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From neglected to dissected: how technological advances are leading the way to the study of *Coxiella burnetii* pathogenesis

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Abstract

Coxiella burnetii is an obligate intracellular bacterial pathogen responsible for severe worldwide outbreaks of the zoonosis Q fever. The remarkable resistance to environmental stress, extremely low infectious dose and ease of dissemination, contributed to the classification of *C. burnetii* as a class B biothreat. Unique among intracellular pathogens, *C. burnetii* escapes immune surveillance and replicates within large autophagolysosome-like compartments called *Coxiella*-containing vacuoles (CCVs). The biogenesis of these compartments depends on the subversion of several host signaling pathways. For years, the obligate intracellular nature of *C. burnetii* imposed significant experimental obstacles to the study of its pathogenic traits. With the development of an axenic culture medium in 2009, *C. burnetii* became genetically tractable, thus allowing the implementation of mutagenesis tools and screening approaches to identify its virulence determinants and investigate its complex interaction with host cells. Here, we review the key advances that have contributed to our knowledge of *C. burnetii* pathogenesis, leading to the rise of this once-neglected pathogen to an exceptional organism to study the intravacuolar lifestyle.

Introducing *Coxiella burnetii*

Coxiella burnetii is a Gram negative obligate intracellular pathogen, and the causative agent of Q fever, a worldwide zoonotic disease (Eldin *et al.*, 2017). Animal coxiellosis is mostly associated with abortion, stillbirths, and weak offspring (Fig. 1). Desiccation of placental materials, excretions of birthing fluids, urine, feces and milk from infected animals contribute to shed *C. burnetii* into the environment (Eldin *et al.*, 2017). Transmission to humans results from exposure to contaminated aerosols and dust (Fig. 1). In humans, *C. burnetii* infections are often asymptomatic and self-limiting, however, 40% of individuals infected with *C. burnetii* develop an acute disease which is associated with a flu-like syndrome, pneumonia, hepatitis and chronic fatigue (Eldin *et al.*, 2017) (Fig. 1). Acute disease may convert into a chronic illness with severe complications, including endocarditis (Eldin *et al.*, 2017). An important risk factor for Q fever outbreaks is the multiple zoonotic reservoirs of *C. burnetii*, which include domestic livestock and wild animals (mammals, reptiles, birds and ticks) (Eldin *et al.*, 2017). *C. burnetii* is extremely infectious, with 1-to-10 bacteria being sufficient to cause disease (Brooke *et al.*, 2013). The low infectious dose coupled to remarkable environmental stability contribute to the significant spreading of *C. burnetii* infections well away from the outbreak source and led to the classification of *C. burnetii* as a category B biothreat (Madariaga *et al.*, 2003).

C. burnetii is a stealth pathogen that actively escapes innate immune recognition by inhibiting the NF- κ B pathway (Mahapatra *et al.*, 2016) and inflammasome activation (Cunha *et al.*, 2015). Infected cells are also protected from apoptosis, thereby preserving the bacterial replicative niche over long periods (Lührmann *et al.*, 2017). *C. burnetii* enters macrophages by phagocytosis through the interaction with α V β 3 integrins (Capo *et al.*, 1999). In contrast, in non-phagocytic cells,

internalization is facilitated by the invasin OmpA (Outer membrane protein A) (Martinez *et al.*, 2014) (Fig. 1). Following internalization, bacteria reside within early endosomal compartments, also called early CCVs, that passively mature along the endocytic pathway by successive fusion events with early and late endosomes and lysosomes (Fig. 1). Maturation is accompanied by the acidification of the CCV lumen (Heinzen *et al.*, 1996), which is required to activate bacterial metabolism (Hackstadt and Williams, 1981) and the translocation of bacterial effector proteins by a Dot/Icm Type 4b Secretion System (T4SS) (Newton *et al.*, 2013). Thus, by 48 hours post-infection, cells display a single, large, mature CCV, where markers of multiple membrane compartments co-exist (Dragan and Voth, 2019), which is indicative of the capacity of *C. burnetii* to hijack multiple host membrane trafficking pathways (Fig. 1). Importantly, the biogenesis of these remarkable compartments is a two-step process requiring both cellular and bacterial factors. This review will focus on the recent technological advances that fostered remarkable progress in our understanding of the complex host/pathogen interplay that controls the generation of the *C. burnetii* replicative niche.

