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Bacterial proteases from the intracellular vacuole niche; protease conservation

and adaptation for pathogenic advantage

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Keywords:

protease, pathogen, intracellular, vacuole

12

Abbreviations

TTSS – type three secretion system

TFSS – type four secretion system

SCV – *Salmonella* containing vacuole

BCV – *Brucella* containing vacuole

ER – endoplasmic reticulum

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1

1   ## Abstract

2   **Proteases with important roles for bacterial pathogens which specifically reside**

3   **within intracellular vacuoles are frequently homologous to those which have**

4   **important virulence functions for other bacteria. Research has identified that**

5   **some of these conserved proteases have evolved specialised functions for**

6   **intracellular vacuole residing bacteria. Unique proteases with pathogenic**

7   **functions have also been described from *Chlamydia*, *Mycobacteria*, and**

8   ***Legionella*. These findings suggest that there are further novel functions for**

9   **proteases from these bacteria which remain to be described. This review**

10   **summarises recent findings of novel protease functions from the intracellular**

11   **human pathogenic bacteria which reside exclusively in vacuoles.**

12

13   ## Introduction

14   Bacterial pathogens display a wide array of tissue trophisms and disease pathologies

15   in humans, including intracellular growth for many pathogens. Most intracellular

16   pathogens have at least a transient association with a vacuole as they are frequently

17   taken into the cell via an endocytic or phagocytic vacuole, however only a select few

18   remain associated with this vacuole. Many others escape to, or directly enter, the

19   cytosol where they replicate, including *Burkholderia*, *Shigella*, *Francisella tularensis*,

20   *Listeria monocytogenes*, and *Porphymonas gingivalis*. In order to be successful within

21   the intracellular vacuole the bacteria must manipulate the host cell to develop and

22   protect the vacuole. Bacterial proteases have critical roles for this pathogen:host cell

23   interface. This review highlights the recent exciting advances in the understanding of

24   the important roles that proteases conduct for bacterial pathogens which require a

25   intracellular vacuole for successful pathogenesis.

2

1

A. Proteases and Bacterial Pathogenesis

3

4　Proteases are known to have crucial roles for all domains of life with functions such

5　as protein processing, cellular functions, signalling, protein maintenance, protein

6　degradation, and liberation of amino acids and have long been implicated in bacterial

7　virulence. It is clear that the specific tissue or niche pathogenic environment of the

8　pathogen directly influences the proteolytic arsenal. An example is the suite of

9　proteases secreted by *Porphyromonas gingivalis* (the major etiological agent for

10　periodontitis) which have been implicated in the tissue destruction which results in the

11　disease pathology (Kumagai*, et al.*, 2005).

12　　　　Intracellular bacterial pathogens often enter cells via endocytosis, followed by

13　rapid escape to the cytoplasm to avoid the lysosomal pathway, or for a specialised

14　group of bacteria, by the establishment of a specific vacuole in which the bacteria

15　reside and replicate. Whilst the composition and localisation of these vacuoles are

16　distinct to each bacterial species, the biogenesis of the vacuole commonly involves

17　both bacterial and host factors. The bacteria manipulate the host cell from within this

18　vacuole; to maintain the integrity of the vacuole, to repress or induce apoptotic and

19　immune signally pathways, to manipulate host cell cytoskeleton, influence cell

20　division, and ensure nutrient supply. There have been a number of comprehensive

21　reviews of host-pathogen interactions and the variety of different bacterial effectors

22　involved in this relationship, including (Knodler*, et al.*, 2001, Bhavsar*, et al.*, 2007,

23　Faherty & Maurelli, 2008, Hybiske & Stephens, 2008).

24

3

1 **B. Bacterial pathogens which replicate and reside within intracellular**

2 **vacuoles**

3

4 There are only six bacterial pathogens which have been described to remain within a

5 vacuole for intracellular replication and survival. For the purpose of this review

6 vacuole resident bacterial pathogens have been defined as: those bacteria which

7 remain intimately associated with, and replicate within, a vacuole for the majority of

8 their intracellular phase either in all or some cell types. The human bacterial

9 pathogens which fit this definition include; *Chlamydia*, *Salmonella*, *Brucella*,

10 *Legionella*, *Coxellia*, and *Mycobacterium*. In order to consider the role of proteases

11 for these intracellular vacuole pathogens, it is important to firstly consider the unique

12 vacuole niche in which these bacteria reside (summarised Fig 1).

13

14 **1.** *Salmonella enterica* **serovar Typhimurium**

15 *Salmonella enterica* serovar Typhimurium is a facultative intracellular pathogen

16 which causes gastroenteritis in humans. The organism encodes two type III secretion

17 systems which inject proteins into the host cell to facilitate invasion and biogenesis of

18 the vacuole (SCV, *Salmonella* containing vacuole)(Brawn*, et al.*, 2007), recently a

19 third type III secretion system encoding a flagella has been described to have an

20 intracellular role, by direct export of flagella components into the host cell to induce a

21 pro-inflammatory response (Hautefort, *et al.*, 2008). The vacuole, which has been

22 described as a stalled late endocytic/lysosomal vesicle, is located close to the golgi

23 apparatus (Ramsden*, et al.*, 2007). Global gene changes observed during different

24 phases of intracellular infection and dependent on the cell type (epithelial or

25 macrophage) suggest *Salmonella* is a versatile intracellular pathogen (Hautefort, *et al.*,

4

1   2008). In spite of the many properties which are yet to be determined Salmonella's

2   survival and manipulation of the host cell from the SCV has many potential areas

3   where novel protease functions may be discovered including, the rapid assembly and

4   removal of distinct protein machinery and factors for the different cell types they

5   infect, and response to and survival in the potentially acidic environment of the late

6   endosome-like vacuole.

