(2)

AD-A250 768

REPORT DOCUMENTATION PAGE

1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution is unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S): NMRI 92-16	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Naval Medical Research Institute; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION: Naval Medical Command
6c. ADDRESS (City, State, and ZIP Code): 8901 Wisconsin Avenue, Bethesda, MD 20889-5055	7b. ADDRESS (City, State, and ZIP Code): Department of the Navy, Washington, DC 20372-5120
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: Naval Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER
8c. ADDRESS (City, State, and ZIP Code): 8901 Wisconsin Avenue, Bethesda, MD 20889-5044	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M161102BS13	AK-111	DA313955

11. TITLE (Include Security Classification)
The genera Coxiella, Wolbachia, and Rickettsiella

12. PERSONAL AUTHOR(S) Williams JC, Weiss E, Dasch GA

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Book chapter	FROM _____ TO _____	1992	14

16. SUPPLEMENTARY NOTATION In: The Prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications. 2nd edition. edited by A. Balows et al. Vol.III New York: Springer-Verlag. 1992 pp.2471-2484

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
rickettsial diseases; Q fever; tick-borne diseases; Coxiella species; Wolbachia species; Rickettsiella species

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Phyllis Blum, Librarian	(301) 295-2188	MRL/NMRI

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

SECURITY CLASSIFICATION OF THIS PAGE
UNCLASSIFIED

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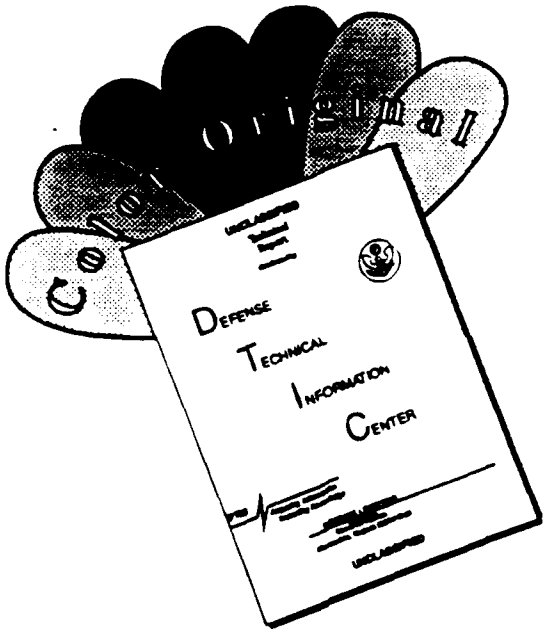

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The Prokaryotes

Second Edition

A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications

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Volume III

Springer-Verlag
New York Berlin Heidelberg London Paris
Tokyo Hong Kong Barcelona Budapest

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Library of Congress Cataloging-in-Publication Data
The Prokaryotes: a handbook on the biology of bacteria:
ecophysiology, isolation, identification, applications/editors, Albert Balows,
2nd ed.
Hans G. Trüper ... [et al.].
p. cm.
Includes bibliographical references and indexes.
ISBN 0-387-97258-7 (set).—ISBN 3-540-97258-7 (set)
1. Prokaryotes—Handbooks, manuals, etc. I. Balows, Albert.
QR72.5.P76 1991
589.9—dc20 91-17256

Printed on acid-free paper.

Production managed by Linda H. Hwang.
Editorial production management by Science Tech Publishers, Madison, WI.
Typeset by Impressions, Inc., Madison, WI.
Printed and bound by Arcata Graphics/Halliday, West Hanover, MA.
Printed in the United States of America.

9 8 7 6 5 4 3 2 1

ISBN 0-387-97258-7 Springer-Verlag New York Berlin Heidelberg
ISBN 3-540-97258-7 Springer-Verlag Berlin Heidelberg New York

CHAPTER 123

The Genera *Coxiella, Wolbachia,* and *Rickettsiella*

J. C. WILLIAMS, E. WEISS, and G. A. DASCH

Derrick (1939), in recognition of the help he received from Frank McFarlane Burnet in identifying the agent of Q fever as a rickettsia, generously named the agent *Rickettsia burnetii*. Philip (1943) recognized that this agent differed considerably from the other rickettsiae and renamed it *Coxiella burnetii*, in honor of Cox, who introduced the technique of yolk sac inoculation for the cultivation of rickettsia (see Chapter 122) and who with Davis (Davis and Cox, 1938) first isolated the Q fever agent in the United States.

The Genus *Coxiella*

As described in Chapter 121, the genus *Coxiella* is not closely related phylogenetically to the other Rickettsiales. *Coxiella burnetii* belongs in the gamma subdivision of the proteobacteria and is specifically, although distantly, related to *Legionella* and *Wolbachia persica*. The genus *Coxiella* has only one species, *C. burnetii*.

The genus *Coxiella* is currently defined by the following characteristics: 1) Obligate intracellular inhabitant of eukaryotes (Weiss and Moulder, 1984). 2) Mildly acidophilic, residing in the acidic compartments (i.e., the phagolysosome) of the host cell (Hackstadt and Williams, 1981a; Thompson, 1988). 3) Morphologically variant infective forms are derived from a developmental cycle by transverse binary fission and sporogenesis; some forms are small enough to pass through 0.2-μm bacteriological filters (McCaul and Williams, 1981) (see Fig. 1). 4) The cells stain by the method of Gimenez (1964, 1965), and may appear either Gram-negative or Gram-positive, with some microorganisms staining acid-fast by the Kinyoun carbolfuchsin method used for tubercle bacilli. The spores stain red by the Dorner method (McCaul and Williams, 1981). 5) Gram-negative envelope with peptidoglycan of the A-one-gamma-type (Amano and Williams, 1984). 6) Genus-specific lipopolysaccharide containing a nontoxic diphosphoryl lipid Cb (cognate to lipid A. Cb = *Coxiella burnetii*) (V. N. Reinhold et al., personal communication) with unusual fatty acid composition (Wollenberger et al., 1985), undefined core, and unique *o*-side chain sugars (Schramek et al., 1985; Amano et al., 1987). 7) Mutational variation in the lipopolysaccharide is linked to virulence shifts (Vodkin and Williams, 1986; Vodkin et al., 1986). 8) GC Content of the DNA of 43 mol% with a genome size of 1.04×10^9 Da and six genomic groups identified by restriction fragment length polymorphisms that are linked to virulence (Mallavia et al., 1991a). 9) Carries an endogenous plasmid of roughly 36 kb that varies in both size and restriction fragment length polymorphisms. This plasmid is linked to various disease potentials (Vodkin et al., 1986; Samuel et al., 1985).

