonally arrayed cylindrical projections.
Utilization of host ATP for synthesis of chlamydial protein.
Small genome.

Table 2
BIOLOGICAL DIFFERENCES BETWEEN ELEMENTARY BODIES AND RETICULATE BODIES

Property	Elementary body	Reticulate body
Infectivity	Yes	No
Multiplication	No	Yes
Inhibition of phagosome-lysosome fusion	Yes	No
Toxic for mice	Yes	No
Toxic for macrophages	Yes	No
ATP transport	No	Yes
Protein synthesis	No	Yes

Modified from Moulder, J. W., *ASM News*, 50, 353, 1984. With permission.

acetate,[19,20] and contain an outer membrane protein that accounts for half of the total protein of that membrane.[10,18,21,22] Chlamydial cell envelopes differ from those of typical Gram-negative bacteria in that they have no peptidoglycan. Electron micrographs show that there is no peptidoglycan layer between the inner and outer membranes,[18] and chemical analysis reveals no muramic acid or any other amino sugar that might have replaced it in the peptidoglycan subunit.[23,24]

Absence of peptidoglycan could mean, as has been suggested for the peptidoglycan-less budding bacteria,[25] that the chlamydiae branched off the main eubacterial tree before peptidoglycan was invented. However, it is more likely that chlamydiae have evolved from ancestors with peptidoglycan because they appear to have retained vestiges of a former peptidoglycan-containing state. Chlamydiae have penicillin-binding proteins similar in location, size, and affinity for the antibiotic to those of host-independent Gram-negative bacteria.[23] In low concentration, penicillin inhibits the growth and division of reticulate bodies and prevents their reorganization into elementary bodies.[26] Growth and multiplication of both *C. trachomatis* and *C. psittaci* are also blocked by D-cycloserine,[27,28] another inhibitor of peptidoglycan synthesis,[29] although *C. trachomatis* strains are usually much more susceptible. Penicillin inhibits the transpeptidation reaction responsible for the closing of the peptide cross-links in peptidoglycan,[29,30] whereas D-cycloserine inhibits both the formation of D-alanine from L-alanine and the synthesis of D-alanyl-D-alanine.[29] Sensitivity of chlamydial multiplication to these two antibiotics implies the presence of a D-alanyl-D-alanine sequence somewhere in the chlamydial cell. For this reason, it has been suggested that chlamydial envelopes contain D-alanyl-D-alanine peptides that are cross-linked to structures other than peptidoglycan.[24] The chlamydial susceptibility to inhibitors of peptidoglycan synthesis in the absence of peptidoglycan is without known parallel. The peptidoglycan-less budding bacteria are, for example, relatively resistant to both penicillin and D-cycloserine.[25]

4. Genus-Specific Lipopolysaccharide

All isolates of *C. trachomatis* and *C. psittaci* so far examined contain a lipid-soluble complement-fixing antigen that is present at all times in the developmental cycle.[31] This genus-specific antigen (formerly called the group antigen) strongly resembles the lipopolysaccharides (LPSs) of host-independent Gram negative bacteria in its location in the outer membrane of the chlamydial cell envelope,[32] in its chemical structure,[33-35] and in its biological activity.[36,37] There is also strong immunologic cross-reaction between the chlamydial LPS and the innermost core of the LPS from *Salmonella* mutants in which that structure is exposed.[38-40] Monoclonal antibodies against chlamydial LPS reveal at least three antigenic domains, two of which are shared with the LPSs of some free-living Gram-negative organisms and one of which is unique to the LPS of chlamydiae.

5. Patches of Hexagonally Arrayed Cylindrical Projections

On the outer membranes of elementary bodies from both *C. psittaci* and *C. trachomatis* there are patches of hexagonally arrayed cylindrical projections that are without obvious counterpart in other bacteria.[41-43] These projections are 10 to 25 nm high and about 25 nm in diameter. On a single chlamydial cell there is never more than one patch, with about 20 cylindrical projections roughly 50 nm apart, center to center. The cylinders extend all the way through the outer membranes, and it has been suggested that they are transmembrane pores connecting the interior of the chlamydial cell with the external environment.[41,44] Other unusual architectural features of the chlamydial surface have also been described, but it has not yet been demonstrated that they occur throughout the genus.