7

8   *2. Brucella*

9   *Brucella* is a human zoonosis which causes a severe and debilitating disease in

10 humans. *Brucella* are low stimulants for the human immune response due to reduced

11 expression of pathogen associated molecular patterns (PAMPs)(Barquero-Calvo, *et*

12 *al.*, 2007). The bacteria produce far fewer classic virulence factors than most other

13 pathogens and this successful pathogenesis is attributed to the localisation within an

14 intracellular vacuole niche environment within a variety of phagocytic cells (reviewed

15 (Ko & Splitter, 2003)). The vacuole maturation into a late autophagosome involves

16 both bacterial and host factors, although little is currently known about the vacuole

17 biogenesis (Qin, *et al.*, 2008). The vacuole is known to inhibit fusion with lysosomes

18 and is associated with the ER. The bacterial factors which have a role for intracellular

19 survival include; SpoT; the ppGpp3'-pyrophosphohydrolase involved in starvation

20 response, a type IV secretion system (VirB), cell wall synthesis, and membrane

21 structure components (Kim, *et al.*, 2003). The limited suite of virulence factors which

22 have been identified for *Brucella* implies that either; the organism requires few

23 virulence factors, or that it has unique virulence strategies which facilitate it's

24 intracellular vacuole niche that remain to be described (potentially including novel

25 proteases).

1

3. *Mycobacterium tuberculosis* and *leprae*

Mycobacterium tuberculosis and *leprae*, causative agents of tuberculosis and leprosy are responsible for considerable human disease burden worldwide. *Mycobacterium,* replicate within a vacuole inside phagocytes during disease, excluding *Mycobacterium ulcerans* which has both intracellular and extracellular phases during infection (Torrado*, et al.*, 2007). The *M. tuberculosis* infected phagocytes are surrounded by a complex of inflammatory cells and material which is termed the granuloma. The aggressive immune response, which causes immunopathology and is thought to be beneficial to the pathogen by increasing transmission, is stimulated by the unique cell wall mycolic acids and other bacterial components. However, the *Mycobacterium* also use multiple means to specifically stimulate or down-regulated the human immune response throughout the course of disease, and are able to persist causing latent disease (reviewed (Doherty & Andersen, 2005)). The *Mycobacterium* vacuole resembles a stalled early endosome, as it is non-acidic and has early endosomal markers. A number of bacterial factors have been identified which mediate the development of this niche vacuole including; SecA2 secreted factors (Kurtz*, et al.*, 2006), urease, lipoarabinomannin, protein kinase G, lipid phosphotase, and cell wall cholesterol composition (Doherty & Andersen, 2005). Proteases are likely to play a variety of critical roles in this host:pathogen interaction, a likely example is the secreted mycosins, discussed further in the next section, and potentially other proteases which function in the modification of both bacterial and host proteins to pathogenic advantage.

4. *Legionella*

6

1   *Legionella* are facultative intracellular pathogens, thought to naturally infect single

2   cell protozoa such as amoebae. *Legionella pneumophila* is the predominant species

3   which cause disease in humans. *Legionella's* biphasic life cycle consists of

4   morphologically distinct variants of the transmissive form (infective) and the

5   intracellular replicative form. The replicative form is found within an intracellular

6   vacuole in alveolar epithelia and macrophages during human disease. *Legionella* and

7   the closely related pathogen C*oxiella* share a distinct type IV secretion system

8   (Dot/Icm, TFSS) which exports bacterial effector proteins into the host cell

9   cytoplasm. These bacterial effector proteins mediate development of the vacuole

10 which traffics to the endoplasmic reticulum (ER) and avoids the lysosomal pathway

11 (reviewed (Roy & Tilney, 2002)). The vacuole is a rich, ribosome surrounded, ER

12 compartment. There appears to be redundancy in the bacterial genes involved in

13 pathogenesis and intracellular survival, due to the presence of multiple homologous

14 genes and also as genetic knockouts in many putative virulence factors do not result in

15 attenuation of virulence. However, many factors have been implicated in virulence

16 including; TFSS, proteases, stationary phase response RpoS, Type II secretion system

17 and iron assimilation genes (reviewed (Roy & Tilney, 2002)). Protease functions for

18 this organism likely include assembly and regulation of bacterial proteins as well as

19 specific degradation and modulation of host proteins. As it has been demonstrated that

20 *Legionella* can survive early acidification of the vacuole proteases which function in

21 stress response may also be critical to the pathogenesis of this organism.

22

23 **5. Coxiella**

24 *Coxiella*, an obligate intracellular pathogen, has many features in common with the

25 closely related *Legionella*. *Coxiella burnetti*, a zoonotic pathogen which causes severe

7

1   flu-like disease and fever in humans, has been identified as a potential bio-terrorism

2   agent. The organism has a biphasic life cycle, consisting of morphologically distinct

3   extracellular environmentally stable form and an intracellular replicative form (within

4   phagocytes during human infections). The intracellular vacuole of *Coxiella* is distinct

5   from that of *Legionella* as it is acidic and is characteristic of a phagolysosome,

6   although both vacuoles locate to the ER (Seshadri*, et al.*, 2003). Co-infection

7   demonstrated that the *Legionella* and *Coxiella* intracellular vacuoles do not fuse, even

8   though the *Coxiella* vacuoles are able to fuse with other pathogenic vacuoles

9   including *Mycobacterium tuberculosis*, indicating that the *Legionella* and *Coxiella*

10   vacuoles are distinct (Sauer*, et al.*, 2005). There is potential for unique proteases

11   which have specific host cell targets to be identified from this organism, given the

12   distinct vacuole development. Stress response and modification of the bacterial

13   proteome between the two bacterial forms both have the potential to involve critical

14   bacterial proteases.

