Chapter 11
Axenic Growth of *Coxiella burnetii*

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Contents

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Abstract Early metabolic studies of *C. burnetii* showed minimal metabolic activity of axenic (host cell-free) organisms in buffers adjusted to neutral pH. However, our understanding of the organism's physiology was greatly improved upon the discovery that *C. burnetii* requires an acidic pH for metabolic activation. Indeed, information gained from acid activation studies coupled with contemporary analyses using transcription microarrays, metabolic pathway reconstruction and metabolite typing, led to an axenic culture system that supports robust growth of *C. burnetii*. While axenic culture of *C. burnetii* can present some technical challenges, the technique is currently facilitating new lines of investigation and development of genetic tools. Axenic cultivation of *C. burnetii* should also prove useful in clinical settings.

Keywords Axenic cultivation • Culture system • Extracellular • Metabolic activity • Microaerobic

11.1 Introduction

The discovery of *C. burnetii* as the causative agent of human Q fever in the late 1930s was followed almost immediately by unsuccessful attempts to cultivate the pathogen in axenic (host cell-free) medium (Cox 1938). Microscopic evaluation established an intracellular vacuolar niche for the organism and contributed to *C. burnetii*'s designation as an obligate intracellular bacterium. Subsequent analyses of the organism's metabolic abilities in buffers adjusted to physiological pH showed substantial activity of central metabolic pathways (e.g., glycolysis and RNA synthesis) in bacterial extracts, but not intact cells (Baca and Paretsky 1983). The demonstration that the *C. burnetii* parasitophorous vacuole (PV) exhibits phagolysosomal characteristics (Burton et al. 1971, 1978) led to the discovery that a moderately acidic pH mimicking that of a phagolysosome (~pH 4.5) is required for metabolic activation of *C. burnetii* (Hackstadt and Williams 1981a). Indeed, *C. burnetii* transport, catabolism and incorporation of both glucose and glutamate are dependent on acidic pH (Hackstadt 1983). Moreover, pH dependent transport of carbon and energy sources is required for *de novo* protein synthesis (Zuerner and Thompson 1983), stability of the ATP pool (Hackstadt and Williams 1981b), and membrane energization (Hackstadt 1983). Acid activation of *C. burnetii* metabolism was presented as a "biochemical stratagem" (Hackstadt and Williams 1981a) manifested as metabolic quiescence and stability at the neutral pH found in the extracellular environment, and metabolic activation and bacterial replication at the acidic pH found in the organism's PV (Akporiaye et al. 1983). With improved knowledge of *C. burnetii* metabolic requirements, researchers revisited the possibility of *C. burnetii* axenic cultivation using more complex buffer systems. While *de novo* nucleic acid and protein biosynthesis were observed for several hours, net biomass increases indicated *C. burnetii* was not replicating (Chen et al. 1990).

11.2 Genomic Insight into *C. burnetii* Metabolic Capacity

The sequencing of multiple *C. burnetii* strains allowed a comprehensive look at *C. burnetii*'s metabolic potential (Beare et al. 2009b; Seshadri et al. 2003). Overall, the ~2 Mb genome of *C. burnetii* is roughly twice the size of most genomes of obligate intracellular rickettsiae (Ellison et al. 2008; McLeod et al. 2004) and chlamydiae (Stephens et al. 1998). Moreover, analysis of coding ORFs shows that *C. burnetii* central metabolic pathways, such as glycolysis, the tricarboxylic acid cycle (TCA), and pathways for nucleic acid biosynthesis, are largely intact. As discussed below, *C. burnetii* does have predicted metabolic peculiarities that may reflect adaptation to intracellular parasitism, including some that may impact overall metabolism.

