1 **Culture under normoxic conditions and enhanced virulence of phase II *Coxiella***
2 ***burnetii* transformed with a RSF1010-based shuttle vector**

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20 Abstract

21 *Coxiella burnetii* is a Gram-negative, facultative intracellular microorganism that can
 22 cause acute or chronic Q fever in human. It was recognized as an obligate intracellular
 23 organism until the revolutionary design of an axenic cystine culture medium (ACCM).
 24 Present axenic culture of *C. burnetii* strictly requires a hypoxic condition (<10%
 25 oxygen). Here we investigated the normoxic growth of *C. burnetii* strains in ACCM-2
 26 with or without tryptophan supplementation. Three *C. burnetii* strains - Henzerling
 27 phase I, Nine Mile phase II and a Nine Mile phase II transformant, were included. The
 28 transformant contains a pMMGK plasmid that is composed of a RSF1010 ori, a
 29 repABC operon, an eGFP gene and a kanamycin resistance cassette. We found that,
 30 under normoxia if starting from an appropriate concentration of fresh age inocula,
 31 Nine Mile phase II can grow significantly in ACCM-2 with tryptophan, while the
 32 transformant can grow robustly in ACCM-2 with or without tryptophan. In contrast,
 33 long-term frozen stocks of phase II and its transformant, and Henzerling phase I of
 34 different ages had no growth capability under normoxia under any circumstances.
 35 Furthermore, frozen stocks of the transformant consistently caused large
 36 splenomegaly in SCID mice, while wild type Nine Mile phase II induced a lesser
 37 extent of splenomegaly. Taken together, our data show that normoxic cultivation of
 38 phase II *C. burnetii* can be achieved under certain conditions. Our data suggests that
 39 tryptophan and an unknown temperature sensitive signal are involved in the
 40 expression of genes for normoxic growth regulated by quorum sensing in *C. burnetii*.

Introduction

Coxiella burnetii is a Gram-negative, facultative intracellular pathogen and the causative agent of human Q fever. The primary transmission route for human is through inhalation of contaminated aerosols from the secretions and excretions of domestic ruminants (1). *C. burnetii* infections in humans may manifest as an acute disease (mainly as a self-limiting febrile illness, pneumonia, or hepatitis) or as a chronic disease (mainly endocarditis in patients with previous valvulopathy) (2). The majority (~50-60%) of human infections are asymptomatic (2, 3). Chronic infections are rare but can be fatal if untreated. *C. burnetii* is a significant cause of culture-negative endocarditis in the United States (4). Treatment of chronic infections is challenging and currently requires a combined antibiotic therapy with doxycycline and hydroxychloroquine for at least 18 months (5). Worldwide only one vaccine for Q fever called Q-Vax is licensed in Australia to protect high risk populations (6).

C. burnetii has two phase variants. Virulent *C. burnetii* isolated from natural sources and infections is defined as phase I. It produces full-length LPS that may play an important role in *C. burnetii* persistent infections by masking toll-like receptor ligands from innate immune recognition by human dendritic cells (7). LPS from phase I *C. burnetii* contains two unique biomarkers of methylated sugars (virenose and dihydrohydroxystreptose) at its O-specific chain. When extensively passaged in immunoincompetent hosts, virulent phase I *C. burnetii* mutates to avirulent phase II (8, 9). LPS from phase II *C. burnetii* is severely truncated and only contains lipid A and partial core oligosaccharide. Lipid A, the basal component of LPS, is essential for *C.*

burnetii growth in macrophage-like THP-1 cells but nonessential in non-phagocytic cells (10).

The recent description of an axenic culture medium and the subsequent modified axenic culture media provide invaluable tools for *C. burnetii* research (11-15). The first generation of axenic culture of *C. burnetii* requires a complex nutrient-rich medium (Acidified Citrate Cysteine Medium, ACCM) and microaerophilic conditions with 2.5% oxygen and 5% carbon dioxide (11). A second-generation medium (ACCM-2) was generated by replacing fetal bovine serum with methyl-beta-cyclodextrin (12), then was improved by supplementing tryptophan (14, 15). A nutritionally defined medium (ACCM-D) further improved *C. burnetii* growth especially with increased bacterial viability (13). The continuous improvement of axenic culture systems significantly facilitates the development of the *C. burnetii* field.

