Targeting mitochondria: how intravacuolar bacterial pathogens manipulate mitochondria

Laura F. Fielden ¹, Yilin Kang ¹, Hayley J. Newton ² [#] & Diana Stojanovski ¹ [#]

¹ Department of Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria, 3010, Australia.

² Department of Microbiology and Immunology at the Peter Doherty Institute for Infection and Immunity, The University of Melbourne, Parkville, Victoria 3000, Australia.

[#] Corresponding Authors:

Diana Stojanovski

E-mail: d.stojanovski@unimelb.edu.au

Hayley Newton

E-mail: hnewton@unimelb.edu.au

Abstract

Manipulation of host cell function by bacterial pathogens is paramount for successful invasion and creation of a niche conducive to bacterial replication. Mitochondria play a role in multiple, important cellular processes including energy production, cellular calcium homeostasis, lipid metabolism, heme biosynthesis, immune signaling and apoptosis. The sophisticated integration of host cell processes by the mitochondrion have seen them emerge as a key target during bacterial infection of human host cells. This review highlights the targeting and interaction of this dynamic organelle by intravacuolar bacterial pathogens and how the modulation of mitochondrial function may contribute to pathogenesis.

Introduction

Mitochondria are essential organelles, described as the powerhouse of the cell and fundamental to eukaryotic cellular function and survival. This semi-autonomous organelle is proposed to have originated when an ancient eukaryotic ancestor internalised α -proteobacteria, approximately 1.5 to 2 billion years ago (Dolezal, et al., 2006, Margulis, 1970). Consistent with this endosymbiotic event, mitochondria have maintained a reduced relic of the eubacterial genome and complementary protein synthesis machinery (Gray, et al., 1999). Furthermore, like bacteria mitochondria have a double-membrane, the outer and inner membranes, which border the two aqueous compartments, the intermembrane space and matrix. This unique architecture underpins the organelles most notable function: the production of cellular energy (ATP) by oxidative phosphorylation. However, mitochondria are also central to many other important cellular processes, including cellular calcium homeostasis, lipid metabolism, heme biosynthesis, immune signaling and apoptosis (Gillies and Kuwana, 2014, Kispal, et al., 1999, Lill, 2009, Rizzuto, et al., 1993, Tait and Green, 2010). With such diverse cellular roles, it is no surprise that an increasing number of virulence factors of both bacterial and viral origin are found to target the mitochondria during infection. During both intracellular and extracellular bacterial infection, virulence factors, termed effector proteins, are secreted into the host cell by a variety of complex secretory machines and many display specific targeting to the mitochondria (Escoll, et al., 2016, Jiang, et al., 2012a, Lobet, et al., 2015, Rudel, et al., 2010). The distinct association displayed by some intravacuolar bacteria with mitochondria suggests a significant contribution to bacterial pathogenesis by this organelle. Through this association, virulence factors aim to modulate mitochondrial function as a means of promoting pathogen survival, replication and pathogenesis.

In recent years, research has focused on understanding the molecular interactions and biochemical functions of individual effector proteins and their role in bacterial pathogenesis. This has been assisted by the development of superior culturing and

genetic manipulation techniques in combination with advanced microscopy, biochemistry and proteomics approaches. This review will primarily focus on bacterial pathogens that replicate within a pathogen-derived vacuole, or intravacuolar pathogens, and their interaction with mitochondria. Even though intravacuolar pathogens are confined within a membrane bound vacuole they exploit specialized secretion systems to introduce effector proteins into the host cell cytosol. From here many effector proteins are targeted to numerous cellular compartments including the mitochondrion, which provides a fascinating example of host-pathogen interactions. A wide array of intravacuolar bacterial pathogens exists, capable of causing diverse disease in human hosts. We will draw examples from the following pathogens; *Legionella pneumophila*, *Chlamydiae spp*, *Simkania negevensis*, *Salmonella enterica* serovar Typhimurium, *Mycobacterium tuberculosis*, *Coxiella burnetii* and *Anaplasma phagocytophilum*, and their interaction with the mitochondria, as features of intracellular pathogenesis (**Table 1**). We will present insight into mitochondrial function and why the organelle is an attractive target for pathogen effectors during cellular infection. We will also discuss the relationship between these pathogens and mitochondria in terms of the mechanisms utilised to control mitochondrial activities and the challenges in studying this phenomenon.

Bacterial virulence factors must traverse multiple membranes to target the host cell

Crossing membranes: from bacterial compartment to the host cytosol

The secretion of specific bacterial effector proteins into the host cell allows efficient manipulation of a diverse range of host cell processes (Aktories, 2011, Alix, et al., 2011, Escoll, et al., 2016, Ho, et al., 2014, Winchell, et al., 2016, Zhou and Zhu, 2015). Intravacuolar pathogens, compared to their cytosolic counterparts, face the additional challenge of targeting these effectors across both bacterial and vacuolar lipid bilayers. This translocation is facilitated by sophisticated secretion systems, which enable the delivery of effector proteins directly into the host cytoplasm (Costa, et al., 2015, Green and Mecsas, 2016). Several classes of bacterial secretion systems exist, which are characterized by the number of membranes spanned as well as the constituent structural and mechanistic