11.2.1 Glycolysis, TCA, and Pentose Phosphate Pathways

Biochemical studies show that *C. burnetii* can catabolize glucose (Hackstadt and Williams 1981a, b). However, the organism does not encode the initial enzyme of glycolysis (glucokinase or hexokinase) needed to convert glucose to glucose-6-phosphate. Because *C. burnetii* lysates can phosphorylate glucose in the presence of carbamoyl phosphate (Paretsky et al. 1962), glucose may be phosphorylated by an alternative carbamoyl phosphate-dependent mechanism that subsequently allows oxidation of glucose via glycolysis. *C. burnetii* encodes a complete TCA cycle and readily metabolizes glutamate, succinate, and pyruvate (Hackstadt and Williams 1981b). However, the organism lacks the glyoxylate bypass enzymes isocitrate lyase and malate synthase, and consequently cannot utilize fatty acids as a sole source of carbon. This is in contrast to the intracellular bacterium *Mycobacterium tuberculosis* that relies on fatty acids as a source of carbon during intracellular growth (Schnappinger et al. 2003). The non-oxidative branch of the pentose phosphate pathway is complete in *C. burnetii*, allowing generation of pentose sugars for nucleic acid biosynthsis. However, the oxidative branch of the pathway is defective due to the lack of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Thus, *C. burnetii* cannot rely on this pathway for synthesis of NADPH, a major source of reducing equivalents for biosynthetic reactions and antioxidant mechanisms. Overall, biochemical and genomic data suggest oxidation of glycolytic and TCA cycle intermediates, and substrates that funnel directly into these pathways, as critical for *C. burnetii* energy production.

11.2.2 Amino Acid Acquisition

Based on reconstruction of amino acid biosynthetic pathways, *C. burnetii* appears auxotrophic for 11 amino acids (Beare et al. 2009b; Seshadri et al. 2003). Thus, during intracellular growth, *C. burnetii* must acquire these amino

acids via transport of free amino acids or peptides from the PV lumen. Autophagosomes, carrying defunct organelles and protein aggregates, and fluid phase endosomes, fuse with the *C. burnetii* PV (Beron et al. 2002; Heinzen et al. 1996). This trafficking likely provides key protein precursors required for *C. burnetii* growth. Indeed, the *C. burnetii* PV lumen is clearly a proteolytic environment as evidenced by the rapid breakdown of bovine serum albumin and the presence of active cathepsins (Howe et al. 2010). This activity is predicted to convert proteins to short peptides and free amino acids that can be transported by *C. burnetii* using several amino acid permeases, di-, tri- and oligo-peptide transporters, and 13 major facilitator superfamily transporters (Beare et al. 2009b). *C. burnetii*'s numerous amino acid auxotrophies suggest the PV has a steady supply of protein precursors, at least during the organism's replicative phase.

11.2.3 Energy Production

The mechanism(s) of *C. burnetii* acid activation is poorly defined. Data from early acid activation studies suggest extracellular acidic pH has multiple beneficial effects on *C. burnetii* physiology including membrane energization (Hackstadt 1983) and activation of metabolite transport (Hackstadt and Williams 1983). With respect to membrane energization, a proton gradient imposed by incubation of host cell-free organisms at acidic pH (i.e., pH 3–5) results in a transient increase of the *C. burnetii* ATP pool (Hackstadt and Williams 1981b). This effect is consistent with proton gradient driven synthesis of ATP via the organism's F_1F_0 ATP synthase. However, maintenance of the ATP pool is dependent on an oxidizable substrate, such as glutamate. Acidic pH clearly assists *C. burnetii* metabolite transport as demonstrated by enhanced transport of glutamate at pH 4.5 (Hackstadt and Williams 1983). Thus, a working model of *C. burnetii* acid activation of metabolism involves stimulation of proton symporters that acquire critical carbon and energy sources.

C. burnetii pH-dependent transport of energy sources and their subsequent oxidation generates reducing equivalents (e.g., NADH) that drives respiratory chain extrusion of protons. This process results in a proton motive force that promotes ATP synthesis. *C. burnetii* encodes a branched respiratory chain with distinct terminal ubiquinol cytochrome *o* (encoded by *cyoABCDE*) or cytochrome *bd* (encoded by *cydAB*) oxidases (Fig. 11.1). Cytochrome *bd*, with its increased affinity for oxygen, is used by bacteria under microaerobic conditions. Thus, *C. burnetii* may adapt to microaerobic levels of oxygen during intracellular growth. In fact, the intracellular oxygen tension of cultured eukaryotic cells and their compartments can be much lower than the extracellular oxygen tension (Hu et al. 1992). Furthermore, the level of oxygen in the spleen, a tissue that can contain *C. burnetii* infected macrophages, has been measured at approximately 3% (Braun et al. 2001).