15

6. Chlamydia

17   *Chlamydia* is an obligate intracellular pathogen, with *Chlamydia pneumoniae* and

18   *Chlamydia trachomatis* causative agents of human disease, including pneumonia,

19   sexually transmitted infections, and trachoma. The bacterium is not able to be

20   genetically manipulated and is typified by a biphasic developmental cycle. The cycle

21   consists of small, metabolically inactive, extracellular, infectious forms (EBs) and

22   intracellular, metabolically active, non infections replicative forms (RBs). The

23   intracellular bacteria replicate within an inclusion vacuole which seems to be entirely

24   exclusive of the endocytic pathway. The biogenesis of the vacuole is mediated by

25   *Chlamydia* interactions with host cell components, including actin, and acquisition of

8

1   vesicles including golgi-derived vesicles containing sphingomyelin (reviewed,

2   (Abelrahman & Belland, 2005)). The manipulation of many host cell pathways to

3   provide an ideal infectious and replicative environment has been demonstrated,

4   including pathways for, immunity, apoptosis, and cell division. Frequently these

5   manipulations have been described to involve TTSS effectors, proteases, and

6   GTPases, however, a number of known effects on the host cell which have been

7   attributed to chlamydial factors remain to be mechanistically elucidated.

8

9   **C. Adaptation of widely conserved bacterial proteases to the**

10 **specialised niche of intracellular bacterial pathogens**

11

12 There are numerous examples of bacterial proteases which are widely conserved

13 throughout the domain bacteria, which have been found to have specialised substrates

14 or functions within some species. There are a number of examples of these

15 modifications for the intracellular vacuole resident bacteria, those with the most well

16 characterised functions are described here.

17

18 **1. Cytoplasmic ATP dependent protease Lon**

19 The cytoplasmic ATP dependent protease Lon (MERNUM: MER000485), which

20 typically functions during stress conditions, and for degradation of ssrA tagged

21 polypeptides referred to as tmRNA or 10aRNA), is important for virulence of some

22 pathogens. Lon, a serine protease of the Clan SJ, Family S17, is important for

23 motility and biofilm formation of *Pseudomonas aeruginosa*, both factors in the

24 virulence of this bacterium (Marr, *et al.*, 2007). Within the intracellular niche, Lon is

25 essential for intracellular survival and epithelial cell invasion of *Salmonella enterica*

9

1   (Boddicker & Jones, 2004). This is at least partially due to Lon's function in

2   differential regulation of the expression *Salmonella* pathogenicity islands during

3   intracellular survival and intracellular infections, by degrading regulatory factors HilC

4   and HilD (Takaya*, et al.*, 2005). This degradation is a specific proteolytic regulatory

5   mechanism, leading to altered transcription of the pathogenicity islands. *Brucella*

6   *abortus* Lon has a stress survival function and a virulence role in the mouse model of

7   infection, although the substrates of *Brucella* Lon involved in mouse infection remain

8   unknown (Robertson*, et al.*, 2000). Given that Lon has been described to have many

9   different functions, and regulatory roles for bacteria, this protease may have important

10   functions for the other vacuole resident pathogens. Interestingly, there are no putative

11   genes for *lon* present on either the *Mycobacterium leprae* or *Mycobacterium*

12   *tuberculosis* genomes, although genes annotated as *lon* are present in the genomes of

13   *Chlamydia, Legionella, Coxiella, and Brucella*. The *Mycobacterium* proteosome

14   activity factor (described below) likely conducts similar functions to Lon. Lon has the

15   potential to have specific regulatory roles for the rapid removal of key proteins which

16   may be important for these pathogens, all of which require specific alterations in their

17   proteomes according to the specific stage of their pathogenesis (ie distinct proteins are

18   likely required for very specific and short time frames during invasion, establishing

19   the vacuole, and preparing to exit the host cell).

20

21   **2.** *Mycobacterium* **Proteasome**

22   A virulence role has also been demonstrated for the *Mycobacterium* proteasome

23   (MERNUM: MER001720 and some of the accessory and co-components. Bacterial

24   proteasomes have only been described in members of the class acintomycetes and are

25   very   reminiscent   of   the   eukaryotic   proteasomes.   These   proteases   are   large

10

1   multisubunit barrel structures which consist of rings of β subunits (catalytic unit)

2   between rings of the α subunit (Wang. T, *et al.*, 2009). The α subunit is the ATPase

3   (Mpa) which is critical for substrate coordination and unfolding. The catalytic subunit

4   has a threonine catalytic residue and belongs to the protease clan PB, family T1

5   according to MEROPs classifications. The proteasome relies on a ubiquitinylation like

6   label for substrate determinants, termed pupylation, which is known to rely on PafA

7   (for some substrates) (Pearce, *et al.*, 2008), a deamidase (PafD) (Striebel, *et al.*, 2009),

8   and the protein which is conjugated to lysines on the substrate proteins (Pup)

9   (reviewed (Darwin, 2009)).   Interestingly, the Mpa and accessory PafA are

10   specifically required for *in vivo* growth and pathogenesis, supporting that, whilst the

11   degradation of damaged proteins (such as those damaged by NO and other oxidants)

12   is an important function of the mycobacterial proteasome both *in vitro* and *in vivo,*

13   some specific substrates may be targeted for regulation or critical proteomic

14   modifications, in a similar scenario to that which has been elucidated for Lon in other

15   bacteria.