The long and winding road (to genetic manipulation)

Together with its high infectivity, the obligate intracellular nature of *C. burnetii* has imposed severe constraints on the study of its pathogenesis. An important step towards the development of tools to investigate *C. burnetii* infection was the isolation of a Phase II variant (NMII) of the Nine Mile Phase I strain (NMI), presenting a single deletion of 21 genes involved in lipopolysaccharide biosynthesis and displaying loss-of-virulence phenotype in animal models (Moos and Hackstadt, 1987; Hoover *et al.*, 2002). Thus, NMII was allowed for manipulation in biosafety level-2 (BSL-2) environments (Fig. 2D), as opposed to other *C. burnetii* strains that require

manipulation in biosafety level-3 (BSL-3) confinement. Importantly, loss-of-virulence of NMII has no impact either on intracellular growth kinetics or development of CCVs in cultured cells, making NMII a relevant model to study host-pathogen interactions (Howe *et al.*, 2010).

Nevertheless, bacterial amplification of *C. burnetii* in embryonated eggs and/or cultured cells represented a real obstacle for its genetic manipulation. Indeed, transposon mutagenesis allowed the generation of *C. burnetii* mutants (Beare *et al.*, 2009); however, amplification and isolation of these from infected cells ruled out the possibility of isolating mutations in virulence genes. Thus, until 2011, *ftsZ* was the only *C. burnetii* gene characterized using Himar1 transposon mutagenesis (Beare *et al.*, 2009) (Fig. 2F).

Whole-genome sequencing of *C. burnetii* NMI RSA493 in 2003 allowed the identification of typical features, including a Dot/Icm secretion system, which is highly homologous to that of *L. pneumophila* (Seshadri *et al.*, 2003) (Fig. 2C). This finding was pivotal to develop bioinformatics-based approaches to predict effector proteins-coding genes. These revealed that similar to *L. pneumophila*, effector proteins translocated by *C. burnetii* encode a C-terminal secretion signal called the E-block motif and are mostly under the control of a PmrA response regulator (Zusman *et al.*, 2007). Due to the genetic intractability of *C. burnetii* at that time, candidate effector proteins were tested for secretion using *L. pneumophila* as a surrogate model, exploiting the homologies between the Dot/Icm secretion systems of the two pathogens. This approach allowed the Roy laboratory to validate the translocation of four *C. burnetii* effector proteins encoding ankyrin repeat homology domains (ARHDs) (Pan *et al.*, 2008) (Fig. 2E). Later work from the Samuel laboratory reported the first

large-scale, bioinformatics-based identification of *C. burnetii* effector proteins and used the *L. pneumophila* system to validate the secretion of 32 new T4SS substrates (Chen *et al.*, 2010) (Fig. 2H). Moreover, using a shuttle plasmid system for the expression of recombinant proteins in *C. burnetii*, Chen and colleagues also demonstrated that the functionality of the Dot/Icm secretion system (Chen *et al.*, 2010).

One year later, a genetic screen of *C. burnetii* proteins carrying C-terminal secretion signals led to the identification of additional effector proteins (Carey *et al.*, 2011) (Fig. 2I). For this study, wild type (wt) *L. pneumophila* or the T4SS-defective $\Delta dotA$ mutant were transformed with a library containing adenylate cyclase enzyme (CyaA)-tagged random fragments of *C. burnetii* genome, leading to the validation of 7 additional *C. burnetii* effectors (Carey *et al.*, 2011). These were further tested for their intracellular localization and function, which indicated a possible implication in the manipulation of host membrane trafficking. Of note, this is the first study to report a replication phenotype associated with a transposon insertion in the *C. burnetii* Dot/Icm gene *icmL* (*icmL*::Tn), thus demonstrating the importance of *C. burnetii* effector protein translocation for infection (Carey *et al.*, 2011) (Fig. 2I). Today, the development of tailored bioinformatics algorithms for the identification of T4SS effector proteins led to the identification of over 140 candidate *C. burnetii* T4SS effectors (Voth *et al.*, 2009; Chen *et al.*, 2010; Carey *et al.*, 2011; Maturana *et al.*, 2013; Weber *et al.*, 2013). Recent genome comparison of the *C. burnetii* strains NMII RSA493, Henzerling RSA331, G Q212, K Q154 and Dugway 5J108-111, revealed that many genes encoding candidate effector proteins are either pseudogenized or missing altogether, leaving only 44 out of the 143 identified effector protein-coding genes intact across all strains (Larson *et al.*, 2016). Despite these significant advances, functional

analysis of *C. burnetii* genes involved in virulence was still limited by the genetic intractability of this pathogen.