6. Utilization of Host ATP for Synthesis of Chlamydial Protein

In adapting to intracellular life, chlamydiae appear to have evolved mechanisms for exploiting the energy-rich compounds of their hosts and to have subsequently lost whatever energy-producing systems they might once have had.[45] Host-free chlamydiae have no respiratory enzymes other than the pyridinoproteins,[46] they catabolize glutamate, glucose, and pyruvate to a limited extent but without producing useful energy,[47,48] and infected host cells do not develop novel energy-generating mechanisms.[49,50] However, host-free reticulate bodies of both *C. psittaci* and *C. trachomatis* move ATP in and ADP out of their intracellular space by means of an ATP-ADP exchange system,[51] and use the host-derived ATP for synthesis of chlamydial protein.[52] Such a mechanism for transport of intact ATP into a cell has otherwise been described only in rickettsiae.[53]

7. Small Genome

Both chlamydial species have genomes consisting of double-stranded DNA molecules with average lengths of 346 μm.[54,55] These lengths correspond to a molecular weight of 660×10^6. Another estimate of chlamydial genome size based on the rate of reassociation of disassociated DNA gives a comparable value.[56] Although the chlamydial genome is much larger than that of the largest viruses, it is among the smallest of all procaryotic genomes (Table 3). Only the *Mycoplasma* genome is smaller. It may be that, once the chlamydiae learned to use host ATP, they no longer needed the genes and gene products associated with energy generation, that these genes disappeared without unfavorable consequences, and that the chlamydial genome shrank accordingly. Perhaps other groups of dispensible genes met similar fates. Lwoff suggested a long time ago that,[62] in the presence of a required metabolite, auxotrophic mutants should have the growth advantage over their prototrophic parents because they have fewer biosynthetic functions to perform. Subsequent discovery of mechanisms for preventing unneeded synthetic activities by means of feedback inhibition and gene repression have cast doubts on Lwoff's suggestion. However, there is still no better explanation for the consistently lower size of genome among procaryotes that live in and on eucaryotic cells (Table 3).

Table 3
SOME COMPARATIVE GENOME SIZES

Bacterial genus or virus	Genome size (M daltons)	Relative size	Ref.
Chlamydia	660	1	54,55
Vaccinia virus	160	0.24	57
Mycoplasma	500	0.76	58
Coxiella	1040	1.6	59
Rickettsia	1100	1.7	60
Neisseria	1300	2.0	56
Escherichia	2840	4.2	61

Modified from Moulder, J. W., *ASM News*, 50, 353, 1984. With permission.

Table 4
DIFFERENTIATION OF *C. TRACHOMATIS* AND *C. PSITTACI*

Character	*C. trachomatis*	*C. psittaci*
Inclusion morphology	Oval, vacuolar	Variable, dense
Accumulation of glycogen in inclusion[a]	Yes	No
Synthesis of folates[b]	Yes	No
Natural hosts[c]	Mice, humans	Birds, nonhuman mammals

[a] Revealed by staining with iodine.
[b] Revealed by growth inhibition with sulfadiazine. There are some exceptions.
[c] Conventional view of host range. There may be exceptions.

Modified from Moulder, J. W., Hatch, T. P., Kuo, C.-c., Schachter, J. and Storz, J., *Bergey's Manual of Systemic Bacteriology*, Vol. 1, Krieg, N. R., Ed., Williams & Wilkins, Baltimore, Md., 1984, 729. With permission.

B. Differentiation of the Two Species of *Chlamydiae*

Page separated *Chlamydia* into two species, *C. trachomatis* and *C. psittaci*, on the basis of these characters: morphology of the inclusion, accumulation of glycogen in the inclusion, and susceptibility to growth inhibition by sulfadiazine (Table 4).[2] Another character not used by Page, natural host range, is also useful.

1. Inclusion Morphology

Individual elementary and reticulate bodies of one species are not readily distinguished from those of the other by either light or electron microscopy. Nevertheless, the intracytoplasmic inclusions in which chlamydiae multiply are obviously different in the two species.[63] Inclusions of *C. trachomatis* are round or oval, relatively rigid, and often not completely filled with chlamydial cells. There tends to be only one inclusion per host cell, and the host cell nucleus is frequently displaced to the periphery. In contrast, *C. psittaci* inclusions are irregular or diffuse, not noticeably rigid, and usually packed with chlamydial cells. There may be several inclusions in one host cell, and the nucleus is not displaced.