These biological and biochemical properties distinguish *C. burnetii* from all known microorganisms. No other bacterium has been described with the unique combination of characters which includes growth within the phagolysosome, synthesis of a poorly endotoxic LPS, and sporulation.

The sporulation process in *C. burnetii* involves the asymmetrical formation of septae and the compartmentation of the cytoplasm of the large cell variant (LCV) mother cell (McCaul et al., 1991b). The cytoplasmic DNA of the mother cell is segregated into an endogenous spore which matures to form the small cell variant (SCV) resting cell. The sequence of events occurring in the mother cell during sporogenesis are as follows: i) a primary septum (S1) is formed by the asymmetric invagination of the cytoplasmic membrane; ii) the layers of membrane-like material circumscribe and engulf the segregated mother cell DNA and cytoplasm; iii) a dense band is formed between the outer face of the S1 and the cytoplasmic face of the cytoplasmic membrane of the mother cell; and iv) a secondary septum (S2) which does not fuse with the S1 separates the endogenous spore from the mother cell cytoplasm. The cell wall material of the spore appearing simultaneously with septate formation shows staining affinity indicative

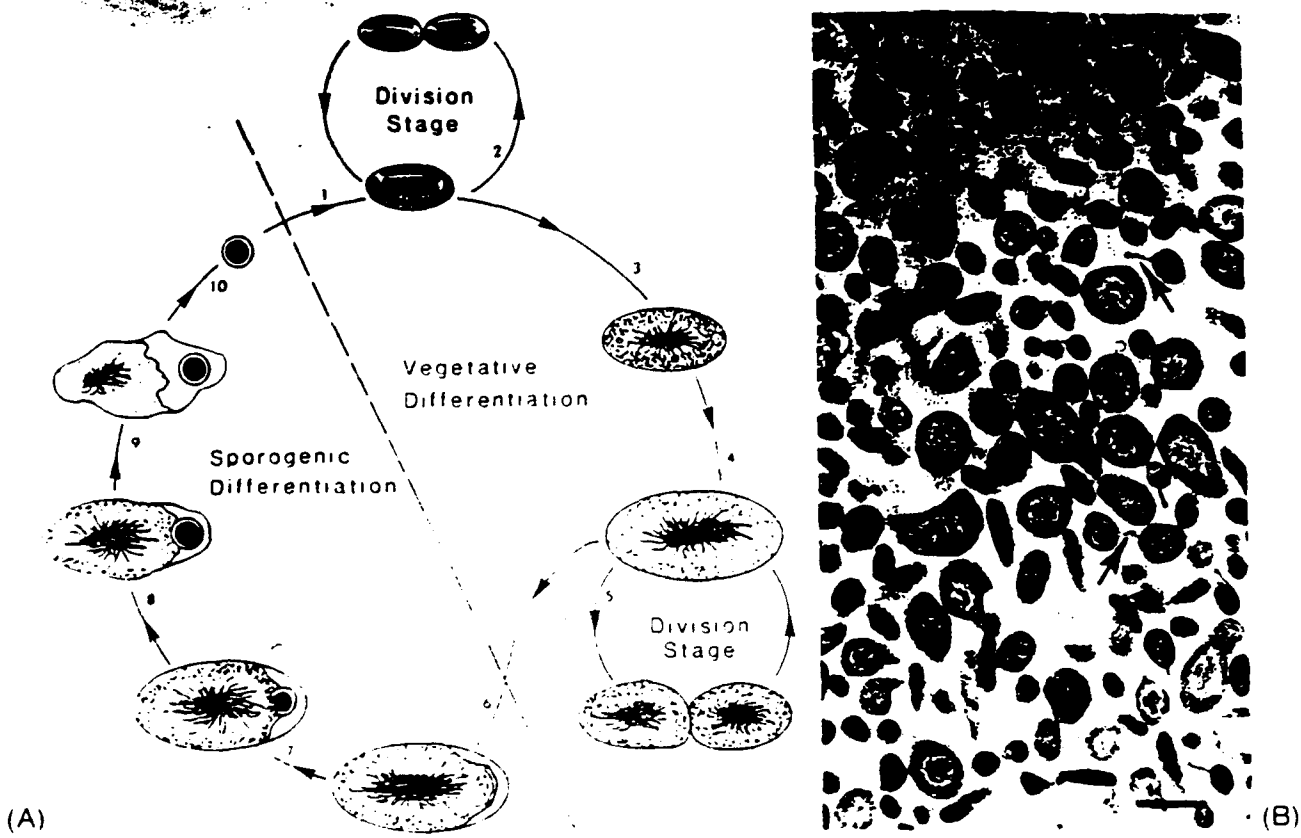

Fig. 1. Developmental cycle of *C. burnetii* within the phagolysosome of eukaryotic cells. (A) Schematic representation. Infection is probably initiated by a spore (stage 10) or by a small cell variant (SCV) (1), which may divide (2) or develop into an intermediate form (3) and a large cell variant (LCV) (4). The LCV may divide (5) or give rise to an endospore and lyse (6–10). (B) Thin section of *C. burnetii* preparation following Renografin gradient separation from yolk sac components. Note pleomorphism of *C. burnetii* cells. Arrows point to typical SCV and LCV. Bar = 0.2 μm. (Reproduced with permission from T. M. McCaul and J. C. Williams, *Journal of Bacteriology* 147:1063–1076, 1981, © American Society for Microbiology, Washington, D.C.)

of polysaccharides, mucopolysaccharides, mucopolysaccharide-protein complexes, peptidoglycan-like substances and non-peptidoglycan-like polymers. The newly formed spore is apparently the precursor to the small cell variant resting cell. In conclusion, the developmental cycle of *C. burnetii* consists of replication of the cell types by transverse binary fission and sporulation.

Habitats of *Coxiella*

Coxiella burnetii occupies an ecological niche in the microbial world that is shared by only a few other parasites (Weiss et al., 1991). Since the entire developmental cycle of this bacterial pathogen is carried out within the phagolysosomal compartment, *C. burnetii* is classified as a moderate acidophile. Growth and sporulation under the acidic condition of the phagolysosome raises questions about bioenergetics and survival in an otherwise microbicidal compartment. The host must elicit a concerted humoral and cellular immune response to control growth of the microorganism in the phagolysosome (Waag, 1990). The physiological adaptations that permit survival and growth within the phagolysosome probably contribute to the broad host range and documented transmission cycles of *C. burnetii*.