A game-changer in the study of *C. burnetii* infections has been the development of a synthetic medium (ACCM for Acidified Citrate Cysteine Medium), followed by the development of ACCM-2, allowing the extracellular culture of this obligate intracellular bacterium (Omsland *et al.*, 2009; Omsland *et al.*, 2011) (Fig. 2G). ACCM-2 development stems from metabolic requirement studies using microarrays, genomic reconstruction of metabolic pathways and metabolite typing (Omsland *et al.*, 2009; Omsland *et al.*, 2011). These studies highlighted that *C. burnetii* requires acid activation buffer (pH 4.75), to reproduce the CCV microenvironment (Heinzen *et al.*, 1996) and increase its metabolic potential as well as low oxygen levels (2.5%) to facilitate the bacterial micro-aerophilic respiration (Omsland *et al.*, 2009; Omsland *et al.*, 2011). This scientific milestone finally enabled genetic manipulation of *C. burnetii*, leading to a new era in our understanding of its pathogenesis (Fig. 2).

From genes to function: characterization of *C. burnetii* effectors involved in vacuole biogenesis

With the development of axenic culture, targeted deletion of *C. burnetii* was formally achievable (albeit remaining extremely challenging), and the role of *C. burnetii* effector proteins in infection could be tested directly. The Heinzen laboratory first exploited axenic culture with the generation of a *dotA* deletion mutant (Beare *et al.*, 2012) (Fig. 2J) and with the identification and characterization of CvpA (for *Coxiella* vacuolar protein A). This effector protein localizes at CCVs and reroutes recycling endosomes to this compartment by interacting with the clathrin adaptor protein AP2 (Larson *et al.*, 2013) (Fig. 1). In a follow-up study, the Heinzen laboratory identified four

additional members of what constitutes today the Cvp sub-class of *C. burnetii* effector proteins (CvpA, CvpB/Cig2, and CvpC-to-E, Fig. 1) (Larson *et al.*, 2015). Accordingly, targeted deletion of *C. burnetii* vacuolar proteins severely affects the biogenesis of CCVs (Larson *et al.*, 2013; Larson *et al.*, 2015).

If targeted deletion of *C. burnetii* genes remains challenging, *Himar1*-based transposon mutagenesis has been extensively applied to the generation of libraries of *C. burnetii* mutants. Thus, by 2013, the Samuel laboratory reported the first study that combined bioinformatics-mediated identification of candidate effector proteins with transposon mutagenesis (Weber *et al.*, 2013) (Fig. 2N). Searching the *C. burnetii* genome for T4SS features (PmrA consensus sequences, E-block motifs, and homologies with known effectors), led to the identification of 234 genes encoding putative *C. burnetii* effector proteins. β -lactamase translocation assay in *L. pneumophila* validated 53 T4SS substrates, most of which were never reported before. Transposon mutagenesis showed that ten effector proteins were involved in the biogenesis of CCVs and bacterial replication (Weber *et al.*, 2013) (Fig. 2N).

The following year, two independent studies reported the large-scale identification of *C. burnetii* virulence determinants based on the generation of two libraries of *C. burnetii* transposon mutants (Martinez *et al.*, 2014; Newton *et al.*, 2014) (Fig. 2O, R). The Bonazzi library consisted of 3000 GFP-tagged mutants, among which over 1000 were sequenced, annotated, and screened using quantitative, multiparametric image analysis to identify bacterial factors involved in host cell invasion, intracellular replication and persistence (Martinez *et al.*, 2014; Martinez *et al.*, 2015). This approach allowed to validate the function of 16 out of the 22 genes constituting the *C. burnetii* Dot/Icm secretion system, characterize the phenotype associated with transposon insertions in 31 genes encoding effector proteins and

identify the first *C. burnetii* invasin OmpA (for Outer membrane protein A), which is necessary and sufficient to trigger internalization by non-phagocytic cells (Martinez *et al.*, 2014) (Fig. 1 & 2P). This study was also the first to report the use of the insect model *Galleria mellonella* to investigate *C. burnetii* virulence *in vivo* (Martinez *et al.*, 2014).