16

17   **3. Cytoplasmic ATP dependent protease complex ClpXP**

18   The cytoplasmic ATP dependent protease complex ClpXP, (ubiquitous Clp (Hsp100)

19   protease/chaperone family, MERNUM: MER00474) have protein maintenance, stress

20   response and virulence roles in many bacteria. ClpP is the serine protease component

21   (Clan SK, Family S14), and is always found with a second subunit, typically ClpA or

22   ClpX which is the ATP binding subunit which also unfolds the substrates. These

23   proteases have been demonstrated to assemble into 7-mer rings. A complex regulatory

24   role of ClpXP has been described for *Salmonella enterica*; the protease regulates the

25   *Salmonella* pathogenicity island I (SPII) TTSS. The regulation occurs via direct

11

1   degradation of the flagella master regulator FliD/C, thus preventing transcription of a

2   gene which encodes a positive regulator (FliZ) of the expression of HilC/HilD which

3   in turn are positive regulators of the SPII (Kage*, et al.*, 2008). *Salmonella enterica*

4   *clpXP* mutants show reduced virulence in the mouse model of disease. *Brucella suis*

5   *clpAB* has been demonstrated to be critical for stress survival, including the types of

6   stress the organism is likely to encounter *in vivo* such as acid stress (Ekaza*, et al.*,

7   2001). The genome sequences for all the vacuole resident bacterial pathogens which

8   are the focus of this Review have at least one set of *clp* genes annotated. This highly

9   conserved protease is likely to be important to most intracellular and extracellular

10   bacteria, and seems unlikely to be a source of unique intracellular adaptations, except

11   in controlling key regulators such as described for *Salmonella*.

12

13   **4. HtrA/DegP**

14   HtrA, a temperature activated stress response protease (MERNUM: MER000266), has

15   been identified to have a virulence role in many bacterial pathogens, by chaperoning

16   or degrading misfolded proteins and in some cases maturation of proteins to allow

17   correct assembly (reviewed, (Clausen*, et al.*, 2002)). HtrA or DegP is a chymotryptic

18   like serine protease with a C-terminal PDZ domain which functions in either gating

19   the active site or substrate coordination depending on the family member. These

20   proteases are frequently found as hexamers but been found in complex with substrates

21   as 24-mers (Krojer*, et al.*, 2008). HtrA is required for virulence of *Salmonella*

22   *enterica* (Lowe*, et al.*, 1999). Interestingly, both the chaperone and protease function

23   of HtrA were important for virulence of Salmonella, although protease activity was

24   more important (Lewis*, et al.*, 2009). HtrA related proteases DegQ and DegS also

25   contribute to *Salmonella* virulence (Farn & Roberts, 2004, Mo*, et al.*, 2006).

12

1   *Legionella* HtrA is critical for virulence in mammalian cells and virulence in the AJ

2   mouse intrapulmonary infection model (Pedersen*, et al.*, 2001, Clausen*, et al.*, 2002).

3   *Brucella* HtrA has been demonstrated to be functionally active, with conflicting

4   reports on an intracellular requirement, although there is consensus that HtrA is

5   important for *Brucella* infection of macrophages *in vitro* (Elzer*, et al.*, 1996, Phillips

6   & Roop, 2001, Roop*, et al.*, 2001, Kim*, et al.*, 2003). HtrA related proteases DegQ

7   and DegS also contribute to *Salmonella* virulence (Farn & Roberts, 2004, Mo*, et al.*,

8   2006). *Chlamydia* HtrA has been demonstrated to be functionally active and present at

9   different levels during intracellular infection model which could imply a role for

10   pathogenesis (Huston*, et al.*, 2007, Huston*, et al.*, 2008). There are three predicted

11   *htrA* for *Mycobacterium tuberculosis* (although in this publication no account for

12   likely DegS and DegQ homologs has been noted, thus we suggest there may be one

13   HtrA, one DegS, and one DegQ) and one of these, HtrA2, was been shown to be

14   important for virulence in the mouse model (Mohamedmohaideen*, et al.*, 2008). The

15   HtrA encoded on the *Coxiella* genome could prove to have a virulence role given the

16   likely high protein stress conditions within the late endo-lysosomal vacuole in which

17   these bacteria reside. A virulence function of HtrA has been demonstrated for many

18   bacteria with considerable diversity of pathogenic niche, and while little is known

19   about the specific substrates of HtrA, the virulence function seems to be due to both

20   the stress response proteolytic activity and protein assembly/chaperone activity for

21   assembly of extra-cytoplasmic proteins (Skorko-Glonek*, et al.*, 1995, Krojer*, et al.*,

22   2002, Skorko-Glonek*, et al.*, 2007). This suggests that while the function of HtrA is

23   unlikely to show unique adaptations to the intracellular niche, the chaperone

24   substrates may be distinct between the vacuole resident and non vacuole resident

13

1   bacterial pathogens and represent key determinants for surviving the intracellular

2   vacuole niche.

3

4   **5. DegS and Site 2 Protease**

5   The proteolytic signal cascade which upregulates *htrA* and other cell envelope stress

6   response components in *E. coli* involves HtrA related protease DegS (MERNUM:

7   MER001373) and the inner membrane Site 2 protease zinc metalloprotease RseP

8   (MERNUM: MER004480). The components of this pathway are conserved and likely

9   conduct a similar function in *Salmonella*. *Mycobacteria* PepD which is one of the

10   three HtrAs, have recently been identified to participate in upregulation of SigmaE

11   during stress, supporting a similar regulatory network in this organism (White*, et al.*,

12   2010). Analysis of the *Legionella, Coxiella,* and *Brucella* genomes are supportive of a

13   potentially similar proteolytic signalling pathway, with many constituents conserved;

14   particularly DegS and RseP, although none of these have been functionally

15   demonstrated. The *Chlamydia* genome encodes a site 2 protease homolog but no

16   DegS homolog suggesting the site 2 protease may be involved in an alternative

17   proteolytic function in this organism. The site 2 protease homolog on the

18   *Mycobacterium tuberculosis* genome (RseP) has been shown to be involved in a

19   proteolytic signal regulating the unique cell envelope composition of *Mycobacterium*,

20   including mycolic acid content (Makinoshima & Glickman, 2007). The role of

21   proteolytic signalling cascades for bacterial pathogens have not been well described

22   and the site 2 protease mediated signalling cascade could play vital and unique roles

23   in the pathogenesis of these intracellular bacteria.