components. Although all systems incorporate a beta-barrel channel, recent progress in this field has revealed a significant degree of structural and mechanistic diversity between each complex (Chandran Darbari and Waksman, 2015, Gold and Kudryashev, 2016, Green and Mecsas, 2016, Nagai and Kubori, 2011, Portaliou, et al., 2016). Briefly, the main secretion systems used by intravacuolar bacteria are the Type 3 Secretion System (T3SS), Type 4 Secretion System (T4SS) and the Type 6 Secretion System (T6SS) (**Table 1**). Additionally, it has been shown that some intravacuolar bacteria, such as species of *Mycobacteria*, encode a Type 7 Secretion System, which assists in mediating protein transport across the waxy, hydrophobic mycomembrane. For detailed reviews on these transport systems refer to the following (Chandran Darbari and Waksman, 2015, Costa, et al., 2015, Galan, et al., 2014, Ho, et al., 2014, Houben, et al., 2014, Portaliou, et al., 2016).

Bacterial effectors are targeted to their specific secretion system by translocation signals encoded within their amino acid sequence, which are unique to different secretion systems. The targeting mechanism distinguishing effector proteins for export does not appear to rely on a definitive amino acid sequence, but instead a specific charge or conformational structure (Costa, et al., 2015). For example, the signal for secretion via the T3SS is found at the N-terminus of the effector protein (Diepold and Armitage, 2015). In contrast, secretion by the T4SS of *L. pneumophila* and *C. burnetii* is believed to be mediated by the final 3-5 C-terminal amino acids, with a particular role for a hydrophobic residue at this location (Nagai, et al., 2005, Voth, et al., 2011). Furthermore, translocation signals appear to be conserved across bacteria that encode analogous secretion systems. This is demonstrated in studies utilising *L. pneumophila* as a surrogate host to screen for *C. burnetii* genes encoding a T4SS translocation signal (Carey, et al., 2011, Voth, et al., 2009). Although encoding distinct effector protein repertoires, it was shown that *L. pneumophila* was capable of translocating *C. burnetii* effector proteins, supporting a conserved translocation signal located at the C-terminus of the T4BSS effector proteins.

Crossing membranes: targeting effector proteins to and within mitochondria

Once released into the host cell cytosol, bacterial effector proteins must then be targeted to the correct cellular location. Typically, effector proteins are fully equipped with this targeting information, allowing the exploitation of the host cell protein trafficking pathways through ‘molecular mimicry’ of eukaryotic signals. Mitochondria possess a highly regulated system of protein sorting. 99% of mitochondrial proteins are nuclear-encoded and synthesized on cytosolic ribosomes (Baker, et al., 2014, Chacinska, et al., 2009, Dolezal, et al., 2006, Harbauer, et al., 2014, Neupert and Herrmann, 2007, Stojanovski, et al., 2012). Nuclear-encoded mitochondrial proteins are post-translationally targeted and imported into the correct sub-compartment, as determined by specific targeting information inherent within the protein sequence (Schmidt, et al., 2010). The translocation and intra-mitochondrial sorting of mitochondrial precursors is coordinated by dynamic translocation systems. Current knowledge indicates the presence of five major import machineries in mitochondria (**Figure 1A**) (Chacinska, et al., 2009, Neupert and Herrmann, 2007, Schmidt, et al., 2010). These include, the general entry gate into mitochondria, or Translocase of the Outer Mitochondrial Membrane (TOM complex), which facilitates translocation of precursor proteins from the cytosol into the intermembrane space (Chacinska, et al., 2009, Neupert and Herrmann, 2007, Schmidt, et al., 2010). Following translocation through TOM precursors are transferred to one of the following molecular machines: (i) the Translocase of the Inner Mitochondrial Membrane (TIM) 23 complex; (ii) the Translocase of the Inner Mitochondrial Membrane (TIM) 22; (iii) the Mitochondrial Intermembrane Space Assembly (MIA) machinery; and the outer membrane Sorting and Assembly Machinery (SAM) complex.

Proteins destined for the mitochondrial matrix and in some instances the inner membrane and intermembrane space, utilize the well-characterised ‘presequence pathway’, which exploits the TIM23 complex. This pathway is defined by the presence of a 20-50 amino acid N-terminal amphipathic helix on the incoming precursor protein (**Figure 1A**) (Mossmann, et al., 2012, Schatz and Dobberstein, 1996, Schmidt, et al., 2010). Presequence-containing proteins interact with the TOM receptors, Tom20 and Tom22 and