The Roy laboratory used a modified-Himar1 expressing mCherry fluorescent protein to generate over 3200 mutants. These were visually screened in HeLa cells to identify genes important in the biogenesis of CCVs and bacterial replication using the lysosomal marker LAMP-1 to visualize the membranes of the *C. burnetii* replicative compartment (Newton *et al.*, 2014) (Fig. 2O). This approach allowed the isolation of mutants characterized by different intracellular phenotypes, including defects in intracellular replication and homotypic fusion of independent CCVs or the appearance of filamentous bacteria. Of note, mutants carrying a transposon insertion in gene encoding *CBU1751* (*cig57*) displayed a severe vacuole biogenesis defect (Newton *et al.*, 2014). The Newton laboratory has further characterized the role of this effector protein in the development of CCVs and intracellular replication, showing that Cig57 subverts clathrin-mediated traffic by interacting with FCHO2, an accessory protein of clathrin-coated pits (Latomanski *et al.*, 2016) (Fig. 1). More recently, CTLC (clathrin heavy chain) has been observed at CCVs, where it plays an essential role in vacuole expansion (Latomanski and Newton, 2018). Importantly, CTLC recruitment to the CCV is related to autophagy, and conversely, the fusion of autophagosomes with CCVs is dependent on CTLC (Latomanski and Newton, 2018). The Roy visual screen also highlighted a multivacuolar phenotype associated with transposon insertions in the gene *CBU0021* (*cig2*), identical to that previously observed following the knockdown of autophagy-related genes (McDonough *et al.*, 2013), suggesting a functional link

between a bacterial effector protein and the manipulation of a specific host cell function (Fig. 2O). This hypothesis was further investigated, demonstrating that Cig2 contributes to the recruitment of the autophagy machinery to CCVs (Fig. 1), thus facilitating their homotypic fusion and contributing to an enhanced bacterial virulence in the *Galleria mellonella* infection model (Kohler *et al.*, 2016).

It takes two to tango: role of host cell pathways in *C. burnetii* vacuole biogenesis

If on one hand effector proteins translocation is critical for pathogenesis, host cell proteins, lipids, and membrane trafficking pathways also play a significant role in *C. burnetii* infections. As mentioned above, the maturation of CCVs along the endocytic pathway is a pre-requisite for effector proteins translocation (Newton *et al.*, 2013)(Fig. 2L); thus, Rab GTPases of the endocytic pathway play a key role in *C. burnetii* intracellular replication (Fig. 1) (Beron *et al.*, 2002; Romano *et al.*, 2007; Campoy *et al.*, 2011). Indeed, silencing of either Rab5 or Rab7 correlates with effector protein translocation defects (Newton *et al.*, 2016) (Fig. 2R).

A comprehensive characterization of the host cell components required for the biogenesis of CCVs was provided for the first time by a genome-wide screen using siRNA targeting eukaryotic genes in *C. burnetii*-infected HeLa cells (McDonough *et al.*, 2013) (Fig. 2M). Host determinants required for *C. burnetii* infection were identified by the analysis of the number and size of CCVs, revealing the importance of several eukaryotic pathways in critical infection events. As expected, the silencing of genes encoding pH-regulating proteins CLN3 and CLCN5, as well as components of the vacuolar ATPase resulted in a defect in the biogenesis of CCVs (McDonough *et al.*, 2013). Besides, the depletion of the retromer cargo complex VPS26-VPS29-VPS35 leads to defective in bacterial replication, revealing a role for retrograde membrane

trafficking in *C. burnetii* infections (McDonough *et al.*, 2013). Interestingly, seminal work from the Colombo laboratory in the past decade demonstrated that the induction of autophagy favors the biogenesis of CCVs and that *C. burnetii* actively manipulates autophagy during infections (Gutierrez *et al.*, 2005; Romano *et al.*, 2007). However, the precise role of autophagy in CCVs' development remained to be characterized. The genome-wide screening approach of the Roy laboratory revealed that knockdown of autophagy proteins syntaxin-17, ATG5 and ATG12 results in the formation of multiple CCVs of smaller size as compared to control cells (McDonough *et al.*, 2013). It was later demonstrated that this multivacuolar phenotype is the result of the defective homotypic fusion of CCVs (Newton *et al.*, 2014; Martinez *et al.*, 2016).