2. Accumulation of Glycogen in Inclusions

A second difference between inclusions of the two chlamydial species is that glycogen accumulates in inclusions of *C. trachomatis* but not in those of *C. psittaci*.[63] The presence of glycogen is customarily demonstrated by staining with iodine, but the red-staining substance in the inclusions of *C. trachomatis* has been unequivocally identified as glycogen.[64] It is generally agreed that glycogen accumulates extracellularly, that is, inside the inclusion but outside the chlamydial cells. The largest accumulations of glycogen are found late in the developmental cycle at about 48 to 72 hr after infection.[65-69] There is little doubt that glycogen is synthesized by chlamydial enzymes and is not the product of a host response to infection. Accumulation of glycogen is inhibited by penicillin[65] and chloramphenicol.[69] *C. trachomatis*-infected cells incorporate adenosine diphosphate glucose, the bacterial glycogen precursor, into glycogen in preference to uridine diphosphate glucose, the mammalian substrate.[70] The role of glycogen synthesis in *C. trachomatis* metabolism and why it is deposited in the extracellular phase (inside the inclusion, outside the chlamydiae) of the inclusion is unknown.[64] The apparent invariant coupling of glycogen accumulation with inclusion morphology suggests both a role for glycogen and a way to explain how chlamydial cells of like morphology produce unlike inclusions. Perhaps glycogen modifies some physical property of the fluid phase of the inclusion in such a way as to produce inclusions of the *C. trachomatis* type.

3. Susceptibility to Growth Inhibition by Sulfadiazine

Susceptibility to sulfadiazine, a manifestation of the ability to synthesize folate,[29] is also significantly associated with inclusion morphology, although not as closely as glycogen accumulation. Most chlamydial strains, classified as *C. trachomatis* on the basis of inclusion morphology, are sensitive to sulfadiazine, whereas most strains similarly designated *C. psittaci* are not.[28] However, there are exceptions. For example, the 6BC strain of *C. psittaci*, widely used in studies on chlamydial biology, is sulfonamide-susceptible,[28] and some isolates of the lymphogranuloma venereum biovar of *C. trachomatis* are partially[71] or completely resistant.[72]

4. Natural Host Range

The orthodox view of the natural host range of *Chlamydia* is that *C. trachomatis*, with the exception of the mouse biovar, is a uniquely human pathogen, whereas *C. psittaci* is a parasite of birds and nonhuman mammals. Conventional wisdom holds that strains of *C. psittaci* indigenous to other mammals are negligible sources of human disease, whereas avian strains may be transmitted to people and cause psittacosis, a chlamydial pneumonia in which person-to-person transfer rarely occurs.

Although this view holds in the great majority of cases, there are exceptions. Strains of *C. psittaci* that are the agents of disease in nonhuman mammals may on occasion cause serious disease in people. The recent well-documented case of acute placentitis and spontaneous abortion due to ovine *C. psittaci* in a farm woman who had helped with lambing is a good example.[73,74] There is also a strain(s) of *C. psittaci* (TWAR) that violates not one but two of the tenets of the conventional wisdom. It produces both conjunctivitis and pneumonia in humans in the absence of any demonstrated nonhuman reservoir.[75,76] Isolates from Taiwan and India appear identical. Serological surveys indicate that antibodies to this agent(s) are prevalent in populations all over the world.[75,76]

C. Differentiation of the Three Biovars of *C. trachomatis*

C. trachomatis has been further divided into biovars, but *C. psittaci* has not.[4] This is not because *C. psittaci* is the more homogeneous of the two species, but rather because it is so heterogeneous that rational subdivision is presently impossible. Each of the three biovars of

Table 5
DIFFERENTIATION OF THE BIOVARS OF *C. TRACHOMATIS*

Characteristic	Biovar		
	Trachoma	Lymphogranuloma venereum	Mouse
Behavior in natural hosts			
Host range	Humans	Humans	Mice
Preferred site of infection	Squamocolumnar epithelial cells	Lymph nodes	Lungs
Behavior in laboratory animals			
Intracerebral lethality for mice	No	Yes	No
Follicular conjunctivitis in primates	Yes	No	No
Behavior in cell culture			
Plaques in mouse fibroblasts	No	Yes	Yes
Entry into host cells markedly enhanced by			
Centrifugation onto cell sheet	Yes	No	No
Treatment of host cells with DEAE[a]	Yes	No	No

[a] Diethylaminoethyl dextran

Modified from Moulder, J. W., Hatch, T. P., Kuo, C.-c., Schachter, J., and Storz, J., *Bergey's Manual of Systemic Bacteriology*, Vol. 1, Krieg, N. R., Ed., Williams & Wilkins, Baltimore, Md., 1984, 729. With permission.

C. trachomatis, mouse, lymphogranuloma venereum (LGV), and trachoma, exhibit the species-defining properties listed in Table 3. The three biovars may be distinguished one from the others according to the criteria of Table 5.