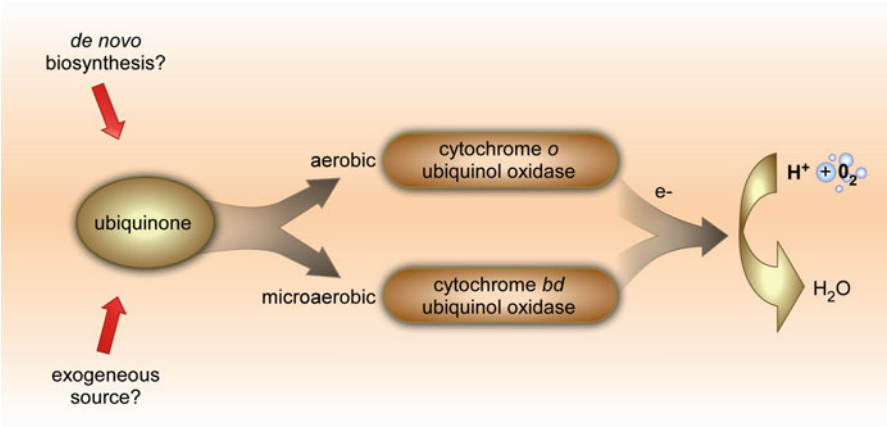

Fig. 11.1 Schematic overview of the *C. burnetii* aerobic respiratory chain. *C. burnetii*'s branched respiratory chain has distinct terminal ubiquinol oxidases incorporating either cytochrome *o* or cytochrome *bd* that are typically used under aerobic and microaerobic conditions, respectively. It is unclear how *C. burnetii* acquires ubiquinone, the predicted major quinone cofactor in electron transport activities, as key biosynthetic enzymes are missing from the pathogen's genome

11.2.4 pH Homeostasis

C. burnetii cytoplasmic pH drops when the organism is incubated in an acidic buffer without an oxidizable energy source. Associated with this pH drop is depletion of the ATP pool and loss of viability (Hackstadt and Williams 1981b). Thus, like other more extreme acidophiles, maintenance of cytoplasmic neutrality by *C. burnetii* is key for survival and the organism likely deploys several mechanisms to maintain pH homeostasis. As described above, proton extrusion via the electron transport chain could contribute to the regulation of cytoplasmic pH. Moreover, energy requiring expulsion of protons could occur by the F_0F_1-ATPase reverse reaction, and by K^+/H^+ and Na^+/H^+ antiporters. Proton consumption via decarboxylation of amino acids can also be utilized as a mechanism to prevent cytoplasmic acidification (Kashiwagi et al. 1992). Ammonia generation during conversion of glutamine to glutamate (Harth et al. 1994) is another example of how *C. burnetii* might couple amino acid metabolism to cytoplasmic pH homeostasis.

11.3 Establishment of a *C. burnetii* Axenic Culture System

Early work establishing conditions that support transient acid activation of *C. burnetii* metabolism provided a foundation on which to further define nutritional and physiochemical conditions that support prolonged axenic metabolic activity. Using

contemporary tools, such as biochemical pathway reconstruction, transcriptomics, and metabolite typing, conditions were defined that not only support prolonged *C. burnetii* metabolic activity, but also axenic replication.

11.3.1 First Generation Medium Supporting Sustained Metabolic Activity

The first stage in designing an axenic culture system for *C. burnetii* was to establish a medium that supported metabolic activity, in the form of protein and ATP synthesis, for several hours (Omsland et al. 2008). The known requirement of a moderately acidic environment (~pH 4.5) for *C. burnetii* metabolic activation (Hackstadt and Williams 1981a) prompted screening of several buffers to identify one with both good buffering capacity at acidic pH and the ability to support *C. burnetii* metabolic activity. Buffers based on acetate and succinate were deleterious to *C. burnetii de novo* protein synthesis. However, a citrate buffer supported high metabolic activity and provided excellent buffering capacity.

Sodium (Na^+), potassium (K^+) and chloride (Cl^-) make up the principal ion gradients used by microbes to transport nutrients and also help establish physiological osmotic pressure in living systems. The eukaryotic cell cytoplasm contains high concentrations of K^+ (~140 mM) relative to Na^+ (~15 mM) and Cl^- (~15 mM). Conversely, these levels are roughly reversed (K^+, ~5 mM; Na^+, ~145 mM; and Cl^-, ~110 mM) in interstitial fluid. Surprisingly, although the *C. burnetii* PV is located in the host cell cytoplasm, pathogen metabolic activity was enhanced in a citrate salts buffer (CSB) containing ion levels resembling interstitial fluid (Omsland et al. 2008). This metabolic effect may reflect PV interactions with fluid phase endosomes that traffic material from the extracellular space to the PV lumen (Heinzen et al. 1996).