The Voth and Heinzen laboratories have further explored the complex interplay between *C. burnetii* and autophagy by. The autophagy-associated cargo receptor p62 is actively recruited at CCVs, independently of LC3-interacting domains (Winchell *et al.*, 2018). Interestingly, *C. burnetii* infections seem to preserve p62 from degradation upon induction of autophagy (Winchell *et al.*, 2018). Accordingly, *C. burnetii* infections inhibit mTORC1, a master regulator of autophagy, by a non-canonical mechanism that does not result in accelerated autophagy, nor a block of the autophagic flux (Larson *et al.*, 2019). Finally, autophagy is also involved in repairing the membranes of CCVs during expansion, which are subject to transitory damage and loss-of-acidification (Mansilla Pareja *et al.*, 2017) (Fig. 2T).

Besides host cell proteins, lipids also play a significant role in the development of CCVs, which are rich in sterols (Gilk *et al.*, 2010). Indeed, cholesterol homeostasis regulates CCVs biogenesis and intracellular survival of *C. burnetii* (Gilk *et al.*, 2013; Mulye *et al.*, 2017) (Fig. 2K). Accordingly, *C. burnetii* actively manipulates cholesterol metabolism via a eukaryotic-like $\Delta 24$ sterol reductase (Gilk *et al.*, 2010). Furthermore,

an image-based supervised machine learning approach led to the identification of CvpB/Cig2 as the first *C. burnetii* effector protein to bind phosphoinositides and manipulate their metabolism (Martinez *et al.*, 2016) (Fig. 2S). Indeed, CvpB/Cig2 binds phosphatidylinositol-3-phosphate (PI(3)P) and phosphatidylserine (PS) and perturbs the activity of the PI3-kinase PIKFYVE. This inhibition results in an enrichment of PI(3)P at CCVs, which is essential for their homotypic fusion (Martinez *et al.*, 2016). Importantly, despite the apparent defect in the biogenesis of CCVs, *cvpB* transposon mutants were unaffected in their capacity of replicating within infected cells. However, *cvpB* mutants are attenuated in the *in vivo* models *Galleria mellonella* (Martinez *et al.*, 2016) and SCID mice (van Schaik *et al.*, 2017), demonstrating that the biogenesis of CCVs can modulate *C. burnetii* virulence, independently of bacterial replication.

To infinity and beyond (conclusions)

As of today, *C. burnetii* stands as the sole example of an obligate intracellular bacterial pathogen for which a specific axenic culture medium has been developed. Consequently, research on this dangerous and complex pathogen has bloomed during this decade, with the development of genetic tools and phenotypic screening approaches to better understand the complex interactions established between *C. burnetii* and its host. The generation of libraries of *C. burnetii* transposon mutants combined with the development of multi-parametric screening approaches have allowed the rapid and unbiased identification of microbial genes involved in specific steps of the infectious process. This has been facilitated by the fact that the majority of *C. burnetii* transposon mutants isolated to date display a significant phenotype during infection (Martinez *et al.*, 2014), suggesting a milder functional gene redundancy as compared to the closely related pathogen *L. pneumophila*. Accordingly,

bioinformatics predicted ~ 150 effector proteins compared to the 300 translocated by *Legionella*.

Nevertheless, understanding how these newly identified virulence determinants manipulate host cell functions remains exceptionally challenging. Probably due to the complex nature of this compartment, a large proportion of the *C. burnetii* effector proteins identified and characterized to date are involved in the biogenesis of the CCV; however, proteins involved in the manipulation of other signaling pathways including apoptosis and inflammation have been identified and partially characterized. With the development of advanced bioinformatics approaches such as unsupervised machine learning, hierarchical clustering and Bayesian network analysis, we can now compare microbial-targeted and host-targeted phenotypic screens, to identify sets of bacterial and eukaryotic genes predicted to be involved in the same biological process during infection.