Phase I *C. burnetii* is a category B select agent with potential for illegitimate use and requires a biosafety level-3 laboratory for culture (16). Phase II *C. burnetii* is approved for use at biosafety level-2 and is widely used for studying the biology and pathogenesis in tissue culture. The caveat of working with phase II *C. burnetii* is the lack of animal models for assessing *in vivo* pathogenesis. To promote the identification of virulence factors in phase II *C. burnetii*, an SCID mouse model was recently established (17). An alternative infection model using larvae of the greater wax moth *Galleria mellonella* was also used to identify virulence factors in phase II *C. burnetii* (18, 19).

In this study, we characterized the growth of various *C. burnetii* strains under different oxygen concentrations in ACCM-2 with or without tryptophan supplementation. We found that, under normoxia if starting from an appropriated concentration of freshly age inocula, *C. burnetii* Nine Mile phase II had significant propagation in ACCM-2 supplemented with tryptophan. Under same conditions, a Nine phase II transformant that contains a RSF1010-based shuttle vector can have robust growth in ACCM-2 with or without tryptophan. Phase I *C. burnetii* had no apparent growth under normoxia in ACCM-2 with or without tryptophan. Long-term frozen stocks of all tested strains unanimously failed to have normoxic growth. In the SCID mouse infection model, compared to wild type Nine Mile phase II, frozen stocks of its transformant caused larger splenomegaly. Our data suggest that *C. burnetii* retains functional genes for aerobic growth, and the expression of these genes might be regulated by a temperature sensitive signal of the quorum sensing system. Our data also suggest that the expression of genes relating to aerobic growth is associated with enhanced *in vivo* virulence in *C. burnetii*.

Materials and Methods

***C. burnetii* strains.** *C. burnetii* Nine Mile phase II and Henzerling phase I are from our laboratory strain collection. *C. burnetii* transformant -NMIIpMMGK was constructed by transforming Nine Mile phase II with a shuttle vector pMMGK by electroporation (20). The pMMGK plasmid is composed of a RSF1010 ori, a repABC operon, an eGFP gene and a kanamycin resistance cassette. Construction of pMMGK was described previously (10).

Cultivation of *C. burnetii*. Seed stocks of *C. burnetii* Nine Mile phase II, Henzerling phase I and phase II transformant NMIIpMMGK were collected from ACCM-2 cultures at 37°C under 2.5% oxygen, and were stored in fresh ACCM-2 at -80°C. Different ages and concentrations of inocula were used to characterize *C. burnetii* growth in two media (ACCM-2 with or without tryptophan supplementation) under hypoxia or normoxia. Inocula were added in 150 µL media per well in 96 well plates (Corning). The plates were placed in a tri-gas CO₂ incubator (2.5% oxygen and 5% CO₂) or a CO₂ incubator (5% CO₂) for seven days at 37°C. Each culture condition has six replicates.

Quantitative PCR. Genome equivalent (GE) of *C. burnetii* was quantified by using qPCR as previously described with minor modifications (21). Bacterial bodies of *C. burnetii* were collected by centrifugation at 15,000 ×g for 30 min. Total DNA was extracted with TIANamp N96 Blood DNA Kit (Tiangen). Genome copy numbers were determined by Taqman probe qPCR specific to *dotA* by using a ViiA™ 7 Real-Time PCR System (Applied Biosystems). PCR conditions were as follows: initial denaturation at 94°C for 10 min, followed by 40 cycles of amplification at 94°C for 15 s, 60°C for 1 min.