Q FEVER. The initial description of "Query" (Q) fever in Queensland Australia led to the identification of the bacterium in human blood and urine after injection of samples into guinea pigs and mice (Derrick, 1937). About the same time, the bacterium was obtained from ticks and grown in the yolk sac of embryonated chicken eggs in the United States (Davis and Cox, 1938). Sero-epidemiological studies have verified the presence of the microorganism in apparently disparate habitats throughout the world (Marrie, 1990). The worldwide distribution of *C. burnetii* is probably a result of the extremely broad host range and the natural transmission cycles that involve wild life, ar-

thropod reservoirs, and vertical transmission by aerosols to domestic animals and humans. Several reviews and individual reports (Berge and Lennette, 1953; Derrick, 1953; Kaplan and Bertagna, 1955; Stoker and Marmion, 1955; Wentworth, 1955; Syrucek and Raska, 1956; Babudieri, 1959; Yunker et al., 1975; Marrie, 1990) have compiled lengthy lists of infected hosts which include the following: human, sheep, goat, cattle, horse, donkey, camel, water buffalo, pig, dog, cat, gerbil, reptile, bird, goose, pigeon, dormouse, rabbit, rat, wild mice, chiggers (of bats and spiny rat), tick, and body louse. Transovarian transmission appears to be a mechanism of maintenance of *C. burnetii* in arthropod reservoirs (Stoker and Marmion, 1955). Experimental transmission from several species of ticks to laboratory animals suggests that ticks play a role in the spread of disease in natural environments. Indeed, infected ticks may excrete 10^{10} infective particles (Cox, 1940; Philip, 1948) which were shown to be infective for almost 1,000 days (Davis, 1943). Other possible vectors are body lice, bedbugs, fleas, and meal-worm larvae, all of which support multiplication of *C. burnetii*. Both lice and bed-bugs excrete the microorganism in feces for months. Also, the microorganism has been isolated from houseflies and maggots collected in or near cages of infected laboratory guinea pigs, but transmission by these potential vectors could not be demonstrated.

Domestic and wild animals play a major role in the airborne transmission of *C. burnetii* to humans (Tigertt et al., 1961). The predominant mechanism of transmission from one species to another is by primary aerosols generated after parturition by an infected animal. Secondary aerosols from contaminated birth fluids, blood, milk, feces, urine, wool, hides, clothes, straw, or other materials spread the microorganism throughout the environment. More recently, a role for cats and rabbits in transmission to humans was documented in isolated outbreaks where no large animal exposure occurred (Marrie, 1990). The environmental sources for possible transmission of *C. burnetii* to humans are numerous. An estimation of human infections based on the prevalence of antibodies against *C. burnetii* ranges from 4% for individuals with no or low risk of infection to >50% for those with occupational exposure. In a recent sero-epidemiological study of anti-*C. burnetii* antibodies in the serum of 495 city dwellers in Baltimore, MD, 6.6% individuals had antibodies against *C. burnetii* (phases I and II) and 28.2% were positive for the phase II whole cell antigen, as assayed by an enzyme-linked immunosorbent assay (J. C. Williams, M. J. England, and H. Paxton, unpublished observations). This survey indicates the potential for widespread Q fever in a locality known to have a high incidence of Q fever in domestic dairy herds (Wagstaff et al., 1965). In addition, 70% of wild mice trapped in Frederick County, Maryland were positive for antibodies against *C. burnetii* (Bolt, 1986). These examples highlight the current incidence of Q fever in humans and mice in selected areas of the United States. Recent sero-epidemiological surveys in Nova Scotia indicate widespread disease among humans and animals (Marrie et al., 1984).

Isolation of *Coxiella*

Infective samples used for isolating the microorganism require special precautionary handling to protect personnel and prevent contamination of the surroundings. These recommended precautions include: 1) biosafety level 2 practices and facilities for nonpropagative laboratory procedures, such as sero-diagnosis; and 2) biosafety level 3 practices and facilities for the homogenization of samples in any menstruum and the propagation of *C. burnetii* in any eukaryotic cell in either tissue culture or animals (Richardson and Barkley, 1988).

PROTECTION OF PERSONNEL. Laboratory-associated infections occur frequently when personnel are not protected by a phase I whole cell vaccine (Smadel et al., 1948; Meiklejohn and Lennette, 1950; Pike, 1976; Ascher et al., 1983; Marmion et al., 1984; Izzo et al., 1988). Q fever is also a concern for individuals at risk of infection while handling animals during and immediately after gestation. While infected animals may shed *C. burnetii*, parturition effectively releases large numbers (i.e., 10^9 microorganisms per gram of placental tissue). Since humans and animals can be infected with as few as 1 to 10 microorganisms (Tigertt et al., 1961), strict adherence to the guidelines must prevail during the attempted isolation of *C. burnetii* from environmental and laboratory samples.

ISOLATION OF *C. BURNETII* IN ANIMALS. Procedures for the isolation and amplification of obligate intracellular bacteria were presented in Chapter 122. The techniques presented here are intended to complement these procedures. To obtain a pure culture of *C. burnetii* from natural habitats (i.e., environmental samples, vectors, mammalian tissues, or clinical specimens), specimens are prepared as homogenates before the intraperitoneal injection of either guinea pigs or mice. The collection of "clean" samples enhances greatly the chances for a successful iso-

lation. Although *C. burnetii* is extremely stable in most environments (McCaul et al., 1981), contaminated samples may contain bacteria which kill the host animal before the outgrowth of *C. burnetii*.

In general, either the guinea pig or the mouse may be selected for the first inoculum prepared as a 1 to 10% homogenate in phosphate-buffered saline or in a nutrient bacteriological medium. After the injection of the guinea pig, a fever may start 3 to 15 days after inoculation, last for 2 to 8 days, and subside without recurrence. The temporal fever curve is dosage dependent and death of the animal rarely occurs unless the inoculum dose is larger than 10^8 microorganisms or the strain is extremely virulent.