24

25   **6. Tail specific protease**

14

1   The tail specific protease (Tsp, MERNUM: MER001295) was first described in *E.*

2   *coli* as a periplasmic protease important for C-terminal processing of substrates

3   including PBP3 or FtsI. The protease is a serine protease of the clan SK family S41

4   which cleaves protein substrates at the C-terminus. There have been few

5   investigations into Tsp from other bacteria although recently a protein processing role

6   in *Borrelia borgdeferi* was demonstrated (Ostberg*, et al.*, 2004). The *Brucella suis*

7   Tsp has a role in cellular morphology suggesting a similar protein processing

8   function, and is important for virulence and intracellular survival (Bandara*, et al.*,

9   2005). Tsp has been shown to have a completely novel function in *Chlamydia*

10 whereby it is exported into the host cell and degrades the protein p65 thus preventing

11 NF-κβ activation of a variety of inflammatory genes (Lad*, et al.*, 2007, Lad*, et al.*,

12 2007). No functional analysis has been reported of the Tsp homologs which are

13 present on the genomes of *Legionella, Coxiella, Salmonella* and no Tsp homolog is

14 present on the genomes of *Mycobacterium*. This highly specialised role of this widely

15 conserved protease for *Chlamydia* demonstrates that the intracellular-vacuole-resident

16 bacteria are a source of novel protease adaptations, even from conserved proteases.

17

18 **7. Deubiquitinases**

19 Only a few pathogens have been shown to produce deubiquitinases, including herpes

20 virus, *Yersinia*, *Burkholderia* and of interest to this review, *Salmonella* and

21 *Chlamydia*. *Chlamydia* was detected to have two ubiquitinases, using an activity

22 based probe (Misaghi*, et al.*, 2006). The deubiquitinases are cysteine proteases which

23 specifically hydrolyse mono- or poly-ubiquitinated substrates, the substrates can be

24 highly specific, meaning that deubquitinases can generally prevent degradation of

25 substrates or conduct specific regulatory functions. The deubiquitinases are a likely

15

1   component of the other vacuole resident pathogens proteolytic suite, and are

2   potentially likely to have unique roles in vacuole development in maintenance as the

3   vacuoles for some of these pathways are derived from manipulations of innate

4   immunity. This role would be unlike the extra-vacuole pathogens where

5   manipulations of the innate immune signalling pathways are more likely to be

6   targeted towards reduced surveillance and reduced secretion of innate immune

7   mediators.

8   **a. *Chlamydia* ChlaDub1**

9   One of the deubiquitinases, ChlaDub1 (CT868, MERNUM: MER011029), was shown

10   to suppress NF-κβ activation by binding to IκBα and inhibiting ubiquination, whilst

11   proteolytic activity is not associated with this suppression; the catalytic domain alone

12   was able to inhibit ubiquitination (Le Gegrate*, et al.*, 2008).

13   **b. *Salmonella* SseL**

14   NF-kb components appears to be a key substrate of the pathogenic ubiquitinases, as

15   the *Salmonella* deubiquitinase (SseL) also de-ubiquitinated IκBα to prevent NF-κβ

16   activation.

17   **c. *Yersinia* YopJ**

18   The *Yersinia* deubiquitinase, YopJ, was demonstrated to have many substrates IκBα

19   was also a key substrate (Zhou*, et al.*, 2005).

20

21

22   **D. Pathogenesis proteases with specialised virulence functions for the**

23   **vacuole resident bacterial pathogens**

24   The specialised niche of these bacteria within the host cell environment means that

25   they have evolved some highly specific and novel proteases, the best characterised

16

1   examples of these are described here. These proteases are often mechanistically

2   related to the conserved proteases but have evolved distinct substrates or roles for the

3   intracellular niche. These exciting findings support that many more novel proteases

4   will be uncovered from this niche.

5

6   **1. *Chlamydia* protease-like activity factor**

7   The *Chlamydia* protease-like activity factor (CPAF, MERNUM: MER028784), is a

8   serine protease which is completely novel to *Chlamydia* and shows some homology to

9   the C-terminal sequence of *Chlamydia* Tsp. CPAF was detected by the proteolytic

10 activity for degradation of transcription factors (RFX5 and USF-1) which upregulate

11 major histocompatibility complex molecules and plays a major role in the cytotoxicity

12 of this pathogen (Zhong*, et al.*, 1999, Zhong*, et al.*, 2000). CPAF is also indirectly

13 responsible for the cleavage of host cell BH3, which prevents the upregulation of

14 apoptosis (Pirbhai*, et al.*, 2006). All members of the chlamydiae, including a distantly

15 related organism amoebal endosymboint *protochlaydia amoebephila* have a CPAF,

16 suggesting that CPAF may have originally evolved for a non-immune target, such as

17 the cytoskeleton proteins which it has been shown to cleave (including keratin 8, and

18 vimentin) (Dong*, et al.*, 2004, Paschen*, et al.*, 2008). The protein is mechanistically

19 related to the tricorn protease (serine catalytic residue, clan SK, family S41) although

20 it is likely that this protease has evolved from gene duplication of Tsp as its protease

21 domain is most closely related to the *Chlamydia* Tsp protease domain. CPAFs

22 mechanism is somewhat reminiscent of the caspases as it is synthesised as a precursor

23 and in a concentration dependent mechanism trans-autocatalysis removes an

24 inhibitory loop which allows activation of the homodimer protease (Huang*, et al.*,

25 2008).