Mouse infection. Female SCID (CB17/Icr-*Prkdc*^{scid}/IcrIcoCrI) mice were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Two separate mouse infection experiments were conducted. Mice were infected with mock or 1×10⁸ GE *C. burnetii* in 200 µL PBS by intra-peritoneal injection. In the first experiment, 18 four weeks old mice were divided into three groups: control (PBS)

group and two infection groups. Fresh age (less than one day old) inocula of Nine Mile phase II and NMIIpMMGK were used. In the second experiment, 9 five weeks old mice were averaged into three infection groups: one month old Nine Mile phase II, one-month old NMIIpMMGK and 19 months old NMIIpMMGK. At indicated days post-infection, mice were weighted and sacrificed to harvest spleens to determine splenomegaly (spleen weight/body weight). Each spleen was homogenized in 2 mL PBS. Total DNAs from 20 μ L of each tissue homogenate were purified with DNeasy Blood & Tissue Kit (Qiagen). *C. burnetii* GEs in spleens were determined by quantitative PCR as described above. All animal procedures were carried out in strict accordance with the guidelines for the Care and Use of Laboratory Animals of the National Ministry of Health of China.

Statistical Analysis. A two-tailed Student *t* test was used for qPCR analysis of *C. burnetii* growth yields under various conditions and was used for comparison of splenomegaly and spleen bacterial loads of different infection groups.

Results

Phase II *C. burnetii* transformants can grow robustly under normoxia in ACCM-2 with or without tryptophan supplementation. Current axenic culture of *C. burnetii* requires a microaerophilic environment (2.5% oxygen) (14). But interestingly, *C. burnetii* encodes terminal oxidases -cytochrome *o* (encoded by *cyoABCDE*) and cytochrome *bd* (encoded by *cydAB*), which are typically associated with aerobic and microaerophilic respiration, respectively (22). The presence of cytochrome *o* suggests the possibility of *C. burnetii* replication under aerobic

conditions. In our attempts to culture *C. burnetii* in regular CO₂ incubators (~20% oxygen), we found that a *C. burnetii* Nine Mile phase II transformant -NMIIpMMGK can grow robustly (~3 logs increase) if starting from a reasonably high inoculum concentration in the regular ACCM-2 medium (without tryptophan supplementation) (Figure 1A). The pMMGK plasmid is composed of the pMMB207 backbone (a RSF1010 *ori* and a repABC operon), an eGFP gene and a kanamycin resistance cassette (10). This initial finding of *C. burnetii* normoxic growth was made in T-flask cultures. To thoroughly characterize the influence of multiple culture parameters on *C. burnetii* growth, 96 well plates were used for *C. burnetii* cultivation. Tryptophan supplementation can improve the viability of *C. burnetii* in ACCM-2 (14). When cultured in ACCM-2 with tryptophan supplementation, NMIIpMMGK displayed similar growth characteristics, though there was a trend of a bigger yield in ACCM-2 with tryptophan supplementation (Figure 1B).

Tryptophan improved axenic growth of phase II *C. burnetii* under normoxia. The axenic growth of wild type phase I and phase II *C. burnetii* in early generations of ACCMs without tryptophan supplementation has been extensively investigated and was reported to be microaerophilic (2.5% oxygen) (11, 12). We repeated the cultivation of wild type phase I and phase II *C. burnetii* in ACCM-2 without tryptophan at different oxygen concentrations (Figure 2AC). In ACCM-2 without tryptophan supplementation, these two strains grew normally at 2.5% oxygen but had no significant growth at 20% oxygen, which is consistent with previous reports. In ACCM-2 with tryptophan at 2.5% oxygen, both strains had similar growth yields

(Figure 2BD). Interestingly, in ACCM-2 with tryptophan at 20% oxygen, wild type phase II *C. burnetii* grew significantly (~2.5 logs increase) if starting from a high concentration of fresh age inocula ($\sim 4 \times 10^6$ GE/mL), while phase I *C. burnetii* consistently had no obvious growth. These results show tryptophan has subtly different effects on the axenic growth of wild type phase I and phase II strains.