ISOLATION OF C. BURNETII IN TISSUE CULTURE. The isolation of individual microorganisms, directly from a field sample or after subpassage, can be accomplished by plaque purification techniques (Ormsbee and Peacock, 1976). Both phase I and phase II variants form plaques in established cell lines and primary cultures of chicken embryo fibroblasts. The plaque purification technique is extremely valuable for the quantification of viable *C. burnetii* and the study of genetic variation between disparate geographical isolates. Since most isolates contain a mixture of phase variants, the plaque purification technique is recommended for the selection of variant forms of *C. burnetii* chosen for further investigation.

INFLUENCE OF VIRULENCE ON ISOLATION. The isolation of *C. burnetii* is greatly influenced by the virulence of the microorganism. Virulent microorganisms in phase I synthesize a "smooth-type" lipopolysaccharide (LPS) which apparently shields the bacterial surface from the destructive activities of the phagolysosome and the bactericidal reactions of the immune response (Baca and Paretsky, 1983). Avirulent microorganisms in phase II synthesize a "rough-type" LPS which is a truncated form of the "smooth" LPS. The absence of the smooth LPS results in the exposure of surface proteins which may be more accessible to the hydrolytic enzymes of the phagolysosome (Williams and Stewart, 1984). Since phase I *C. burnetii* produce a systemic disease in immunocompetent animals, the microorganisms are easily isolated from the spleen, liver, and kidney of infected animals. However, avirulent phase II microorganisms usually do *not* survive the host immune response thereby producing a self-limited disease. A vigorous host immune response generally precludes the isolation of phase II *C. burnetii* from organs of infected animals (Williams et al., 1986b). Yet, the phase II microorganisms grow profusely in antibiotic-free yolk sacs of embryonated eggs and eukaryotic cells in culture.

INFLUENCE OF ANTIBIOTICS ON ISOLATION. The use of antibiotics to prevent the growth of unwanted microbes during the isolation procedure is not generally recommended, but when the specimen is heavily contaminated, clindamycin, erythromycin, viomycin, cycloserine, cepholathin, chloramphenicol, streptomycin, or penicillin(s) may be used as bacteriostats of various microbes, while allowing the growth of *C. burnetii* (Spicer et al., 1981). Rifampin, trimethoprim, doxycycline, and oxytetracycline are effective inhibitors of the growth of *C. burnetii* in animals and tissue culture. Several quinolone compounds, such as ofloxacin, pefloxacin, difloxacin, oxolinic acid, and ciprofloxacin, are effective inhibitors of growth of the Nine Mile strain of *C. burnetii* (Baca, 1989). Also, gentamicin sulfate is effective in preventing the growth of *C. burnetii* in infected BHK-21 cells passed as few as six times in culture (J. C. Williams and M. J. England, unpublished observations). The strains implicated in the continuance of chronic Q fever are resistant to elimination in vivo by antibiotic therapy, and these strains are also more resistant to antibiotics in vitro (Baca, 1989). The isolation of naturally antibiotic resistant strains is rare. However, a strain resistant to tetracycline was isolated and identified as the cause of tetracycline treatment failure in an epidemic in humans exposed to aerosols following abortions in sheep (Spicer et al., 1981). Therefore, one must be cautions when using antibiotic selection for the isolation and enrichment of *C. burnetii*.

AMPLIFICATION OF AN ISOLATE. Attenuation of virulent strains by an undefined genetic mechanism referred to as phase variation occurs upon subpassage of *C. burnetii* in vitro in either tissue culture or the yolk sacs of embryonated eggs (Stoker and Fiset, 1956). Subpassage of phase I microorganisms in immunocompetent animals maintains the phase I character. Thus, microorganisms in phase I can be recovered from a mixed population of phase I and phase II cells by subpassage in animals. The outcome of repeated subpassage, in the absence of an immunologic selective pressure, is the isolation of a pure culture of genetically stable phase II microorganisms which do *not* revert to phase I in immunocompetent animals (Williams et al., 1986b). These properties of *C. burnetii* led scientists to speculate that phase I is the naturally occurring variant, while the phase II variant is

a laboratory artifact. This assumption is unlikely because there is evidence that phase II variants can be selected in animals after the injection of phase I microorganisms. The most likely scenario is that the current isolation procedures select for the phase I variant while eliminating the phase II microorganism. The failure to obtain an isolate after injection of immunocompetent animals should be followed by isolation procedures carried out in tissue culture or yolk sacs of embryonated eggs.

Identification of *Coxiella*

The clinical and laboratory diagnosis of Q fever is a formidable challenge because of the nonspecific symptoms and clinical signs and the difficulty of propagation and characterization of the microorganisms. The symptoms may include malaise, chills and fever, myalgia, severe headache, atypical pneumonia with dry unproductive cough, and chest pains (Sawyer et al., 1987; Marrie, 1988). Therefore, in clinical diagnosis of acute infections, Q fever is often confused with influenza. Chronic Q fever usually goes unrecognized for many years because symptoms are nonspecific and they vary significantly from patient to patient. Established procedures for the laboratory diagnosis are accomplished by serological detection of a three to four fold rise in antibody against whole cell phase I and phase II antigens. More direct diagnostic methods which will eventually eliminate the use of the whole-cell antigens are currently in the early stages of development. These include the detection of 1) antibodies against *C. burnetii*-specific LPS, protein antigens, and antigenic peptides; 2) antibodies against strain-specific peptides; and 3) DNA sequences encoding specific strain differences.

The host immune response to infection by *C. burnetii* is first recognized by antibody formation to surface proteins of the avirulent phase II and the virulent phase I microorganism. Antibodies directed against phase I smooth LPS occur later in the infection, and high titers to phase I cells and smooth LPS are diagnostic for chronic disease (J. C. Williams, and T. H. Marrie, unpublished observations). However, most individuals with acute Q fever resolve the infection without the formation of detectable antibody to the smooth LPS (J. C. Williams, unpublished observations). Importantly, individuals vaccinated with inactivated phase I whole cells and considered to be immune usually do not develop titers to the smooth LPS (J. C. Williams, unpublished observations).