the linear precursor is then threaded through the beta-barrel channel formed by Tom40. Cooperation of the intermembrane space domain of Tom22 with the intermembrane space domains of the TIM23 complex subunits, Tim50, Tim21 and Tim23, facilitates transfer of the precursor to the TIM23 translocon (Chacinska, et al., 2005, Schulz, et al., 2011). Interestingly, two modular assemblies of the TIM23 complex exist: the TIM23^{SORT} and TIM23^{MOTOR} forms (Chacinska, et al., 2009, van der Laan, et al., 2010). The TIM23^{SORT} complex is characterized by its association with complexes III and IV of the mitochondrial respiratory chain, via the Tim21 subunit (van der Laan, et al., 2006, Wiedemann, et al., 2007). TIM23^{SORT} directed precursors possess a hydrophobic transmembrane segment, or stop-transfer signal, downstream of the N-terminal presequence, which halts precursor translocation during transit through the TIM23 channel. This event requires the membrane potential across the inner membrane as a driving force and initiates the lateral release of the precursors into the inner membrane. (Chacinska, et al., 2010, van der Laan, et al., 2007). Alternatively, complete translocation of precursor proteins into the matrix requires the recruitment of an additional molecular machine, the PAM complex and formation of the TIM23^{MOTOR} complex. The central component of PAM, the matrix 70kDa heat shock protein (mtHsp70) is proposed to mediate precursor protein transfer into the matrix through a series of ATP-dependent reactions, assisted by five additional PAM co-chaperone proteins (Chacinska, et al., 2009, Neupert and Herrmann, 2007, Stojanovski, et al., 2012). Once in the matrix the presequence is cleaved by the mitochondrial processing peptidase (MPP) (**Figure 1A**) (Chacinska, et al., 2005).

Multiple bacterial proteins of both intracellular and extracellular bacterial origin have been identified to hijack the 'presequence pathway'. For example, the effector protein Ats-1, produced by *A. phagocytophilum*, the causative agent of anaplasmosis, localizes to the mitochondrial matrix due to the presence of an N-terminal presequence, which is cleaved following import into this compartment (**Table 1**) (**Figure 1B**) (Niu, et al., 2010). The T3SS effector protein VopE, translocated into host cells by the extracellular bacterium *Vibrio cholera*, similarly contains an N-terminal targeting sequence and is dependent on the

mitochondrial membrane potential for import into the organelle (Suzuki, et al., 2014). Curiously, VopE was demonstrated to interact with Miro GTPases at the mitochondrial outer membrane (discussed further below). Due to the highly organized nature of the mitochondrial import pathways, this disconnection between VopE localisation and function raises the interesting possibility that this effector uses elements of the presequence pathway for import to the outer membrane. Recently, the biogenesis of certain outer membrane proteins in *Saccharomyces cerevisiae* has been shown to be dependent on a mitochondrial targeting sequence and membrane potential for import (Sinzel, et al., 2016, Song, et al., 2014, Wenz, et al., 2014), indeed such a mechanism could be exploited by VopE.

In addition to the TIM23 complex, the inner membrane of mitochondria is also residence to the TIM22 complex. Mitochondrial precursor proteins that possess internal targeting information, such as the transmembrane segments of mitochondrial carrier proteins are targeted to the TIM22 complex (**Figure 1A**) (Chacinska, et al., 2009, Neupert and Herrmann, 2007, Rehling, et al., 2003, Schmidt, et al., 2010). Hydrophobic carrier precursors are threaded through the TOM complex in a loop conformation and are captured by intermembrane space chaperones belonging to the small TIM family (Curran, et al., 2002, Webb, et al., 2006, Wiedemann, et al., 2001). The small TIM's shuttle the hydrophobic precursor through the aqueous intermembrane space to the TIM22 complex. The core of the translocase contains the channel-forming Tim22 protein, which inserts the precursors into the inner membrane in a membrane potential-dependent manner (Rehling, et al., 2003, Schmidt, et al., 2010). Studies of the effector repertoire of *L. pneumophila*, which is the causative agent of Legionnaire's disease (Brenner, et al., 1979), have revealed a novel mitochondrial carrier protein, which acts as a unidirectional nucleotide transporter (**Table 1**) (Dolezal, et al., 2012). The effector, known as LncP (discussed further below), contains six transmembrane domains, effectively targeting it to the TIM22 translocase, which facilitates integration of LncP into the mitochondrial inner membrane (**Figure 1B**).

Finally, beta-barrel proteins of the outer membrane are inserted from the intermembrane space side by the SAM complex (**Figure 1A**) (Schmidt, et al., 2010, Wiedemann, et al., 2003). Proteins that utilise the SAM complex possess a C-terminal signal referred to as the beta-signal (Hohr, et al., 2015, Kutik, et al., 2008). The receptor protein, Sam35, recognises the C-terminal beta-signal and mediates the insertion of the precursor into the channel of the outer membrane complex, Sam50 (Kutik, et al., 2008). Finally, the accessory subunit, Sam37 assists with the release of the precursor from the proteinaceous pore of Sam50 into the outer mitochondrial membrane (Chan and Lithgow, 2008, Hohr, et al., 2015). It is interesting to note that the signal targeting bacterial beta-barrel proteins to the outer membrane of Gram-negative bacteria are functional in eukaryotic mitochondria, indicating a conservation of the targeting pathway (Walther, et al., 2009a, Walther, et al., 2009b). Considering the prokaryotic origin of the mitochondrion, it is likely that bacteria can exploit this evolutionary relationship during infection to direct virulence factors to mitochondria (Jiang, et al., 2012b, Lucattini, et al., 2004). For instance, recognition and import of the pro-apoptotic protein PorB, an ATP-binding, beta-barrel porin encoded by *Neisseria meningitidis* may be due to structural similarities between it and the endogenous mitochondrial porin, voltage dependent anion-selective channel (VDAC) (Jiang, et al., 2011, Muller, et al., 2000, Muller, et al., 2002). Similar to the import of VDAC, PorB is translocated into mitochondria by the TOM complex and is then shuttled through the intermembrane space by the small TIM chaperones to the SAM complex (Jiang, et al., 2011, Muller, et al., 2002). Despite lacking a C-terminal beta-signal, PorB is capable of insertion into the outer membrane by the main component of the SAM complex, Sam50, independent of the partner proteins Sam35 and Sam37 (Jiang, et al., 2011). This targeting mechanism is reminiscent of the BAM complex within the prokaryotic outer membrane (Jiang, et al., 2011, Robert, et al., 2006). It is plausible that PorB exploits this evolutionary connection to allow recognition and insertion into the outer mitochondrial membrane.