CSB was then supplemented with three complex nutrient sources to compensate for *C. burnetii*'s auxotrophies and to exploit the organism's otherwise sophisticated metabolic machinery: (1) neopeptone, a combination of peptides, nucleotides and vitamins, (2) fetal bovine serum (FBS), and (3) RPMI cell culture medium. Supplementation of CSB with individual nutrient sources only marginally improved *C. burnetii* metabolic activity. However, a considerable increase in metabolic activity was observed when the nutrient sources were combined, creating a first generation medium called Complex *Coxiella* Medium (CCM) (Omsland et al. 2008). *C. burnetii* metabolic activity was optimal within a narrow range of nutrient concentrations. In fact, addition of extra nutrients, such as succinate or pyruvate, actually caused a decline in *C. burnetii* ATP pools and *de novo* protein synthesis. Incomplete oxidation of excess substrate resulting in generation of inhibitory intermediates may explain *C. burnetii* sensitivity to certain carbon sources (Teixeira de Mattos and Neijssel 1997). The negative effects of succinate and pyruvate on

C. burnetii metabolism may also reflect suboptimal biochemical routing of the oxidized substrate (Tesh et al. 1983).

11.3.2 Second Generation Medium Supporting Axenic Replication

Although CCM supported prolonged *C. burnetii* metabolic activity, increases in genome equivalents (GE) indicating cell division were not observed. Therefore, transcription microarray technology was used to assess the overall metabolic state of *C. burnetii* in CCM with the goal of defining potential medium nutritional deficiencies (Omsland et al. 2009). When compared to the transcriptome of *C. burnetii* replicating in Vero host cells, ribosomal gene expression of *C. burnetii* in CCM was dramatically downregulated, suggesting suboptimal protein synthesis could be blocking replication. Thus, an additional source of amino acids (casamino acids) was added to CCM in an attempt to boost pathogen *de novo* protein synthesis (Omsland et al. 2009). A high concentration of L-cysteine was also added as this supplement is necessary for axenic growth of *Legionella pneumophila* (Ewann and Hoffman 2006), a close phylogenetic relative of *C. burnetii*. The new medium called Acidified Citrate Cysteine Medium (ACCM), supported a considerable increase in *C. burnetii de novo* protein synthesis. However, cell division was still not observed.

As discussed, the presence of a branched respiratory chain in *C. burnetii* suggests the organism might adjust to alterations in oxygen concentration during intracellular growth. Previous testing of media had been conducted under ambient (~20%) oxygen conditions. Therefore, the effect of oxygen availability on *C. burnetii* metabolic activity was probed using Phenotype Microarrays incubated in 20, 5 or 2.5% oxygen environments. The number of oxidized substrates increased as oxygen level decreased, suggesting *C. burnetii* is a microaerophile. Subsequent incubation of *C. burnetii* in ACCM in a 2.5% oxygen environment resulted in robust growth with approximately a 3 $\log_{10}$ increase in *C. burnetii* GE observed over a 6 day incubation (Omsland et al. 2009). Importantly, the infectivity of axenically propagated bacteria for Vero cells was similar to host cell-propagated bacteria. *C. burnetii* colony growth was also achieved using semi-solid ACCM-agarose plates.

11.4 Practical Aspects of *C. burnetii* Axenic Culture

Axenic cultivation of *C. burnetii* is enabling new lines of investigation that are difficult or impossible to conduct with bacteria propagated in host cells. However, axenic growth can be challenging and attention to detail is important for success. In this section, technical issues that are important to successful axenic culture of *C. burnetii* are discussed.

11.4.1 Preparation of Acidified Citrate Cysteine Medium (ACCM)

ACCM supports substantial growth of *C. burnetii*. However, the organism is highly sensitive to medium variations so care must be taken during medium preparation. For example, *C. burnetii* tolerates a moderate range of nutrient concentrations with elevated concentrations of certain nutrients, including some carbon sources, readily inhibiting *C. burnetii* metabolic activity and growth (Omsland et al. 2008). Moreover, ion conditions, especially the concentration of chloride, have considerable effects on metabolic activity (Omsland et al. 2008). The ability of *C. burnetii* to grow in ACCM is even affected by the water used to prepare the medium. Reproducible culture can be achieved using regular deionized water. The sensitivity of *C. burnetii* to ion and nutrient concentrations also means that a humidified incubator should be used for cultivation of *C. burnetii* in ACCM to prevent medium evaporation. Finally, *C. burnetii* acidophilic metabolism is highly sensitive to extracellular pH (Hackstadt and Williams 1981a). Therefore, care should be taken to adjust ACCM to pH 4.75 as moderate (e.g., 0.5 pH units) deviations from this value may compromise growth. The acidic pH of ACCM may contribute to medium instability as culture is inconsistent if the medium is more than 1 week old.