Long-term frozen stocks of *C. burnetii* strains failed to have apparent propagation under normoxia. The cultivation of NMIIpMMGK at 20% oxygen was constantly repeated. Its viability at 2.5% oxygen in ACCM-2 with or without tryptophan had no obvious variation after long-term frozen (>three months) in -80°C (Figure 3AB). Interestingly, its growth ability at 20% oxygen in either medium was completely abolished after long-term frozen. Similar with NMIIpMMGK, wild type Nine Mile phase II also failed to replicate at 20% oxygen in ACCM-2 with tryptophan (Figure 3CD). The growth deficiency of wild type Henzerling phase I at 20% oxygen in either medium was consistent (Figure 3EF). Compared to growth of freshly cultivated inocula (Figure 2), lower fold change of growth yield of long-term frozen inocula was observed in either medium (Figure 3C-F), suggesting low viability of both wild type frozen stocks. Taken together, our results suggest that a temperature sensitive factor is associated with the expression of genes involved in *C. burnetii* aerobic growth and viability.

Phase II *C. burnetii* transformants show enhanced virulence in the SCID mouse infection model. The SCID mouse model allows for assessing *in vivo* pathogenesis of phase II *C. burnetii* (17). Our *C. burnetii* phase II strains with or without the pMMGK

plasmid showed variable growth capability under normoxia. We next intended to investigate whether pMMGK affects the virulence of phase II *C. burnetii* in the SCID mouse model. Firstly, fresh (one day old) inocula of 1×10^8 genome equivalents (GE) of NMII or NMIIpMMGK were used to infect four weeks old female SCID mice by peritoneal (IP) injections. 16 days post infection similar magnitude of splenomegaly was detected in both infected groups ($p=0.10$) (Figure 4A, C). Notably, two cases of exceptional splenomegaly were observed in the NMIIpMMGK group. To examine the potential influences of inoculum age on *in vivo* virulence, another infection experiment using frozen bacterial stocks and five weeks old female SCID mice were conducted. Compared to the results of using one day old inocula, 18 days post infection one-month-old frozen stock of NMII caused similar size of splenomegaly (Figure 4B, C). Unexpectedly, however, frozen stocks of both one month old and 19 months old of NMIIpMMGK induced similar large splenomegaly in all infected mice (Figure 4B, C). The sizes of splenomegaly are in accordance with genome equivalents in all infected mice (Figure 4D). Taken together, frozen stocks of phase II *C. burnetii* transformants showed enhanced virulence in the SCID mouse model.

Discussion

The development of the axenic culture medium significantly advanced *C. burnetii* research (11). The axenic culture medium for *C. burnetii* was continuously improved (11-14). These medium studies found that hypoxic conditions are strictly required for *C. burnetii* axenic replication. In this study, we characterized the axenic growth of different *C. burnetii* strains in ACCM-2 with or without tryptophan supplementation,

especially their growth under normoxic conditions. Our goal was to further improve the axenic culture of *C. burnetii* under normoxia.

We found that, under normoxic conditions if starting from appropriate concentrations of fresh inoculum, *C. burnetii* transformants of pMMGK can have robust growth in ACCM-2, regardless of tryptophan supplementation. Similarly, wild type *C. burnetii* phase II can grow significantly in ACCM-2 with tryptophan supplementation. Clearly, the *C. burnetii* transformants have a greater capability of growing under normoxia. The mechanism of *C. burnetii* normoxic growth is unknown, but likely its expression of *cyoABCDE* and *cydAB* is coordinately regulated in a reciprocal fashion in response to oxygen concentration as in *E. coli* (23).

C. burnetii has been recognized as an intracellular parasite in nature. Inside the infected cells, *C. burnetii* replicates in a hypoxic, phagolysosome-like vacuole (24). The cytochrome *bd* oxidase, with its increased affinity for oxygen, is likely used by *C. burnetii* under hypoxic conditions. The alternative terminal ubiquinol oxidase in *C. burnetii* -cytochrome *o* (typically used for aerobic respiration), correlates with our finding of normoxic growth. Intriguingly, the normoxic growth is affected by multiple factors including the shuttle vector pMMGK, the age and concentration of inoculum, and the phase of *C. burnetii*. The differential normoxic growth of different strains suggests that, in *C. burnetii* phase II, a temperature-sensitive quorum sensing signal molecule like RNA might be involved in the regulated expression of genes for aerobic respiration, especially cytochrome *o*.