17

1

2. Exported metalloproteases – *Legionella* MspA/ProA

Exported metalloproteases have long been the focus of research efforts for *Legionella* and has been demonstrated that the major secreted zinc metalloprotease (MspA/ProA, MERNUM: MER001039) of *Legionella* has a host of virulence roles, including cleavage of various immune molecules (including IL2), and processing of bacterial factors, however, it is not essential for virulence (Moffat*, et al.*, 1994, Rossier*, et al.*, 2008). The protease has been demonstrated to be an active protease of the Clan MA, Family M4 class, and may yet have critical virulence functions, as there is numerous metalloproteases of the same class and other metalloproteases encoded on the genome of *Legionella* which would allow for redundancy. A diverse range of metalloproteases are present on the genomes of all the intracellular bacterial vacuole pathogens, and are likely to conduct a variety of processes, including as exported proteases with specific host cell targets.

3. Plasminogen activator protease - *Legionella*

A Plasminogen activator protease, Pla, homologous to that from *Yersinia pestis* has been described from *Legionella pneumophila* (Vranckx*, et al.*, 2007). The surface located protease was demonstrated to be important for survival within amoebae and likely has a distinct function from the *Yersinia* homolog (which is thought to facilitate invasion through endothelia) given the different pathogenic lifestyles of these two organisms.

4. Subtilisin-like serine proteases – *Mycobacterium*

a. Mycosin

18

1   The presence of five putative exported subtilisin-like serine proteases (mycosins)

2   (clan S8, family S8 MEROPS classification) on the genome of *Mycobacterium*

3   *tuberculosis* has been reported (Dave, *et al.*, 2002). The role of these proteases

4   remains unknown although one (mycosin-1; MERNUM: MER003060) was

5   demonstrated to be cell wall and culture media associated. These mycosins may have

6   a function in the modifications of the cell wall of *Mycobacteria* during the different

7   stages of cellular invasion or even between acute and persistent phases, another

8   function which it is tempting to speculate could be for the targeted release of cell wall

9   compounds to stimulate the immune response and facilitate development of the

10   granuloma.

11

12   **b. Cell wall protease Rv2224c**

13   *Mycobacterium* also produces a cell wall protein (serine protease Rv2224c; annotated

14   as a dipeptidylpeptidase), which has been biochemically demonstrated to have

15   esterase activity, however, a direct role of this protein in secretion of two forms of

16   GroEL has been demonstrated which supports that it is a protease. Rc2224c mutants

17   were impaired for virulence, intracellular survival, resistance to immune compounds,

18   and showed reduced resistance to cell wall stressors (Rengarajan, *et al.*, 2008).

19

20   **c. Transmembrane serine protease Rv3671c**

21   A putative transmembrane serine protease (Rv3671c, MEROPS PA Clan S1) from

22   *Mycobacterium tuberculosis* was described to have a critical role in the acid resistance

23   required for the organism to survive the acidification of the phagosome in activated

24   macrophages. Rv3671c mutants were impaired for virulence and persistence in an *in*

25   *vivo* mouse model (Vandal, *et al.*, 2008). However, the biochemical mechanism of

19

1   this protease and it's substrates remain to be characterised although it seems most

2   likely to have a role in cell wall integrity.

3

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III. Conclusions

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7   The intracellular vacuole resident bacterial pathogens share many crucial virulence

8   components, including proteases, with their extracellular and cytoplasmic bacterial

9   relatives. The distinct niche environments of each of these pathogens will clearly have

10 differing requirements for proteases. Proteases which function for stress protection,

11 nutrient acquisition, regulation, cell division, and growth are all likely to be important

12 for these pathogens similarly to other pathogens. Highly specific manipulations of

13 host cell pathways to develop and maintain the vacuole niche environment and

14 supportive host cell conditions also likely involves specialised bacterial proteases

15 which remain to be characterised. However, the finding of novel adaptations of both

16 conserved and pathogenic specific proteases described here support that there are

17 likely further novel proteases from these bacteria. Future experimental analysis of

18 both the conserved and unique proteases found within each of these bacterial

19 pathogens will provide insights into bacterial pathogenesis and likely uncover novel

20 mechanisms for host-pathogen interaction to pathogenic advantage.

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IV. Acknowledgements

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1 **V. References**

2

3 Abelrahman YM & Belland RJ (2005) The chlamydial developmental cycle. *FEMS*

4 *Microbiol Rev* **29**: 949-959.

5 Bandara AB, Sriranganathan N, Schurig GG & Boyle SM (2005) Carboxyl-terminal

6 protease regulated *Brucella suis* mophology in culture and persistence in

7 macrophages and mice. *J Bacteriol* **187**: 5756-5775.

8 Barquero-Calvo E, Chaves-Olarte E, Weiss DS, *et al.* (2007) *Brucella abortus* uses a

9 stealthy strategy to avoid activation of the innate immune system during the

10 onset of infection. *PLOS One* **2**: e631.

11 Bhavsar AP, Guttman JA & Finlay BB (2007) Manipulation of host-cell pathways by

12 bacterial pathogens. *Nature* **449**: 827-834.

13 Boddicker JD & Jones BD (2004) Lon protease activity causes down-regulation of

14 *Salmonella* pathogenicity island 1 invasion gene expression after infection of

15 epithelial cells. *Infect Immun* **72**: 2002-2013.