1. Differentiation of Biovar Mouse from Biovars LGV and Trachoma

In addition to a lesser degree of DNA homology,[78] mouse may be readily distinguished from LGV and trachoma in at least two other important ways. First, its natural hosts are mice, not people, and, second, its antigens (the genus-specific antigen excepted) cross-react only minimally with antigens of the other two biovars. There is no cross-reaction at all in the serological reactions usually used to identify chlamydiae,[79,80] but when the major outer membrane protein of the mouse biovar is denatured with sodium dodecyl sulfate or oxidized with periodate, it reacts with monoclonal antibodies that recognize epitopes on the major outer membrane proteins of the LGV and trachoma biovars.[81]

2. Distinction Between Biovars LGV and Biovars Trachoma

These two biovars are much closer to each other than either of them is to mouse. Not only do their DNAs exhibit nearly complete homology,[78] their antigens also extensively cross-react at the species-specific, subspecies-specific, and serovar-specific levels when tested with monoclonal antibodies by micro-immunofluorescence.[80] These antigens are located mainly, if not exclusively, on the major outer membrane protein.[82,83] There are also structural differences between the outer membrane proteins of biovars LGV and trachoma.[84,85] Each biovar is uniquely defined by its behavior in natural human hosts, in laboratory animals, and in cell culture (Table 5).

In humans, the LGV biovar infects mainly cells of the lymphatic system, and, although its clinical manifestations are protean, they are all lumped together as a single disease entity, lymphogranuloma venereum.[4,86,87] In contrast, the trachoma biovar infects chiefly squamocolumnar epithelial cells in various tissues and organs of its human hosts to give rise to a whole spectrum of pathology that is described in terms of a number of distinct disease entities such as follicular conjunctivitis (trachoma), urethritis, cervicitis, salpingitis, and infant pneumonia[4,86,87] (Refer also to Chapter 8 "Overview of Human Diseases").

Table 6
CHLAMYDIA-LIKE MICROORGANISMS IN INVERTEBRATES

Host			
Common name	**Latin binomial**	**Phylum**	**Ref.**
Hydra	*Hydra viridis*	Coelenterata	91
Clam	*Mercenaria mercenaria*	Mollusca	92
Scallop	*Argopecten irradians*	Mollusca	93
Tellina	*Tellina tenuis*	Mollusca	94
Oyster	*Crassostrea angulata*	Mollusca	95
Spider	*Coelotes luctuosus*	Arthropoda	96
Scorpion	*Buthus occitanus*	Arthropoda	97
Isopod	*Porcellio scaber*	Arthropoda	98
Crab	*Cancer magister*	Arthropoda	99

In laboratory animals, the two biovars may be unequivocally separated.[86] Only the LGV biovar kills mice by the intracerebral route, and only the trachoma biovar causes a follicular conjunctivitis when instilled into the eyes of nonhuman primates.

In cell culture, the LGV biovar forms plaques on monolayers of susceptible cells such as the mouse L cell,[88] but the trachoma biovar has not been observed to produce plaques on any known host cell. Failure to produce plaques is an expression of the low efficiency with which this biovar establishes secondary infections in the cells adjacent to the primarily infected cell. Even with susceptible cell lines such as HeLa[221] or McCoy, infection is so inefficient that entry-promoting procedures are used both in initial infection of cell cultures with clinical specimens and in subsequent serial propagation. These procedures include centrifugation of the inoculum onto the host-cell monolayer[66] and pretreatment of the monolayer with polyanions.[89] Entry of the LGV biovar may be modestly enhanced (less than two-fold), but entry of the trachoma biovar is increased 10- to 100-fold.[90]

III. CHLAMYDIAL PHYLOGENY

Each step toward a better understanding of evolutionary relationships among the chlamydiae broadly defined satisfies a deep-seated human longing to know where things come from. For those of us concerned with pathogenesis of chlamydial disease and host resistance to infection, there are also practical benefits. Good phylogenetic information provides a rational basis for choosing the best animal models of human chlamydial disease and for predicting the behavior of one chlamydial agent of disease from that of another.

A. Some Matters of Phylogenetic Importance

1. Invertebrate Hosts

A casual search of the literature unearthed nine reports of chlamydia-like organisms living intracellularly in invertebrate hosts (Table 6). Only one of these microorganisms has been cultured outside its natural hosts,[94] and with the exception of the clam agent,[100] the evidence for kinship with *Chlamydia* is entirely morphological. The presence of a developmental cycle is inferred from electron microscopic observation of cell types resembling elementary bodies and reticulate bodies. The clam agent contains the genus-specific antigen[100,101] and its inclusions stain positively with iodine.[100] The agents from isopods[98] and hydras[91] do not react with genus-specific antibody. Rare observation of a chlamydia-like organism in an invertebrate host might be ascribed to chance association with a chlamydia of vertebrate provenance, but the repeated observation of such organisms in a wide range of invertebrates cannot be brushed aside. These invertebrate-dependent agents must be considered chlamydiae *sensu lato*, and a place must be found for them in chlamydial phylogeny.