Recent observations of the antigenic structure of *C. burnetii* have revealed the complexity of the interaction of the morphological cell types with the host immune system (McCaul et al., 1991a; Williams et al., 1990). The antigenic structure of *Coxiella burnetii* morphological cell types was investigated by post-embedding immunoelectron microscopy and immunoblotting. Polyclonal antibodies produced in rabbits against phase I whole cells, the chloroform:methanol residue of phase I whole cells, the cell walls of the large and small cells, and the peptidoglycan-protein complexes labelled antigenic epitopes of both of the cell types. Murine monoclonal antibodies against the phase I lipopolysaccharide labelled the cell walls of the small cells and a subpopulation of the highly organized large cells. Murine monoclonal antibodies against a 29.5-kDa outer membrane protein labelled only the large cells. The absence of the 29.5-kDa protein in purified and pressure-resistant small cells was verified by the inability to detect the protein, by Coomassie brilliant blue staining of SDS-polyacrylamide gels, and immunoblotting. Neither the polyclonal nor the monoclonal antibodies were effective labels of the spore within the large mother cell. The pattern of immunolabels obtained with the monoclonal antibodies clearly indicated that the observed ultrastructural differences between the cell types reflected different cell-associated antigens. The antigenic differences between the spore, the small cells, and the large cells may relate to the events of either stage-specific sporulation or growth by transverse binary fission, both of which constitute the developmental cycle.

Expression of different surface antigens throughout the developmental cycle probably confers a biological advantage for *C. burnetii*. Antigenic variation of parasite surface structures during stage-specific differentiation is a mechanism of evading host immunological responses. *Coxiella burnetii* readily infects humans, thereby causing, not only subclinical or acute Q fever (classical Q fever), but also chronic disease (endocarditis or granulomatous hepatitis) (Marrie 1988). Properties of the microorganism, such as the expression of antigenically distinct forms might also confer biological advantage for the survival of *C. burnetii* in an immunologically hostile environment. Other biological factors that may contribute to the persistence of *C. burnetii* infections are the immune-suppressive complex (Waag, 1990), the lipopolysaccharide (Amano et al., 1987), and acid activation of metabolism (Hackstadt and Williams, 1981a). These virulence factors could contribute to the establishment of chronic and recrudescent infections in both immune and immunocompromised individuals.

DETECTION OF ANTIBODIES. The serological identification of antibodies directed against *C. burnetii* has been accomplished by use of several diagnostic tests, namely, complement fixation (CF) (Stoker and Fiset, 1956), microagglutination (MA) (Fiset et al., 1969), capillary-tube agglutination (CA) (Luoto, 1956), microimmunofluorescence (MIF) (Peacock et al., 1983), radioimmunoprecipitation (RIP) (Tabert and Lackman, 1965), enzyme-linked immunosorbent assay (ELISA) (Williams et al., 1986a), or immunoblot assay (IB) (Williams and Stewart, 1984). The CF, MA, and CA are the least sensitive tests for the detection of antibody and therefore they are *not* recommended. The MIF test and the ELISA are recommended for the detection of specific antibodies because 1) they are more sensitive than the other tests; 2) the class and subclass of antibodies against specific antigens are easily quantitated; and 3) large numbers of serum samples can be tested at one time. Additionally, the ELISA can be used with soluble and particulate antigens. Both the microimmunofluorescence test and the ELISA are commercially available from several companies. The RIP and IB assays are research tools that are currently not available for serodiagnosis.

The prototype strain of choice for the preparation of antigens is the isogenic Nine Mile variants in either phase I (clone 7) and phase II (clone 4) (Williams et al., 1981). The phase II variant is a semi-rough LPS mutant with more protein epitopes exposed on the surface than the smooth LPS parental phase I strain (Williams and Stewart, 1984). The absence of the smooth LPS renders the phase II strain a more sensitive indicator of antibody against *C. burnetii*. The phase II whole-cell antigen also has "common antigen" protein exposed on the surface (Vodkin and Williams, 1988; Williams et al., 1990). This property of the phase II antigen also gives more cross-reactions and, therefore, more false positives, than the phase I whole-cell antigen. Thus, for the standard serological diagnosis of Q fever one must *not* rely solely on the phase II whole-cell antigen. Both phase I and phase II whole-cell antigens are recommended for the detection of antibodies against *C. burnetii*.

Since Q fever can progress from the acute disease to chronic hepatitis and endocarditis, an antibody-based serologic test to distinguish the various disease entities is desirable. The ratio of antibody titer and the antibody class reacting with a specific antigen have proven utility in the diagnosis of the disease progression (Peacock et al., 1983). Both the MIF test and the ELISA are effective in the detection of specific antibodies of the acute or the chronic forms of Q fever. The ELISA is the most sensitive assay for class-specific antibodies against whole-cell antigens and soluble antigens (Williams et al., 1986b). Studies conducted with *C. burnetii*-specific synthetic peptides indicate that peptide-based serodiagnosis is feasible (J. C. Williams et al., unpublished observations). Current methods demonstrate highly specific antibody reactions with the purified LPS of the Nine Mile strain (Williams et al., 1986b).

PREPARATION OF DIAGNOSTIC ANTIGENS. The preparation of the antigens for the above antibody tests are carried out by purifying viable microorganisms free of host materials (Williams et al., 1981). Purification by Renografin gradients, as the final step in the procedure, is recommended for the removal of host membranes and mitochondria. The purified viable microorganisms may be treated with 1% formalin to cross-link and stabilize the surface antigens. Treatment of the whole-cell antigens with higher concentrations of formalin only serves to cross-link and denature valuable protein antigens without inactivating *C. burnetii*. Complete inactivation of the cells is accomplished by the delivery of 2.1 mega rads of gamma irradiation (i. e., cobalt 60) while the microorganisms are held on dry ice (Scott et al., 1989). This procedure is required to inactivate the endogenous spore which develops within the mother cell (T. F. McCaul, H. A. Thompson, and J. C. Williams, unpublished observations).

The soluble LPS of the phase I cell is purified by a hot phenol method (Amano et al., 1987). After dialysis against water to remove phenol and salts, the antigen may be used in the ELISA (Williams et al., 1986a, 1986b).

The preparation of diagnostic antigens by methods which change the phase I antigen to phase II antigen by chemical treatments of whole cells have been described. The phase I whole-cell antigen is easily converted to the phase II whole-cell antigen by treatments with trichloroacetic acid (Anacker et al., 1962; Brezina and Urvolgyi, 1961) or potassium periodate (Schramek et al., 1972). These chemically derived antigens are poor substitutes for the native undenatured antigens described above.