Another example of effector targeting to mitochondria can be drawn from *Mycobacterium tuberculosis*, the causative agent of tuberculosis, which is a facultative

intracellular bacterium that replicates in a unique phagosome compartment within human macrophages (Schlesinger, 1996). In contrast to before mentioned Ats-1 and LncP, the signal targeting the *M. tuberculosis* effector protein PE_PGRS33 has been associated with the highly conserved 'linker' region of the protein consisting of a putative transmembrane domain (**Table 1**) (Cadieux, et al., 2011, Palucci, et al., 2016). Thus, understanding the targeting and import of bacterial effector proteins not only assists in elucidating their function, but may also provide insight into both characterised and novel import pathways of endogenous mitochondrial proteins.

Mitochondrial functions targeted by bacterial effector proteins

Once bacterial effector proteins have reached mitochondria they can then carry out their destined function and modulate organelle activity. Herein we will discuss the mitochondrial functions that are targeted by numerous effector proteins and how this influences bacterial pathogenesis and host cell survival.

Apoptosis

Mitochondria mediate the intrinsic pathway of apoptosis by coordinating both the release of pro-apoptotic proteins into the cytosol and acting as signaling platforms for the activation of the caspase cascade (Tait and Green, 2010). Mitochondrial outer membrane permeabilisation (MOMP), regulated by the Bcl-2 family of proteins, induces the release of intermembrane space proteins, such as cytochrome *c*, Smac/DIABLO and Hrt/Omi, which culminates in the activation of the caspase cascade and cell death (Gillies and Kuwana, 2014, Tait and Green, 2010, Vaux, 2011). Perforation of the outer mitochondrial membrane occurs by oligomerisation of Bax, after translocation from the cytosol to mitochondria, as well as Bak into homo- and hetero-oligomers (**Figure 2A**) (Gillies and Kuwana, 2014). Furthermore, intracellular calcium exchange at the endoplasmic reticulum and mitochondrial interface has been implicated in mitochondrial-mediated apoptosis through the upregulation of VDAC1 and the mitochondrial permeabilisation transition pore (mPTP) (De Stefani, et al., 2012, Keinan, et al., 2013, La Rovere, et al., 2016, Weisthal, et

al., 2014). Oscillating mitochondrial calcium uptake maintains both mitochondrial and cytosolic calcium at homeostatic levels and is important for mitochondrial bioenergetics (La Rovere, et al., 2016).

Apoptosis is perhaps the most commonly targeted pathway upon bacterial infection. Recognition of pathogen associated molecular patterns by intracellular receptors such as Nod-like receptors (NLRs) effectively allows a cell autonomous innate immune response that can eliminate some infections. As such, some intravacuolar bacteria have evolved approaches to control this signaling process using effectors to interact with anti-apoptotic or pro-apoptotic cellular proteins. Comparative proteomic studies of *M. tuberculosis* virulent (H37Rv) and avirulent (H37Ra) strains have revealed the pathogen suppresses the progression of apoptosis during intracellular infection (Abarca-Rojano, et al., 2003, Park, et al., 2006). Jamwal et al., (2013) revealed distinct mitochondrial phenotypes associated with either the virulent or avirulent infection. Specifically, mitochondria of H37Rv infected cells showed normal ultrastructure and increased activity, whilst mitochondria in cells infected with H37Ra were less electron dense and appeared 'exhausted' (Jamwal, et al., 2013). Proteomics approaches revealed upregulation of several proteins in the virulent strain, including the voltage dependent anion-selective channel 2 (VDAC2), which interacts with the anti-apoptotic protein Bak to maintain the protein in an inactive conformation (Cheng, et al., 2003, Lazarou, et al., 2010). Whilst the molecular basis for this anti-apoptotic phenotype is unclear, it is highly likely to be regulated by uncharacterised mitochondrial targeted effector proteins encoded by *M. tuberculosis* as demonstrated by *A. phagocytophilum*. During intracellular infection of neutrophils, *A. phagocytophilum* effector protein Ats-1 localises to the mitochondria and inhibits Bax-induced apoptosis (**Figure 2A**) (Niu, et al., 2010). Typically, within 6-12 hours after release into the peripheral bloodstream, neutrophils undergo apoptosis (Akgul, et al., 2001). When Ats-1 transfected cells were challenged with etoposide, a known inducer of Bax-mediated cell death, Bax remained diffusely localised in the cytosol rather than at mitochondria (Niu, et al., 2010). Niu et al., (2010) propose that this was due to the