The capability of normoxic growth in ACCM-2 without tryptophan

supplementation is restricted to *C. burnetii* transformants. Their enhanced virulence is likely associated with normoxic growth. How might the shuttle vector pMMGK confers these “gain-of-function” phenotype to *C. burnetii*? The shuttle vector pMMGK brings new proteins (RepA, RepB, RepC, eGFP and Kan^R) and new RNAs to *C. burnetii*. The potential effects of these proteins and RNAs on bacterial physiology are impossible to predict (25, 26). The RSF1010 *ori*-based shuttle vectors are commonly used for *C. burnetii* transformation (27, 28). Whether pMMGK or other similar vectors can restore normoxic growth of other *C. burnetii* phase I and phase II strains will be worthy of further investigation.

The normoxic cultures in this study were performed under static conditions. Given that oxygen supply in static cultures is much less than in shaken cultures, it is necessary to test *C. burnetii* propagation in normoxic shaken cultures. This will help determine the influence of inoculum concentration on *C. burnetii* growth under normoxia, as one may argue that high concentrations of inoculum might reduce the physical concentration of oxygen in media. Despite this possibility, static cultures of *C. burnetii* under normoxic and hypoxic conditions have distinct outcomes in infected macrophages (29). *C. burnetii* replication in macrophages is prevented in static hypoxic cultures, but not in static normoxic cultures (29).

In conclusion, we characterized the growth of various *C. burnetii* strains in ACCM-2 with or without tryptophan under normoxic and hypoxic conditions. Consistent with previous reports, *C. burnetii* had robust growth under hypoxia. What is surprising is that we found a *C. burnetii* phase II transformant and the wild type *C.*

burnetii phase II can have significant to robust growth under certain normoxic circumstances. Compared to the wild type strain, the transformant displayed enhanced virulence in the SCID mouse model. Normoxic growth of *C. burnetii* is affected by the shuttle vector pMMGK, the age and concentration of inoculum, and the phase of *C. burnetii*. Our findings suggest that current axenic culture of *C. burnetii* under hypoxia might possibly be further modified to allow routine axenic culture under normoxia.

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References

1. Raoult D, Marrie T, Mege J. 2005. Natural history and pathophysiology of Q fever. *Lancet Infect Dis* 5:219-26.
2. Maurin M, Raoult D. 1999. Q fever. *Clin Microbiol Rev* 12:518-53.
3. Hechemy KE. 2012. History and prospects of *Coxiella burnetii* research. *Adv Exp Med Biol* 984:1-11.
4. Mulye M, Samanta D, Winfree S, Heinzen RA, Gilk SD. 2017. Elevated Cholesterol in the *Coxiella burnetii* Intracellular Niche Is Bacteriolytic. *MBio* 8.