DETECTION OF ANTIGENS. Antigen capture methods using monoclonal and specific polyclonal antibodies are currently being explored in several laboratories. Studies involve the use of polyclonal or monoclonal antibodies against unique surface antigens. The sugars unique to the *C. burnetii* LPS are excellent targets for the development of monoclonal antibody capture methods (Schramek et al., 1985). Through recombinant DNA techniques, specific protein

antigens (Vodkin and Williams, 1988) are being studied for the development of whole molecule or synthetic peptide-based monoclonal antibody capture diagnostic techniques.

DETECTION OF GENOMIC AND PLASMID DNA. Recombinant DNA technology has revolutionized the development of diagnostic methods (Tenover, 1988, 1989). Unique nucleotide sequences of the *C. burnetii* chromosome and plasmid have been identified that 1) distinguish *Coxiella* from other microorganisms; 2) are indigenous to the species; and 3) are appropriate for the detection of strains which cause either acute or chronic diseases (Mallavia et al., 1991a). A repetitive sequence has been identified as a possible species-specific probe (T. A. Hoover et al., unpublished observations). The development of these specific nucleotide sequences as probes when coupled with the polymerase chain reaction (Guatelli et al., 1989) will greatly improve the detection of *C. burnetii* in samples.

Ecophysiology of *Coxiella*

Entry of *C. burnetii* into a host cell is sometimes described as a passive event on the part of the parasite, achieved by phagocytosis (see Baca and Paretsky, 1983). However, the efficiency by which *C. burnetii* infects animals and enters both professional and nonprofessional phagocytes in culture casts some doubt on the assumption that phagocytosis is the only mechanism. Williams and Vodkin (1987) presented data which suggest that some of the defense proteins of the host, discharged into serum, facilitate entry of *C. burnetii* into the cells and, more specifically, into their phagolysosomes. Thus, *C. burnetii* exploits the host's natural defense mechanisms to become established in its ecological niche, a phenomenon not unlike that encountered in other parasites of the phagolysosome (Weiss et al., 1991).

Although axenic growth of *C. burnetii* has not been achieved, a variety of host-independent metabolic activities have been demonstrated by several investigators using cell-free extracts or whole cells (Baca and Paretsky, 1983). Hackstadt and Williams (1981a) have shown that the optimum pH for such activities by whole cells is 5.0 or below and is negligible at pH 7.0. That such a restriction prevails in the natural environment was demonstrated by their finding that chemical reagents which raise the pH of the *C. burnetii*-containing phagolysosomes, prevent growth. The chief source of energy is derived from the aerobic oxidation of glutamate, but also intermediates of the citric acid cycle can be used. Also, in contrast to the rickettsiae, glucose can be used by *C. burnetii*. Significant protein synthesis has been demonstrated in a high potassium-low sodium medium containing the naturally occurring amino acids (Zuerner and Thompson, 1983). As a remarkable adaptation to its acidic environment, it was shown that at pH 7.0, the ATP pool of *C. burnetii* is quite stable, even in the absence of glutamate, but at pH 4.5, without substrate the ATP level declines rapidly (Hackstadt and Williams, 1981b). Thus, *C. burnetii* has two mechanisms that explain its remarkable stability in the environment: one is its sporogenic cycle, the other its metabolic inactivity at neutral pH.

Applications of *Coxiella*

The production of DNA probes, primers, and other reagents for the rapid identification of *C. burnetii* infection has been discussed. Of particular interest is the development of tests that distinguish between strains that only cause acute infection from those that are likely to persist in the patient and in many instances lead to life-threatening hepatitis and endocarditis. Patients with the latter conditions require close attention. It was shown by Mallavia et al. (1990b) that chronic strains have a specific plasmid, QpRS, or, if they do not carry the plasmid, they carry the same base sequence in their chromosomal genome. Thus, it should be possible to develop a two-step polymerase chain reaction (PCR) amplification (Guatelli et al., 1989), the first step identifying *C. burnetii* infection, followed, if positive, by a second step identifying the base sequence typical of the chronic strains.

Ormsbee et al. (1964) have clearly shown that a whole-cell Q fever vaccine is far more effective when obtained from phase I than from phase II cells. However, because of its immunomodulatory complex (IMC) content (Waag, 1990), it is a relatively toxic preparation. Subunit protein antigens, free of LPS and IMC, are now being tested for their vaccinogenic potential (Williams et al., 1990).

The genus *Wolbachia*

The generic designation *Wolbachia* honors S. Burt Wolbach, who described the rickettsia of Rocky Mountain spotted fever and, in collaboration with Marshall Hertig (1924), studied the rickettsia-like microorganisms of insects (see Weiss et al., 1984). The species of this genus probably have very little in common. One feature that they share is a habitat in blood-sucking arthropod hosts, which derive no obvious benefit or harm from their presence. Of the three

species, only *W. persica* has been studied extensively, although unevenly. *W. pipientis* has aroused considerable interest, but investigations have been hampered by limitations in methods of propagation. *W. melophagi* is only of historic interest, since observations on this microorganism have not been reported for almost three decades.

Habitat of *Wolbachia persica*

W. persica was isolated by Earl C. Suitor, Jr. from the Malpighian tubules and gonads of the tick *Argas persicus* (= *A. arboreus*) collected in Egypt by Harry Hoogstraal (Suitor and Weiss, 1961). Although seen in most *Argas* ticks received from Egypt, isolations were successful only from those that had fed on the buff-backed heron, *Bubulbus ibis*. Isolation attempts failed when the ticks were parasites of chickens or were fed on chickens in the laboratory (Suitor, 1964b). *W. persica* isolates, morphologically and antigenically very similar to the Suitor isolate, were also obtained from the wood tick, *Dermacentor andersoni*, collected in Montana (Burgdorfer et al., 1973). Cory et al. (1975) described the isolation of five wolbachia-like agents from the blood of the chipmunk, *Eutamias ruficaudus*, presumably a host of the tick carrying the wolbacniae. Isolations were successful in moth (*Antherea eucalypti*) and mosquito (*Aedes albopictus*) cell lines, but not by the injection of chicken embryos, guinea pigs, or voles. Intracellular bacteria, identical or similar to *W. persica*, have been observed in ticks quite often in many parts of the world, ever since Cowdry (1925) first described them. See, for example, Trager (1939), Sixl-Voigt et al. (1977), Hayes and Burgdorfer (1981), and Amiressami (1988). Whether all these symbionts of ticks are related to *W. persica* is not known.