maintenance of the mitochondrial membrane potential by the matrix-localised Ats-1, preventing Bax oligomerisation and subsequent apoptosis. Interestingly, Ats-1 has also been implicated in initiating autophagosome formation through an interaction with BECN-1 suggesting effector targeting and thus function, is dependent on the host cell environment (Niu, et al., 2008). Despite extensive characterization of the putative functions of Ats-1, the importance of this effector during *A. phagocytophilum* infection remains to be genetically tested. *A. phagocytophilum* is an obligate intracellular pathogen which significantly restricts the ability of researchers to apply traditional genetic manipulation techniques. Recent advances have led to the development of new strategies to genetically manipulate other obligate intracellular bacteria (Bastidas and Valdivia, 2016, Mueller, et al., 2016, Noriega, et al., 2015). Applying these techniques to *A. phagocytophilum* may eventually lead to creation of an Ats-1 mutant, which can be assessed for both the apoptotic phenotype and overall virulence.

Another example of a mitochondrial effector manipulating apoptotic signaling can be taken from the early stages of intracellular infection by *Salmonella enterica*, where the soluble form of the fimbrial subunit FimA associates with the outer mitochondrial membrane and interferes with apoptotic induction (**Figure 2B**) (Sukumaran, et al., 2010). Here, FimA tightens the association of mitochondrial hexokinase (HK) with VDAC1, preventing HK release. The interaction of HK with the N-terminal region of VDAC1 is believed to exert an anti-apoptotic effect through reduction in channel conductance, possibly preventing calcium influx and/or cytochrome *c* release (Abu-Hamad, et al., 2009, Arzoine, et al., 2009). Sukurmaran et al., (2010) note that dissociation of FimA from the mitochondria at later stages of infection may suggest temporal coordination of apoptosis by the bacterium. Intravacuolar bacterial pathogens are likely to demonstrate redundancy in effector protein function, encoding proteins capable of targeting different levels of the apoptotic cascade. *C. burnetii*, the causative agent of the zoonotic disease Q fever, has been shown to target multiple points of the apoptotic pathway including preventing the release of cytochrome *c*, modulating the interplay between Beclin-1 and Bcl-2 and

regulating the mitochondrial-nuclear trafficking of p32 (Luhmann and Roy, 2007, Vazquez and Colombo, 2010) The *C. burnetii* T4SS substrates AnkG, CaeA, CaeB have been identified as having a role in down-regulating host cell apoptosis and it is plausible the bacterium encodes further anti-apoptotic effectors with the total number of effectors being approximately 150 (Eckart, et al., 2014, Klingenberg, et al., 2013, Luhmann, et al., 2010). AnkG and CaeB both localise to mitochondria although modulate apoptosis by different mechanisms. AnkG binds to p32 and due to this interaction, is trafficked to the nucleus upon apoptosis induction from which it is believed to exert an anti-apoptotic effect (Eckart, et al., 2014). Meanwhile, the molecular nature of CaeB apoptosis control remains unknown but it has been demonstrated to occur downstream of Bax activation and mitochondrial translocation yet upstream of caspase 9 activation (Klingenberg, et al., 2013). Importantly, these studies have been performed by ectopically expressing effector proteins and thus the importance of these virulence factors during infection remains unclear. Researchers have recently developed axenic growth conditions for *C. burnetii*, paving the way for successful genetic manipulation of this previous obligate intracellular pathogen (Moffatt, et al., 2015, Omsland and Heinzen, 2011). Targeted gene deletion has now been demonstrated (Beare, et al., 2012, Cunha, et al., 2015) and thus it will be interesting to resolve the importance of AnkG and CaeB during *C. burnetii* infection.

Mitochondrial dynamics

Mitochondria form dynamic reticular networks within the cell, maintained by complementary fission and fusion events (Baker, et al., 2014). Mitochondrial morphology is implicated in a variety of cellular processes including calcium signaling, autophagy, apoptosis and necrosis (Baker, et al., 2014, Kasahara and Scorrano, 2014). The remodeling of mitochondrial cristae is also recognised as a feature of mitochondrial dynamics. Cristae structure, tightness and abundance depends on the environmental state of the cell, for example, during apoptosis, cristae widen and cristae junctions open to assist in the release of pro-apoptotic molecules from the mitochondria (Pernas and Scorrano, 2016). Mitochondrial localisation of the effector SipB during *S. Typhimurium*

infection of macrophages distorts mitochondrial cristae morphology and is believed to promote autophagy-mediated cell death (Hernandez, et al., 2003). Mitochondria exhibited swelling and loss of cristae structures, morphology consistent with apoptotic cells. It is proposed that *S. Typhimurium* may facilitate dissemination from the intestine to systemic tissues by initially mediating macrophage cell death, resulting in the release of inflammatory cytokines and further influx of macrophages (Guiney, 2005). Cell death of these later recruited macrophages is delayed, allowing migration from the intestine to other tissues (Guiney, 2005).