11.4.2 Modifications of ACCM

ACCM is a complex nutrient medium containing chemically undefined components, namely FBS, neopeptone, and casamino acids. We recently found that 1% FBS can be replaced with 1 mg/ml of synthetic methyl-β-cyclodextrin (Mβ-CD) (Omsland et al. 2011). Mβ-CD is used in other bacteriologic media where it may sequester toxic metabolites (Marchini et al. 1995; Imaizumi et al. 1983). The modified medium termed ACCM-2 supports improved growth of *C. burnetii* in liquid medium and as colonies in solid medium.

11.4.3 Growth of C. burnetii in ACCM

C. burnetii cultures can be established in ACCM with or without shaking. Gentle shaking (75 RPM) improves *C. burnetii* growth in ACCM while vigorous shaking can cause protein precipitation. Robust growth occurs in shaken 125 ml Erlenmeyer flasks containing 20 ml of medium (Fig. 11.2a) or 250 ml Erlenmeyer flasks containing 40 ml of medium. *C. burnetii* appears highly sensitive to several detergents (A. Omsland and R. Heinzen, unpublished observations). Thus, disposable polyethylene terepthalate glycol (PETG) Erlenmeyer flasks with 0.2 μm filtered screw top caps are preferred. Without shaking, culture vessels should provide a large air-liquid interface to promote gas exchange. T-25 and T-75 tissue culture flasks containing 7 and 20 ml of medium, respectively, work well in this manner. Although liquid cultures

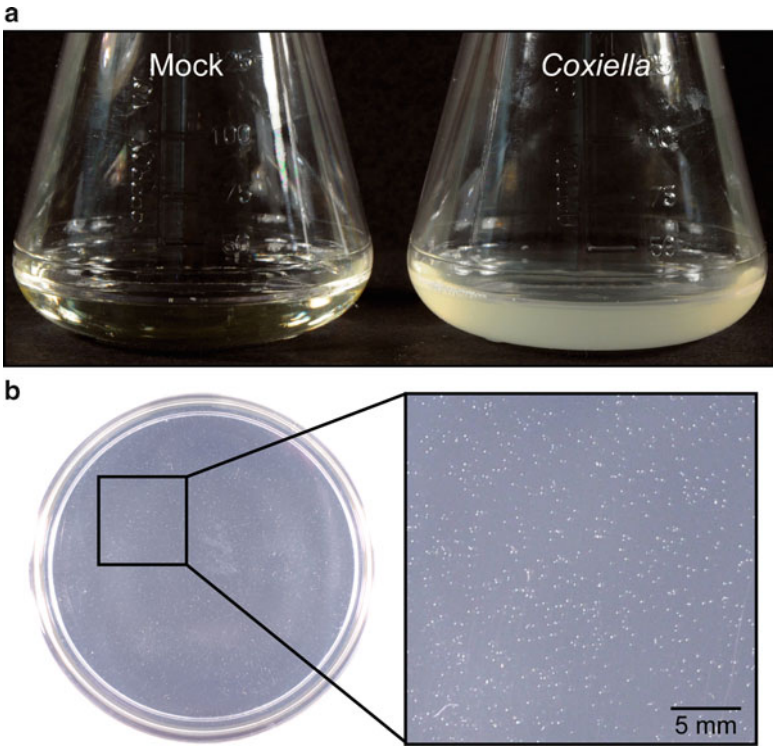

Fig. 11.2 Axenic culture of *C. burnetii*. (**a**) Mock and *C. burnetii* Nine Mile (phase II)-inoculated ACCM-2 following 6 days of incubation with shaking in a 2.5% oxygen environment. (**b**) Representative colony formation by *C. burnetii* Nine Mile (phase II) in ACCM-agarose following 6 days of incubation in a 2.5% oxygen environment

can be started using fewer than 10 organisms, an inoculum of 1.0×10^6 organisms/ml will develop to stationary phase (~10^9 organisms/ml) in approximately 6 days (Omsland et al. 2009), similar to the organism's growth kinetics in cultured cells (Coleman et al. 2004). A critical parameter that affects *C. burnetii* axenic growth is oxygen concentration. *C. burnetii* replicates in ACCM when oxygen levels are between 1% and 5%. Declines in growth are observed in 7.5% oxygen while no growth is observed in 10% oxygen. An incubator with automatic monitoring and gas adjustments ensures a reliable oxygen environment.