- 283 5. Angelakis E, Raoult D. 2010. Q Fever. *Vet Microbiol* 140:297-309.
- 284 6. Chiu CK, Durrheim DN. 2007. A review of the efficacy of human Q fever
285 vaccine registered in Australia. *N S W Public Health Bull* 18:133-6.
- 286 7. Shannon JG, Howe D, Heinzen RA. 2005. Virulent *Coxiella burnetii* does not
287 activate human dendritic cells: role of lipopolysaccharide as a shielding
288 molecule. *Proc Natl Acad Sci U S A* 102:8722-7.
- 289 8. Hackstadt T. 1988. Steric hindrance of antibody binding to surface proteins of
290 *Coxiella burnetii* by phase I lipopolysaccharide. *Infect Immun* 56:802-7.
- 291 9. Beare PA, Jeffrey BM, Long CM, Martens CM, Heinzen RA. 2018. Genetic
292 mechanisms of *Coxiella burnetii* lipopolysaccharide phase variation. *PLoS*
293 *Pathog* 14:e1006922.
- 294 10. Wang T, Yu Y, Liang X, Luo S, He Z, Sun Z, Jiang Y, Omsland A, Zhou P,
295 Song L. 2018. Lipid A Has Significance for Optimal Growth of *Coxiella*
296 *burnetii* in Macrophage-Like THP-1 Cells and to a Lesser Extent in Axenic
297 Media and Non-phagocytic Cells. *Front Cell Infect Microbiol* 8:192.
- 298 11. Omsland A, Cockrell DC, Howe D, Fischer ER, Virtaneva K, Sturdevant DE,
299 Porcella SF, Heinzen RA. 2009. Host cell-free growth of the Q fever
300 bacterium *Coxiella burnetii*. *Proc Natl Acad Sci U S A* 106:4430-4.
- 301 12. Omsland A, Beare PA, Hill J, Cockrell DC, Howe D, Hansen B, Samuel JE,
302 Heinzen RA. 2011. Isolation from animal tissue and genetic transformation of
303 *Coxiella burnetii* are facilitated by an improved axenic growth medium. *Appl*
304 *Environ Microbiol* 77:3720-5.

- 305 13. Sandoz KM, Beare PA, Cockrell DC, Heinzen RA. 2016. Complementation of
306 Arginine Auxotrophy for Genetic Transformation of *Coxiella burnetii* by Use
307 of a Defined Axenic Medium. *Appl Environ Microbiol* 82:3042-51.
- 308 14. Vallejo Esquerra E, Yang H, Sanchez SE, Omsland A. 2017. Physicochemical
309 and Nutritional Requirements for Axenic Replication Suggest Physiological
310 Basis for *Coxiella burnetii* Niche Restriction. *Front Cell Infect Microbiol*
311 7:190.
- 312 15. Sanchez SE, Vallejo-Esquerra E, Omsland A. 2018. Use of Axenic Culture
313 Tools to Study *Coxiella burnetii*. *Curr Protoc Microbiol*
314 doi:10.1002/cpmc.52:e52.
- 315 16. Atlas RM. 2003. Bioterrorism and biodefence research: changing the focus of
316 microbiology. *Nat Rev Microbiol* 1:70-4.
- 317 17. van Schaik EJ, Case ED, Martinez E, Bonazzi M, Samuel JE. 2017. The SCID
318 Mouse Model for Identifying Virulence Determinants in *Coxiella burnetii*.
319 *Front Cell Infect Microbiol* 7:25.
- 320 18. Norville IH, Hartley MG, Martinez E, Cantet F, Bonazzi M, Atkins TP. 2014.
321 *Galleria mellonella* as an alternative model of *Coxiella burnetii* infection.
322 *Microbiology* 160:1175-81.
- 323 19. Selim A, Yang E, Rousset E, Thiery R, Sidi-Boumedine K. 2018.
324 Characterization of *Coxiella burnetii* strains from ruminants in a *Galleria*
325 *mellonella* host-based model. *New Microbes New Infect* 24:8-13.
- 326 20. Beare PA, Heinzen RA. 2014. Gene inactivation in *Coxiella burnetii*. *Methods*