Mitochondrial dynamics is targeted during both *V. cholerae* and *Listeria monocytogenes* infection. For instance the T3SS effector protein VopE, translocated into host cells by the extracellular bacterium *Vibrio cholera*, localises to mitochondria and binds to the GTPase domain of the mitochondrial Rho GTPases Miro 1 and 2 (Suzuki, et al., 2014). Miro GTPases assist in the attachment and movement of mitochondria along microtubules, a process that has been demonstrated to play a role in lymphocyte adhesion and polarity (Morlino, et al., 2014, Reis, et al., 2009). Additionally, secretion of the bacterial toxin lysteriolysin O (LLO) during *L. monocytogenes* infection induces mitochondrial fragmentation independently of the mitochondrial fission and fusion components Opa1 and Drp1 (Stavru, et al., 2013). Ongoing research is likely to further identify processes of mitochondrial dynamics similarly targeted by intravacuolar bacteria pathogens.

Mitochondrial coordination of immune signaling

Mitochondria are also key players in initiating and coordinating immune signaling and the inflammatory response (Arnoult, et al., 2011). Increasing evidence implicates mitochondria as critical players in cellular antibacterial immunity through the production of mitochondrial reactive oxygen species (mtROS) and activation of signaling pathways (West, et al., 2011a). Although replication occurs within the PCV, secreted bacterial effector proteins and the components of their secretion systems may be recognised by host cell immune surveillance mechanisms (Zhao and Shao, 2015). A subset of Toll-like

receptors (TLRs) are known to localize to endocytic compartments (TLR1, 2 and 4) and activation of these TLRs results in translocation of TRAF6 to the mitochondria where it interacts with ECSIT (evolutionary conserved signaling intermediate in Toll-pathways), promoting the release of mtROS and induction of the pro-inflammatory and bactericidal signaling pathways (West, et al., 2011b). Currently there are no defined intravacuolar bacterial effector proteins that function to control mitochondrial immune signaling pathways, however due to the importance of the organelle in the coordination of immunity, it is likely that they exist. However, a nice example can be taken from the extracellular bacterium *Vibrio cholera*, where the interaction of VopE with Miro (described above) perturbs mitochondrial perinuclear clustering and indirectly reduces MAVS-mediated NF κ B signaling (Suzuki, et al., 2014). Through the alteration of mitochondrial dynamics, VopE inhibits host inflammatory responses.

Mitochondrial metabolism

Mitochondrial synthesis of ATP by oxidative phosphorylation is integral to eukaryotic cell survival. Furthermore, mitochondria participate in lipid synthesis, fatty acid metabolism and contribute to cellular calcium homeostasis. Important contacts between the mitochondria and the endoplasmic reticulum, such as those formed at the mitochondrial associated membrane (MAM) facilitates the exchange of both metabolites and lipids between organelles (Phillips and Voeltz, 2016, Tatsuta, et al., 2014, van Vliet, et al., 2014). During infection, pathogens must acquire essential nutrients for survival and proliferation. For intracellular pathogens, a rich metabolite pool may be found in the host cytosol, whilst intravacuolar pathogens must employ different mechanisms to facilitate access and uptake of nutrients into the vacuole. As mitochondria present a hub of metabolic processes, regulation of these functions may provide the bacteria with an increase in available nutrient sources. For instance, *Chlamydiae* are referred to as 'energy parasites'. These pathogens lack genes encoding the F₁F₀ATP synthase and instead must import host-derived ATP into the bacterial cell by an ATP/ADP translocase (Fuchs, et al., 2012, Wyllie, et al., 1998). Increasing the ATP accessible within the host cell would favor Chlamydial establishment and proliferation. The *L. pneumophila* effector protein LncP,

described above as a TIM22 translocase substrate, is a carrier protein that is capable of depleting ATP from the mitochondrial matrix (Dolezal, et al., 2012). This function would alter host cell ATP dynamics, however the precise contribution of LncP toward *L. pneumophila* pathogenesis remains to be elucidated. Manipulation of metabolism may also impact the function of the host cell. Expanding knowledge of the metabolic requirements of immune cells, coupled with the observation that several intravacuolar pathogens infect cells of the innate immune system, presents the interesting possibility of influencing the activation of the host immune response through mitochondrial control of metabolism (Ganeshan and Chawla, 2014).

Mitochondrial protein trafficking

As highlighted earlier, mitochondria utilise dynamic translocation machineries to import nuclear-encoded precursors. Indeed, mitochondrial function is highly dependent on the organelles proteome, which places an enormous pressure on these import pathways. Genome wide siRNA screens have highlighted the importance of mitochondrial translocation machinery in *Chlamydia caviae* and *C. burnetii* infection (Derre, et al., 2007, McDonough, et al., 2013). Upon depletion of two subunits of the TOM complex (Tom40 and Tom22) during *C. caviae* infection, a reduction in the size of chlamydial inclusions was observed and a subsequent proteomic screen identified putative interactions between five inclusion membrane proteins and the TIM-TOM complex (Derre, et al., 2007, Mirrashidi, et al., 2015). During *C. burnetii* infection, the silencing of the intermembrane space chaperone proteins Tim8b, Tim10 and Tim13 reduced the size of the *Coxiella*-containing vacuole and intracellular bacterial replication (McDonough, et al., 2013). The interaction between bacterial effector proteins and the mitochondrial import machinery may promote the import of other effectors into the organelle, thus providing another mechanism by which bacteria may modulate organelle function.