11.4.4 Storage of ACCM Cultured C. burnetii

ACCM-cultivated *C. burnetii* are sensitive to repeated freeze-thaw cycles, but excellent viability is observed when stocks are stored in ACCM containing a cryoprotectant, such as 10% dimethyl sulfoxide. Nonetheless, for optimal viability, *C. burnetii* stocks should be stored as single-use aliquots.

11.4.5 C. burnetii Colony Growth in ACCM-Agarose

An important advance enabled by axenic growth is the ability to obtain *C. burnetii* clones by colony formation in ACCM-agarose. Minute colonies form when organisms are embedded in semi-solid medium using a soft agarose overlay method (Fig. 11.2b). *C. burnetii* will not grow directly on the surface of solid ACCM. This behavior may reflect a requirement by *C. burnetii* to be submersed in an acid bath for proper membrane energization and transporter function. The use of a thin overlay medium promotes outgrowth of *C. burnetii* colonies in one focal plane, thus facilitating identification and picking of isolated colonies.

To prepare ACCM-agarose plates, combine 10 ml 2× ACCM (pH 4.75, 37°C) with 10 ml 0.5% UltraPure Agarose preheated to 50°C, then pour the mixture into 100 mm Petri dish creating a 0.25% ACCM-agarose base. After the medium base has solidified, prepare the 0.1% agarose overlay medium by combining 1.25 ml 2× ACCM (37°C), 0.75 ml water (37°C), and 0.5 ml 0.5% UltraPure Agarose (50°C). Add the *C. burnetii* inoculum (maximum 0.1 ml) to the overlay medium, mix, then pour over the ACCM-agarose base. Incubate plates at 4°C for 30 min to promote solidification of the low percentage overlay medium. Remove excess liquid in ACCM plates by placing them in a biosafety cabinet, lids cracked, for 20–30 min or until condensation is gone.

Colonies develop in ACCM-agarose in 4–7 days when incubated in a 2.5% oxygen environment. Colonies may be as small as 0.05 mm in diameter (Omsland et al. 2009). The reason for small colony size is unclear, but electron microscopic analysis reveals that 6 day old colonies are primarily comprised of *C. burnetii* small cell variants (SCV) which are non-replicative, stationary phase cells (Coleman et al. 2004; Omsland et al. 2011). Nutrient depletion in the colony microenvironment may drive *C. burnetii* into stationary phase and halt further colony expansion.

11.4.6 Selection and Recovery of C. burnetii Genetic Transformants Using ACCM-Agarose

Axenic culture in both ACCM and ACCM-agarose greatly facilitates isolation of *C. burnetii* genetic transformants. Compared to cell culture-based methods, where clonal isolation of *C. burnetii* transformants can take 8–12 weeks (Beare et al. 2009a), transformants can be isolated in as little as 16 days using axenic techniques (Beare et al. 2010; Omsland et al. 2011). Picking of *C. burnetii* colonies is facilitated by first locating isolated colonies using a dissecting microscope. Colonies can then be extracted from solid ACCM-agarose with a micro-pipette tip with an enlarged opening (approximately 0.5 mm). The clonal populations of *C. burnetii* can then be expanded by culture in ACCM following release of embedded organisms by repeated pipetting.

11.4.7 Determination of C. burnetii Viability Using ACCM-Agarose

Enumeration of *C. burnetii* in stock preparations is commonly conducted using an infectious focus-forming unit (FFU) assay or by determining the number of GE by quantitative PCR (Q-PCR) (Coleman et al. 2004). The FFU assay relies on immunofluorescent detection of PVs in cultured host cells. This method is complicated by the fact that phase I and phase II *C. burnetii* have dramatically different infectivity for cultured host cells. For example, the infection efficiency of phase II *C. burnetii* for Vero cells is approximately 5 GE per FFU while several hundred phase I *C. burnetii* are required to produce a single FFU (Cockrell et al. 2008; R. A. Heinzen, unpublished data). While the GE assay is sensitive and accurate, it does not distinguish between live and dead bacteria. The plating efficiency of the *C. burnetii* Nine Mile phase II strain in ACCM-agarose is nearly 1 GE per colony (Beare et al. 2010). Thus, quantification of *C. burnetii* by a conventional colony forming unit assay using ACCM-agarose plates is an alternative method to accurately enumerate the number of live organisms in a given preparation.