16 Brawn LC, Hayward RD & Koronakis V (2007) *Salmonella* SPI1 effectro SipA

17 persists after entyr and cooperates with a SPI2 effector to regulate phagosome

18 maturation and intracellular replication. *Cell Host & Microbe* **1**: 63-75.

19 Clausen T, Southan C & Ehrmann M (2002) The HtrA family of proteases:

20 Implications for protein composition and cell fate. *Mol Cell* **10**: 443-455.

21 Darwin KH (2009) Prokaryotic ubiquitin-like protein (Pup), proteasomes and

22 pathogenesis. *Nature Rev Micro* **7**: 485-491.

23 Dave JA, Gey van Pittius NC, Beyers AD, Ehlers MRW & Brown GD (2002)

24 Mycosin-1 a subtilisin-like serine protease of *Mycobacterium tuberculosis*, is

1   cell wall associated and expressed during infection of macrophages. *BMC*

2   *Microbiol* **2**.

3   Doherty TM & Andersen P (2005) Vaccines for Tuberculosis: Novel Concepts and

4   Recent Progress. *Clin Microbiol Rev* **18**: 687-702.

5   Dong F, Su H, Huang Y, Zhong Y & Zhong G (2004) Cleavage of host keratin 8 by a

6   *Chlamydia* secreted protease. *Infect Immun* **72**: 3863-3868.

7   Ekaza E, Teyssier J, Ouahrani-Bettache S, Liautard JP & Kohler S (2001)

8   Characterisation of the *Brucella suis clpB* and *clpA* mutants and participation of

9   the genes in stress responses. *J Bacteriol* **183**: 2677-2681.

10   Elzer PH, Phillips RW, Robertson GT & Roop RM (1996) The HtrA stress response

11   protease contributes to resistance of *Brucella abortus* to killing by murine

12   phagocytes. *Infect Immun* **64**: 4838-4841.

13   Faherty CS & Maurelli AT (2008) Staying alive: bacterial inhibition of apoptosis

14   during infection. *TRENDS Microbiol* **16**: 173-180.

15   Farn J & Roberts M (2004) Effect of inactivation of the HtrA-like serine protease

16   DegQ on the virulence of *Salmonella enterica* serovar typhimurium in mice.

17   *Infect Immun* **72**: 7357-7359.

18   Hautefort I, Thompson A, Eriksson-Ygberg S, *et al.* (2008) During infection of

19   epithelial cells *Salmonella enterica* serovar Typhimurium undergoes a time-

20   dependent transcriptional adaptation that results in simultaneous expression of

21   three type 3 secretion systems. *Cell Microbiol* **10**: 958-984.

22   Huang Z, Feng Y, Chen D, *et al.* (2008) Structural basis for activation and inhibition

23   of the secreted *Chlamydia* protease CPAF. *Cell Host and Microbe* **4**: 529-542.

24   Huston WM, Theodoropoulos C, Mathews SA & Timms P (2008) *Chlamydia*

25   *trachomatis* responds to heat shock, penicillin induced persistence, and IFN-γ

23

1 induced persistence by alterhing levels of extracytoplasmic stress resopnse

2 protease HtrA. *BMC Microbiol* **8**: 190.

3 Huston WM, Swedberg JE, Harris JM, Walsh TP, Mathews SA & Timms P (2007)

4 The temperature activated HtrA protease from pathogen *Chlamydia trachomatis*

5 acts as both a chaperone and protease at 37°C. *FEBS Lett* **581**: 3382-3386.

6 Hybiske K & Stephens RS (2008) Exit strategies of intracellular pathogens. *Nature*

7 *Rev Microbiol* **6**: 99-110.

8 Kage H, Takaya A, Ohya M & Yamamoto T (2008) Coordinated Regulation of

9 Expression of *Salmonella* Pathogenicity Island 1 and Flagellar Type III

10 Secretion Systems by ATP-Dependent ClpXP Protease. *J Bacteriol* **190**: 2470-

11 2478.

12 Kim S, Watarai M, Kondo Y, Erdenebaatar J, Makino S & Shirahata T (2003)

13 Isolation and characterisation of mini-Tn5Km2 insertion mutants of *Brucella*

14 *abortus* deficient in internalization and intracellular growth in HeLa cells. *Infect*

15 *Immun* **71**: 3020-3027.

16 Knodler LA, Celli J & Finlay BB (2001) Pathogenic trickery: deception of host cell

17 processes. *Nature Review Molecular and Cellular Biology* **2**: 578-588.

18 Ko J & Splitter GA (2003) Molecular Host-Pathogen interaction in Brucellosis:

19 Current understanding and future approaches to vaccine development for mice

20 and humans. *Clin Microbiol Rev* **16**: 65-78.

21 Krojer T, Garrido-Franco M, Huber R, Ehrmann M & Clausen T (2002) Crystal

22 structure of DegP (HtrA) reveals a new protease-chaperone machine. *Nature*

23 **416**: 455-459.

24

1 Krojer T, Sawa J, Schäfer E, Saibil HR, Ehrmann M & Clausen T (2008) Structural

2   basis for the regulated protease and chaperone function of DegP. *Nature* **453**:

3   885-890.

4 Kumagai Y, Yagishita H, Yajima A, Okamoto T & Konishi K (2005) Molecular

5   Mechanism for connective tissue destruction by dipeptidyl aminopeptidase IV

6   produced by the periodontal pathogen *Porphyromonas gingivalis*. *Infect Immun*

7   **73**: 2655-2664.

8 Kurtz S, McKinnon KP, Runge MS, Ting JPY & Braunstein M (2006) The SecA2

9   scretion ractor of *Mycobacterium tuberculosis* promotes growth in macrophages

10   and inhibits the host immune response. *Infect Immun* **74**: 6855-6864.