Mitochondria are closely associated with pathogen-occupied vacuoles during infection

Upon successful uptake into the host cell, intravacuolar pathogens reside and replicate within a membrane-bound compartment, frequently referred to as the

parasitophorous vacuole (PV). The biogenesis and physiological characteristics of this replicative niche are unique to the pathogen (Creasey and Isberg, 2014, Ham, et al., 2011). For instance, *L. pneumophila* actively evades endocytic maturation and acidification of the phagosomal compartment and instead directs fusion with ER-derived vesicles (**Figure 3A**) (Horwitz and Maxfield, 1984). During maturation, pathogen-occupied vacuoles traffic to distinct cellular locations permitting association with specific organelles. Such intracellular positioning provides ready access to the organelle for acquisition of essential nutrients and host biochemical pathways essential for replication of the bacterium. Close associations between the PV of multiple intravacuolar pathogens such as *L. pneumophila*, *Chlamydia psittaci* and *S. negevensis*, and mitochondria have been observed by fluorescence and electron microscopy (**Figure 3A**) (Horwitz, 1983, Matsumoto, 1981, Matsumoto, et al., 1991, Mehlitz, et al., 2014).

The formation of the *Legionella*-containing vacuole (LCV) involves a unique series of cytoplasmic events occurring over 8 hours post-infection (**Figure 3A**) (Horwitz, 1983). Early in infection (1 hour), the LCV is observed in close apposition to the mitochondria however, over time this association declines and LCVs become predominately lined with ribosomes in an ER-derived vacuole (**Figure 3A**) (Horwitz, 1983). This phenomenon is also demonstrated during cellular infection by *Chlamydia psittaci* and *Simkania negevensis* (**Figure 3, B and C**) (Matsumoto, 1981, Matsumoto, et al., 1991, Mehlitz, et al., 2014). *C. psittaci* and *S. negevensis* both belong to the phylum *Chlamydiae* and are associated with respiratory infection (Herweg and Rudel, 2016). All *Chlamydiae* organisms display a biphasic development cycle, forming both infectious elementary bodies (EB) and replicative reticulate bodies (RB) (Herweg and Rudel, 2016). Over 20 years ago, Matsumoto noted that the inclusion (the *Chlamydial* PV) of *C. psittaci* comes into close association with the mitochondria (5.1 nm) (**Figure 3B**) (Matsumoto, 1981, Matsumoto, et al., 1991). Although the precise reason for this interaction remains to be elucidated, it has been proposed as a method by which *C. psittaci* acquires ATP from the host cell (Knittler and Sachse, 2015).

During *S. negevensis* infection, the ER-associated *Simkania*-containing vacuole (SCV), containing both EB and RB bacteria, forms contact sites with the mitochondria at 48 hours post-infection (**Figure 3C**) (Mehlitz, et al., 2014). Mehlitz et al (2014) observed the SCV using both fluorescence and electron microscopy and found mitochondria interact with the vacuolar-ER surface (Mehlitz, et al., 2014). This interaction appeared dependent on the growth stage of the bacterium as earlier vacuoles were ‘intertwined’ with the mitochondrial network, whilst those of a later stage appeared ‘lined’ by the mitochondrial network (Mehlitz, et al., 2014). Mitochondrial – ER contact sites are dynamic metabolic and signaling hubs (Phillips and Voeltz, 2016, Raturi and Simmen, 2013, Vance, 2014) and the authors’ postulate that the close interaction of the SCV with these sites may provide *S. negevensis* with the lipids required for replication. Indeed as noted above, mitochondria are important players for the biosynthesis and trafficking of lipids including phosphatidylserine, phosphatidylethanolamine and cholesterol (Martin, et al., 2016, Tatsuta, et al., 2014, Vance, 2015). Interestingly, this association was rarely observed during infection of the natural protozoan host, *Acanthamoeba castellanii*, possibly indicating that it represents a host-specific trait.

Conclusion and perspectives

The molecular pathogenesis of intravacuolar bacterial pathogens is an emerging area in the field of host-pathogen research. Understanding how and why these pathogens target and manipulate mitochondria provides an exciting platform on which we can dissect both bacterial pathogenesis and survival, but also interrogate mitochondrial function in the context of infection. Expanding capacity to culture and genetically manipulate intravacuolar pathogens has created the possibility to investigate the contribution of individual effector proteins to bacterial pathogenesis. As much of the current knowledge has relied on ectopic expression of effector proteins within the host, these advances will allow an appreciation of the importance of virulence factors in the context of infection. In addition, progress in the field of host-pathogen proteomics has revealed more extensive bacterial effector

repertoires, targeted host protein interactions and the modulation of host cell processes. The combination of advanced microscopy such as cryo-electron microscopy (EM) and structural knowledge has shed light on the macromolecular complexes utilised by bacterial pathogens to secrete proteins across multiple membranes. Thus the study of the specific and coordinated intracellular targeting of bacterial effector proteins has gained momentum.