11.5 Summary

Axenic culture has removed the severe experimental obstacles associated with *C. burnetii*'s obligate reliance on a eukaryotic host cell for growth. Perhaps most importantly, ACCM culture techniques are aiding development of new genetic tools for *C. burnetii* (Chen et al. 2010; Voth et al. 2011; Beare et al. 2010). Moreover, biochemical and physiological studies can now be conducted without the metabolic background of the host cell.

C. burnetii axenic growth requirements have *in vivo* correlates. Oxygen gradients that occur within biological systems, including across biological membranes, can be associated with oxygen concentrations that are considerably lower than atmospheric oxygen levels (i.e., ~20%) (Hu et al. 1992; Khan et al. 2003). *C. burnetii* microaerophilic metabolism was hypothesized based on the organism's branched respiratory chain containing terminal oxidases typically associated with bacterial responses to normoxic and microaerobic levels of oxygen. The physiological basis of enhanced *C. burnetii* metabolism in a microaerobic environment is unclear, but may include sensitivity to oxidative stress. Technical difficulties in measuring ion levels in acidic vacuoles have prevented direct measurement of PV Na^+, K^+, and Cl^- concentrations. However, *C. burnetii*'s requirement for specific levels of these ions in ACCM likely correlates with their *in vivo* roles in physiological processes such as metabolite transport and pH homeostasis. Finally, a pH of ~4.75 is firmly established for the *C. burnetii* PV (Akporiaye et al. 1983; Maurin et al. 1992), a value that correlates with optimal pathogen growth in ACCM.

Several advances in *C. burnetii* genetic transformation have been made using ACCM (Chen et al. 2010; Voth et al. 2011; Beare et al. 2010). For example, secretion into the host cell cytoplasm of several potential *C. burnetii* effector molecules was recently demonstrated with a shuttle vector developed using axenic procedures (Chen et al. 2010; Voth et al. 2011). Continued development of genetic methods should soon allow fulfillment of molecular Koch's postulate for putative *C. burnetii* virulence genes. ACCM culture will also aid investigations of mechanisms used by *C. burnetii* to persist in the harsh conditions of its PV, and the biological bases of developmental form transitions. For instance, *C. burnetii* is highly sensitive to certain short chain fatty acids, such as propionate, during growth in ACCM (unpublished data). In *L. pneumophila*, nutritional cues including amino acid availability and exposure to short chain fatty acids can trigger a stringent response that drives transition of replicative to transmissive forms that are biologically reminiscent of *C. burnetii* large cell variant (LCV) and SCV developmental forms (Sauer et al. 2005; Edwards et al. 2009; Dalebroux et al. 2009). Thus, these molecules may also trigger stringent response genes that regulate *C. burnetii* morphological transitions, a hypothesis that is testable with ACCM culture techniques.

C. burnetii can be grown to high numbers in embryonated eggs (Williams et al. 1981). However, bacteria purified from eggs or mammalian host cells (Cockrell et al. 2008) contain eucaryotic host cell contamination, making these preparations suboptimal for biochemical analyses or vaccine production. Additionally, the homogenization and centrifugation steps involved in purification can generate infectious aerosols, making the procedure potentially hazardous. Purification of *C. burnetii* from ACCM is simple, safe and results in organisms free of host cell contamination.

ACCM cultures of the avirulent *C. burnetii* Nine Mile phase II isolate can be started from tissue samples of infected SCID mice that contain as few as 10 organisms (Omsland et al. 2011). Virulent *C. burnetii* isolates also grow well in ACCM; thus, it should be possible to isolate these organisms from infected immunocompetent animals and human patients using ACCM culture techniques. Recent Q fever outbreaks (Gilsdorf et al. 2008; Schimmer et al. 2009) highlight the need for improved clinical and epidemiological methods. Axenic isolation of *C. burnetii* should prove valuable in these settings.

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