Despite the giant leaps taken since the development of axenic culture, many technical barriers still exist today. Directed mutagenesis remains challenging, limiting our studies to the mutants available in the transposon libraries hosted in the laboratories that undertook this endeavor. Nevertheless, our knowledge of this once-neglected pathogen is ever increasing. This will allow, in the near future, to address burning questions on *C. burnetii* pathogenesis, including a characterization of the strategies used to evade immune recognition, cell-to-cell spread, and, consequently, dissemination to distant organs (heart and liver) following infection of alveolar macrophages.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Overview of *C. burnetii* infections. *C. burnetii* is an obligate intracellular pathogen which infects wild and farm animals. Bacteria are shed in the environment with birth products and excretions leading to the contamination of hay and dust. Human infection occurs 2-3 weeks after the inhalation of contaminated particles, followed by the internalization of *C. burnetii* by alveolar macrophages. Human Q fever remain asymptomatic in 60% of infected individuals, whereas 40% develop an acute, febrile disease, which can turn into a chronic disease with more severe symptoms including endocarditis. *C. burnetii* invades eukaryotic cell through phagocytosis, which is facilitated by the bacterial invasin OmpA. Early CCVs mature along the endocytic pathway by successive fusion events with early endosomes (EEs), late endosomes (LEs) and lysosomes. Acidification of the CCV activates bacterial metabolism and the translocation of bacterial effector proteins (purple circles and ovals) by the Dot/Icm secretion system. Several effector proteins are collectively beneficial for the biogenesis of the mature CCV where bacterial replication occurs. These include the Cvp family (for *Coxiella* vacuolar proteins) of effectors that localize at CCV membranes and manipulate host membrane trafficking pathways. CvpA interacts with the clathrin adaptor AP2 at recycling endosomes (REs), re-routing these compartments to the forming CCV. CvpB binds PI(3)P at early endosomes and enriches this phospholipid at CCVs. This is required to favor the autophagy-mediated homotypic fusion between CCVs. Although not a Cvp, Cig57 interacts with the clathrin adaptor FCHO2 at clathrin-coated pits and re-routes clathrin-mediated membrane traffic to the CCV.

Figure 2. Milestone discoveries that have contributed to the knowledge of *C. burnetii* pathogenesis.

A 1937 **Discovery of the Q-fever pathogen - *Coxiella burnetii***

B 1956 **Isolation of *C. burnetii* Nine Mile phase II**

C 2003 **First genome sequencing of *C. burnetii* NMI**

E 2008 **Identification of the first *C. burnetii* effector proteins**

G 2009 **Development of an axenic culture medium for *C. burnetii***

I 2010 **Screen for Dot/Icm secretion signals**

K 2011 **Importance of Dot/Icm T4SS in *C. burnetii* infection highlighted by *icmL::Tn* mutant**

M 2012 **Genome-wide siRNA screen of host factors involved in *C. burnetii* infection**

O 2013 **Importance of cholesterol in *C. burnetii* internalisation and CCV biogenesis**

R 2014 **Role of autophagy in CCV biogenesis**

T 2015 **Visual screen of a library of *C. burnetii* Tn mutants**

S 2016 **siRNA screen of RabGTPases required for T4SS functionality**

U 2017 **Involvement of autophagy in CCVs repairing**

V 2017 **Manipulation of PI metabolism by a *C. burnetii* effector protein**

W 2017 **Phenotypic profiling based on multi-parametric high content screening**

X 2017 **Phenotypic screen of a library of *C. burnetii* Tn mutants**

Y 2017 **Identification of the invasin OmpA**

Z 2017 **Use of *Galleria mellonella* as an infection model for *C. burnetii***

AA 2017 **Identification of *C. burnetii* effectors based on bioinformatics analysis & phenotypic screening**

AB 2017 **CCV acidification required for Dot/Icm activation and secretion**

AC 2017 **Use of Cre-lox-mediated recombination on T4SS component DotA**

AD 2017 **Identification of *C. burnetii* effector proteins by bioinformatics analysis**

AE 2017 **Evidence of Dot/Icm functionality**

AF 2017 **Generation of the first *C. burnetii* mutant using transposon mutagenesis**

AG 2017 **Role of *ftsZ* in bacterial cell division**

AH 2017 **Role of autophagy in CCV biogenesis**

AI 2017 **Visual screen of a library of *C. burnetii* Tn mutants**

AJ 2017 **siRNA screen of RabGTPases required for T4SS functionality**

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