The sophisticated import machineries of mitochondria present an additional barrier to translocated effector proteins, many of which are hijacked by the bacteria to deliver effector proteins to the correct sub-mitochondrial compartment. By studying this targeting, we may gain an insight into non-canonical mitochondrial targeting signals and import pathways, such as those of outer membrane proteins. The impact of bacterial effectors on seemingly disparate mitochondrial functions allows the elucidation of the links between mitochondrial processes. These interactions not only provide us with a greater understanding of the molecular mechanisms by which pathogens modulate the mitochondria, but also highlight the complexity and importance of this unique organelle.

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Figure and table legends

Table 1:

Examples of intravacuolar bacterial pathogens that target the mitochondria.

Figure 1: Mitochondrial translocation machineries hijacked by bacterial pathogens during infection. (A) Mitochondria contain a sophisticated system of protein sorting, encompassing five major import pathways. The Translocase of the Outer Membrane (TOM) complex, facilitates translocation across the mitochondrial outer membrane (OM), into the intermembrane space, where precursor pathways diverge. The Sorting and Assembly Machinery (SAM) complex inserts β -barrell proteins into the outer membrane. The Mitochondrial Intermembrane space Assembly (MIA) machinery coordinates the oxidative folding of many intermembrane space proteins with the assistance of the sulfhydryl oxidase Essential for Respiration and Viability 1 (Erv1). Precursor proteins containing an N-terminal presequence are transferred through the Translocase of the Inner Membrane (TIM) 23 complex and are either laterally released into the inner membrane or transported into the matrix with the assistance of the Presequence translocase-Associated Motor (PAM) complex. Presequences are cleaved by the Mitochondrial Processing Peptidase (MPP). Finally, hydrophobic precursor proteins are transported through the intermembrane space by the hexameric chaperone complexes: Tim9-Tim10 and delivered to the TIM22 complex for insertion into the inner membrane. **(B)**

Mitochondrial import pathways and translocation machineries can be manipulated by bacterial pathogens to target and sort bacterial effector proteins into a specific mitochondrial sub-compartment. Upper panel: Import of *Anaplasma phagocytophilum* effector protein Ats-1 is via the presequence pathway. Ats-1 contains a cleavable, N-terminal presequence, which targets the protein to the TIM23 complex and enables import into the mitochondrial matrix. Lower panel: The *Legionella pneumophila* effector protein LncP is translocated into the mitochondria by the TOM complex and is targeted to the TIM22 complex, by the hexameric Tim9-Tim10 complex. The TIM22 complex then mediates assembly of the protein within the mitochondrial inner membrane.

Figure 2: Targeting of mitochondrial apoptotic pathways by intravacuolar bacterial effector proteins. Top panel: During *Anaplasma phagocytophilum* infection, Ats-1 is imported into the mitochondrial matrix via the TOM and TIM23 machineries. Ats1 is believed to stabilise the mitochondrial membrane potential ($\Delta\psi$) and cristae structure, preventing Bax oligomerisation and the release of apoptotic molecules, such as cytochrome c into the cytosol.

Bottom panel: The soluble form of the pilus protein, FimA, encoded by *Salmonella enterica* localises to the mitochondria through an association with the outer mitochondrial membrane protein Voltage Dependent Anion Channel-1 (VDAC1). Here, FimA stabilises the interaction between VDAC1 and hexokinase (HK), preventing dissociation of HK from VDAC1 and mediating short-term suppression of apoptosis within host cells. This may prevent VDAC1 mediated influx of calcium ions (Ca^{2+}) into the mitochondria, resulting in mitochondrial swelling and rupture of the outer membrane. In each case, the normal pathway of intrinsic apoptosis is shown on the left, whilst the modification during infection is shown to the right.

Figure 3: Entry and formation of the pathogen occupied vacuole and its association with the mitochondria. Intravacuolar pathogens gain entry to the host cell by endocytosis

and display unique vacuole biogenesis pathways, culminating in the formation of a replicative niche. Several intravacuolar pathogens display a distinct association with the mitochondria. Panels depict the biogenesis pathway of each individual bacterium, highlighting the interaction with the mitochondria. **(A)** *Legionella pneumophila* evades endocytic maturation and instead directs fusion of the *Legionella*-containing vacuole (LCV) with ER-derived vesicles. At 1 hour post-infection, the LCV is observed in close apposition with the mitochondria. This association declines and the endoplasmic reticulum (ER)-derived LCV becomes predominately lined with ribosomes. Both **(B)** *Chlamydia psittaci* and **(C)** *Simkania negevensis* display a biphasic lifecycle, with the infectious elementary body (EB) invading the cell before conversion into the replicative reticulate body (RB). The replicative vacuole of *Chlamydia psittaci*, termed the Chlamydial inclusion, is located at the peri-Golgi region and is associated with the mitochondria. Similarly, the ER-associated *Simkania negevensis*-containing vacuole (SCV) forms contact sites with the mitochondria 48 hours post-infection.

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Loss of cristae structure

membrane; LncP: *Legionella* nucleotide carrier Protein; OMM: Outer mitochondrial membrane; PE_PGRS33: pro-glu_polymorphic GC-rich sequence 33; SipB: Salmonella invasion protein B; T3SS: Type 3 Secretion System; T4SS: Type 4 Secretion System; T7SS: Type 7 Secretion System

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Author/s:

Fielden, LF;Kang, Y;Newton, HJ;Stojanovski, D

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