Isolation and Identification of *Wolbachia persica*

W. persica cells are spherical, approximately 0.7 μm in diameter, but quite variable in size. Gram-negative and best visualized in smears fixed with Carnoy's fixative and stained with Giemsa's stain. Most electron micrographs of *W. persica* and wolbachia-like agents have demonstrated that the cells are located in the vacuoles of their host cells, where they multiply by binary fission and achieve a high-population density. The cell envelope is typical of Gram-negative bacteria, but exhibits significant variation in thickness, smoothness, and rigidity of the outer membrane, which is not always clearly separated from the cytoplasmic membrane. The wolbachiae, unlike the typhus and spotted fever rickettsiae, do not seem to have a microcapsular layer (Suitor and Weiss, 1961; Suitor, 1964c; Burgdorfer et al., 1973; Sixl-Voigt et al., 1977; Hayes and Burgdorfer, 1981). Whether differences encountered reflect genetic variation among the wolbachiae or variation in physiologic state as observed for *R. rickettsii* (Hayes and Burgdorfer, 1982) is not known.

W. persica is cultivated most satisfactorily in the yolk sac of chick embryos, by procedures identical to those used for rickettsiae. Although there is every indication that it grows only intracellularly, heavily infected yolk sac suspensions contain numerous *W. persica* cells in their lipid fraction. The cells can be released, during purification procedures, by treatment with trypsin. The identity of the isolate with the microorganisms seen in the Malpighian tubules of the ticks was demonstrated by fluorescent antibody staining, using antibodies elicited in rabbits by injection of the yolk sac isolate (Suitor and Weiss, 1961; Suitor, 1964c). *W. persica* has also been grown in the human body louse, the mealworm (*Tenebrio molitor*), and the tick *Ornithodoros moubata* (Weyer, 1973). Attempts to cultivate this microorganism in a variety of cell-free media, including those designed for the growth of *Mycoplasma*, have failed (Suitor, 1964c). Yolk sac grown *W. persica* is pathogenic for the tick or laboratory animals only when injected in large numbers. The range of antibiotic susceptibility is approximately the same as that of the rickettsiae (Suitor and Weiss, 1961).

The antigenic structure of *W. persica* has been investigated with the aid of monoclonal antibodies (Dasch et al., 1990). A *Wolbachia*-specific 50 kDa protein antigen and a lipopolysaccharide were detected with antibodies which did not react with typhus or spotted fever rickettsiae, or with *Rochalimaea*, *Ehrlichia*, *Chlamydia*, *Legionella*, or *Proteus*. The presence in *Wolbachia* of the common bacterial 60-kDa protein was also detected with broadly cross-reactive monoclonal antibodies.

Ecophysiology of *Wolbachia persica*

The in vitro metabolic activities of *W. persica*, separated from host constituents, have been studied extensively by measuring O_2 consumption, $^{14}CO_2$ production from radiolabeled substrates, and incorporation of radiolabeled carbon into various macromolecules (Weiss et al., 1962, 1964; Neptune et al., 1964a, 1964b). In contrast to rickettsiae, *W. persica* actively metabolizes glucose, to approximately the same extent that *Rickettsia prowazekii* uses glutamate. Other substrates used by *W. persica* are serine, pyruvate, glutamate, glutamine, glycerol, and

acetate. *W. persica* also displays an active in vitro lipid metabolism. In the presence of metabolizable substrates, such as glucose or serine, it incorporates added palmitate, as well some of the carbons from glucose or serine, into phosphatides. The GC content of the DNA is approximately 30 mol% (Kingsbury and Weiss, 1968). As mentioned in Chapter 121, 16S rRNA sequence analysis indicates that *W. persica* belongs in the gamma subdivision of the proteobacteria and is distantly related to the *Coxiella-Legionella* cluster.

Applications of *Wolbachia persica*

Because of relative ease of cultivation and lack of virulence for its hosts, *W. persica* is a convenient bacterium to use for basic studies of endosymbiosis, for the preparation of reagents that distinguish it from rickettsial pathogens, and for the study of bacterial phylogeny.

Habitat of *Wolbachia pipientis*

W. pipientis was first described by Hertig (1936) as occurring in the gonads and gut epithelium of the mosquito *Culex pipiens*. In the mature insect, *W. pipientis* is found primarily in the cells of gonads and is inherited by offspring via the egg cytoplasm (Yen and Barr, 1974). Morphologically identical organisms have been reported in virtually all strains of *C. pipiens* collected from various parts of the world, and from several other species of the genus *Culex* (Irving-Bell, 1977; Curtis, 1983; Larsson, 1983). Similar, if not identical, organisms have been seen with variable frequency in other insects, such as mosquitoes of the *Aedes scutellaris* group (Beckett et al., 1978; Wright and Barr, 1980), and the almond moth, *Ephestia cautella*, a cosmopolitan pest of dry fruits, grains, nuts, and other stored vegetable products (Kellen et al., 1981). The intracellular bacterium seen in tsetse flies (*Glossina*) and cultivated on a mosquito cell line (Maudlin and Ellis, 1985; Welburn and Maudlin, 1987) is probably not related to *W. pipientis*.

Identification of *Wolbachia pipientis*

W. pipientis is a Gram-negative rod ranging in size from 0.25 to 1.5 μm. It is located in vacuoles, presumably phagosomes, of host cells and multiplies by binary fission. The fine structure is typical of Gram-negative bacteria, except that the cell wall displays some plasticity. The cytoplasm sometimes contains phage particles (Wright et al., 1978). Attempts to grow *W. pipientis* outside the host have not been successful and differentiation of strains by immunological methods or macromolecular migration patterns has not been reported.

Ecophysiology and Applications of *Wolbachia pipientis*

The chief interest in *W. pipientis* is derived from the observation that it plays a role in reproductive incompatibility between strains (or subspecies) of the host. Mating of insects from different geographic areas sometimes produce eggs that do not hatch, since females cannot be fertilized with the infected sperm of some strains. When the male mosquitoes are rendered aposymbiotic (deprived of their intracellular bacteria) by addition of 25 μg/ml tetracycline to the larval rearing water or by maintaining the larvae at elevated temperature (32–33°C) for 5 to 7 days, incompatibility is eliminated. The female insects can be fertilized whether infected or not. Cytoplasmic incompatibility has also been demonstrated in the *Aedes* mosquitoes and in the almond moth, and microbial reproductive incompatibility has been suggested as a possible approach to pest control (Yen and Barr, 1974; Fine, 1978; Wright and Wang, 1980; Kellen et al., 1981). Infection of the midgut cells of the tsetse fly does not appear to be associated with its susceptibility to *Trypanosoma congolense* infection (Moloo and Shaw, 1989).