- 327 Mol Biol 1197:329-45.
- 328 21. Coleman SA, Fischer ER, Howe D, Mead DJ, Heinzen RA. 2004. Temporal
329 analysis of *Coxiella burnetii* morphological differentiation. J Bacteriol
330 186:7344-52.
- 331 22. Omsland A, Heinzen RA. 2011. Life on the outside: the rescue of *Coxiella*
332 *burnetii* from its host cell. Annu Rev Microbiol 65:111-28.
- 333 23. Tseng CP, Albrecht J, Gunsalus RP. 1996. Effect of microaerophilic cell
334 growth conditions on expression of the aerobic (cyoABCDE and cydAB) and
335 anaerobic (narGHJI, frdABCD, and dmsABC) respiratory pathway genes in
336 *Escherichia coli*. J Bacteriol 178:1094-8.
- 337 24. Minnick MF, Raghavan R. 2012. Developmental biology of *Coxiella burnetii*.
338 Adv Exp Med Biol 984:231-48.
- 339 25. Baltrus DA. 2013. Exploring the costs of horizontal gene transfer. Trends Ecol
340 Evol 28:489-95.
- 341 26. Yano H, Shintani M, Tomita M, Suzuki H, Oshima T. 2019. Reconsidering
342 plasmid maintenance factors for computational plasmid design. Comput Struct
343 Biotechnol J 17:70-81.
- 344 27. Chen C, Banga S, Mertens K, Weber MM, Gorbasljeva I, Tan Y, Luo ZQ,
345 Samuel JE. 2010. Large-scale identification and translocation of type IV
346 secretion substrates by *Coxiella burnetii*. Proc Natl Acad Sci U S A
347 107:21755-60.
- 348 28. Voth DE, Beare PA, Howe D, Sharma UM, Samoilis G, Cockrell DC,

Omsland A, Heinzen RA. 2011. The *Coxiella burnetii* cryptic plasmid is enriched in genes encoding type IV secretion system substrates. *J Bacteriol* 193:1493-503.

29. Hayek I, Fischer F, Schulze-Luehrmann J, Dettmer K, Sobotta K, Schatz V, Kohl L, Boden K, Lang R, Oefner PJ, Wirtz S, Jantsch J, Luhrmann A. 2019. Limitation of TCA Cycle Intermediates Represents an Oxygen-Independent Nutritional Antibacterial Effector Mechanism of Macrophages. *Cell Rep* 26:3502-3510 e6.

Figure legends

Figure 1. Growth of *C. burnetii* NMIIpMMGK in ACCM-2 (A) and ACCM-2 with tryptophan supplementation (B) at 2.5% and 20% oxygen. At 2.5% oxygen, regardless of its inoculum concentration, the NMIIpMMGK strain consistently has robust growth in ACCM-2 and ACCM-2 plus tryptophan. At 20% oxygen, in both ACCM-2 and ACCM-2 plus tryptophan, the growth yield of NMIIpMMGK ranges from 0-3.6 logs, depending on its inoculum concentration.

Figure 2. Growth of wild type *C. burnetii* phase I and phase II strains in ACCM-2 and ACCM-2+Trp at 2.5% and 20% oxygen. In ACCM-2, at 20% oxygen, the wild type NM II and Henzerling I strains have no significant growth. In ACCM-2 plus tryptophan, at 20% oxygen, the growth of NM II ranges from 0-2.5 logs, depending on its inoculum concentration, while the Henzerling I strain consistently fails to grow.

Figure 3. Growth of frozen stocks of *C. burnetii* strains -Henzerling phase I and Nine Mile phase II and NMIIpMMGK in ACCM-2 with (A, C, E) or without tryptophan (B,

D, F) at 2.5% and 20% oxygen. In both ACCM-2 and ACCM-2 plus tryptophan, frozen stocks of three *C. burnetii* strains have significant growth at 2.5% oxygen. However, frozen stocks of all strains fail to grow at 20% oxygen, regardless of the media and their inoculum concentrations.

Figure 4. Intra-peritoneal challenge of SCID mice with different inoculum ages of *C. burnetii* strains -Nine Mile phase II and NMIIpMMGK. **(A, B)** Spleens were removed from challenged and control mice. **(C)** Splenomegaly calculated as spleen weight as a percentage of total body weight at the time of necropsy after infection. **(D)** Genome equivalents calculated using Taqman real-time PCR with DNA purified from infected lungs. Error bars represent standard deviation from the mean.

NM II

Henzerling I

NMIIpMMGK

NM II

Henzerling I

□ day 0

▨ day 7, 20% O₂

▩ day 7, 2.5% O₂

A

B

C

D