Wolbachia melophagi

W. melophagi is found in the wingless fly, *Melophagus ovinus*, a parasite of sheep commonly called sheep ked. It adheres to the gut epithelium but is not intracellular. Nöller (1917) reported its cultivation on blood-glucose-bouillon agar, on which colonies 0.4 to 0.6 mm in diameter appeared after 35 to 40 days. Hertig and Wolbach (1924) detected minute colonies after 3 to 5 days. Kligler and Ascher (1931) cultivated microorganisms not only derived from the sheep ked, but also from diptera infecting the goat, horse, and dog. It has been grown in the yolk sac of chicken embryos by Steinhaus (1946) and by Henneberg and Wolff (1963). Interest in this microorganism stemmed from its similarity to *Rochalimaea* in morphology, staining properties, and host-cell interaction, and the possibility that it might serve as an axenic model for the rickettsiae. Interest waned when *Rochalimaea* was cultivated in an axenic medium (see Chapter 122).

The Genus *Rickettsiella*

The generic designation *Rickettsiella* has been applied to a variety of intracellular bacteria that are pathogenic for their invertebrate hosts. Ex-

cept for a recent study of genome size (Frutos et al., 1989), most of the available information has been derived from light and electron microscopy and infection of other invertebrate and vertebrate hosts. Much of the information that is generally used for bacteria characterization and classification is not available. Because of this lack of information, a conservative approach to taxonomy has been adopted (Weiss et al., 1984).

Habitats of *Rickettsiella*

R. popilliae is a parasite of the Japanese beetle, *Popillia japonica* in the USA (Dutky and Gooden, 1952), of the cockchafer, *Melolontha melolonthae*, and of the meals worm, *Tenebrio molitor*, in Germany (Krieg, 1955, 1965) and several other insects in Europe and Africa. *R. grylli* is a parasite of the cricket, genus *Gryllus*, of the isopod, genus *Armadillidium*, in France (Vago and Martoja, 1963; Vago et al., 1970), and other insects and crustaceans in the Near East and the USA. *R. chironomi* is a parasite of the midge, genus *Chironomus* (Weiser, 1963), of the spider, genera *Argyrodes* and *Pisaura* (Meynadier et al., 1974; Morel, 1977), and of the scorpion, genus *Buthi* (Morel, 1976) in Europe. Numerous other specific names, recognizing the host, and some other generic names have been proposed, among them, *R. phytoseiuli*, a parasite of the mite, genus *Phytoseiulus*, in Czechoslovakia (Šuťáková and Ruttgen, 1978), and *R. (Porochlamydia) buthi* for the parasite of the scorpion (presumably a *Rickettsiella*. In addition, several of these bacteria have not been named, among them, parasites of marine invertebrates in North American and European coasts (Bonami and Pappalardo, 1980; Elston and Peacock, 1984), a habitat similar to that of bacteria generally regarded as chlamydiae (Page and Cutlip, 1982).

Identification of *Rickettsiella*

Most of these bacteria appear to have a cycle of development somewhat similar to that of the chlamydiae see Chapter 202). For example, *R. popilliae* infection is started by rod-shaped particles, 0.2 to 0.6 μm in size, which gain entrance into vacuoles of cells of the fat body or hepatopancreas of their hosts. There they enlarge to particles, at least 1.0 μm in diameter, and divide by binary fission. Eventually small particles are reformed which escape from the cell to start a new cycle. In some of the species, such as *R. chironomi*, the infectious particle is disk-shaped, 0.06 by 0.6 μm in size, rather than rod-shaped. A unique feature among some *Rickettsiella* species is the formation of giant round cells from which arise bipyramidal crystalline bodies, 0.8 by 1.8 to 3.8 μm in size. These bodies possibly derive from the albuminoid reserve of the host as the result of a disturbance of host cell metabolism (Huger, 1959; Krieg, 1959; Federici, 1980; Šuťáková, 1988). Rickettsiellae generally have outer membranes typical of Gram-negative bacteria and stain well by methods used for rickettsiae and chlamydiae.

Virulence of rickettsiellae for their hosts varies considerably. Interestingly, crickets infected with *R. grylli* survive better when reared at 30°C than at lower temperatures. They do even better when reared in a temperature gradient, where they select a temperature of 33°C when infected and 26.6°C when not infected, an excellent example of protective thermoregulatory behavior (Louis et al., 1986). Intraperitoneal injection of *R. grylli* into mice does not elicit obvious effects, but eventually lesions form in the spleen and liver, often leading to tumor formation (Delmas et al., 1985, 1986). Limited growth of *R. popilliae* has been obtained in chicken embryo entodermal cell cultures and in McCoy cells incubated at 28 or 32°C, the numbers of the rickettsiellae increasing during the first 3 weeks of incubation, but no growth was demonstrated after four or five passages (Suitor, 1964a). Limited growth of *R. popilliae* has also been obtained in other mammalian cell lines (Pourquier et al., 1963) and of *R. grylli* in cricket cardiac cell cultures (Meynadier et al., 1967). For antigen preparations, rickettsiellae are often cultivated in laboratory strains of arthropods. For example, *R. phytoseiuli* was successfully grown in *Dermacentor reticulatus* ticks (Šuťáková and Reháček, 1989).

Ecophysiology of *Rickettsiella*

The genome size of four species of *Rickettsiella* was determined by pulse-field gel electrophoresis (Frutos et al., 1989). The sizes ranged from 1,550 kb for *Porochlamydia buthi* to 2,650 kb for *R. chironomi*, with intermediate sizes for *R. popilliae* and *R. grylli*. The genome size of two species of *Chlamydia* determined in the same experiment was 1,450 kb. The restriction enzyme digest patterns for each of the DNAs also differed for each species.

Applications of *Rickettsiella*

The chief interest in these microorganisms stems from their effect on laboratory insectaries and other animals. Some have been considered as control agents for agricultural pests, since they are maintained in the soil for years, and infection of offspring is effected through soil contamination, rather than transovarian passage.

Acknowledgments

The preparation of this chapter was supported in part by the Naval Medical Research and Development Command, Department of the Navy, work unit #3M161102.BS13.AK.111.

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