11 Lad SP, Li JL, Correia JD, Pan QL, Gadwal S, Ulevitch RJ & Li EG (2007) Cleavage

12   of p65/Re1A of the NF-kappa B pathway by *Chlamydia*. *PNAS* **104**: 2933-2938.

13 Lad SP, Yang G, Scott DA*, et al.* (2007) Chlamydial CT441 is a PDZ domain-

14   containing tail-specific protease that interferes with the NF-kappa B pathway of

15   immune response. *J Bacteriol* **189**: 6619-6625.

16 Le Gegrate G, Krieg A, Faustin B, Loeffler M, Godzik A, Krajewski S & Reed JC

17   (2008) *Chla*Dub1 of *Chlamydia trachomatis* suppressses NF-κβ activation and

18   inhibits IκBα ubiquitination and degradation. *Cell Microbiol* **10**: 1879-1892.

19 Lewis C, Skovierova H, Rowley G*, et al.* (2009) *Salmonella enterica* serovar

20   typhimurium HtrA: regulation of expression and role of the chaperone and

21   protease activities during infection *Microbiol* **155**: 873-881.

22 Lowe DC, Savidge TC, Pickard D, Eckmann L, Kagnoff MF, Dougan G & Chatfield

23   SN (1999) Characterization of candidate live oral *Salmonella typhi* vaccine

24   strains harboring defined mutations in *aroA, aroC, and htrA*. *Infect Immun* **67**:

25   700-707.

25

1 Makinoshima H & Glickman MS (2007) Regulation of *Mycobacterium tuberculosis*
2 cell envelope composition and virulence by regulated intramembrane
3 proteolysis. *Nature* **436**: 406-409.

4 Marr AK, Ovehage J, Bains M & Hancock RE (2007) The Lon protease of
5 *Pseudomonas aeruginosa* is indcued by aminoglycosides and is involved in
6 biofilm formation and motility. *Microbiol* **153**: 474-482.

7 Misaghi S, Balsara ZR, Catic A, Spooner E, Ploegh HL & Starnbach MN (2006)
8 *Chlamydia trachomatis*-derived deubiquitinating enzymes in mammalian cells
9 during infection. *Mol Microbiol* **61**: 142-150.

10 Mo E, Peters SE, Willers C, Maskell DJ & Charles IG (2006) Single, double and
11 triple mutants of *Salmonella enterica* serovar Typhimurium *degP* (htrA), *degQ*
12 (hhoA) and *degS* (hhoB) have diverse phenotypes on exposure to elevated
13 temperature and their growth *in vivo* is attenuated to different extents. *Microbial*
14 *Pathogenesis* **41**: 174-182.

15 Moffat JF, Edelstein PH, Regula DPJ, Cirillo JD & Tompkins LS (1994) Effects of an
16 isogenic Zn-metalloprotease-deficient mutant of *Legionella pneumophila* in a
17 guinea-pig pneumonia model. *Mol Microbiol* **12**: 693-705.

18 Mohamedmohaideen NN, Palaninathan SK, Morin MP, *et al.* (2008) Structure and
19 function of the virulence-associated high temperature requirement A of
20 *Mycobacterium tuberculosis*. *Biochemistry* **47**: 6092-6102.

21 Ostberg Y, Carroll JA, Pinne M, Krum JG & Mathews S (2004) Pleiotropic effects of
22 inactivating a chaperone and protease at 37oC. *FEBS Lett* **186**: 3382-3386.

23 Paschen SA, Christian JG, Veier J, Schmidt F, Walch A, Ojcius DM & Hacker G
24 (2008) Cytopathicity of *Chlamydia* is largely reproduced by expression of a
25 single chlamydial protease. *JCB* **182**: 117-127.

26

1   Pearce MJ, Mintseris J, Ferreyra J, Gygi SP & Darwin KH (2008) Ubiquitin-like

2   protein invovled in the proteasome pathway of *Mycobacterium tuberculosis*.

3   *Science* **322**: 1104-1107.

4   Pedersen LL, Radulic M, Doric M & Abu Kwaik Y (2001) HtrA homologue of

5   *Legionella pneumophila*: an indispensable element for intracellular infection of

6   mammalian but not protozoan cells. *Infect Immun* **69**: 2569-2579.

7   Phillips RW & Roop RM (2001) *Brucella abortus* HtrA functions as an authentic

8   stress response protease but is not required for wild-type virulence in BALB/c

9   mice. *Infect Immun* **69**: 5911-5913.

10 Pirbhai M, Dong F, Zhong Y, Pan KZ & Zhong G (2006) The Secreted Protease

11 Factor CPAF Is Responsible for Degrading Pro-apoptotic BH3-only Proteins in

12 *Chlamydia trachomatis*-infected Cells. *J Biol Chem* **281**: 31495-31501.

13 Qin Q, Pei J, Ancona A, Shaw BD, Ficht TA & de Figueiredo P (2008) RNAi screen

14 of endoplasmic reticulum-associated host factros reveals a role for IRE1α in

15 supporting *Brucella* replication. *PLOS Pathog* **4**: 1000110.

16 Ramsden AE, Mota LJ, Munter S, Shorte SL & Holden DW (2007) The SPI-2 type III

17 secretion system restricts motility of *Salmonella*-containing vacuoles. *Cell

18 Microbiol* **9**: 2517-2529.

19 Rengarajan J, Murphy E, Park A, *et al.* (2008) *Mycobacterium tuberculosis* Rv224c

20 modulates innate immune response. *PNAS* **105**: 264-269.

21 Robertson GT, Kovach ME, Allen CA, Ficht TA & Roop RM (2000) The *Brucella

22 abortus* Lon functions as a generalized stress response protease and is required

23 for wild-type virulence in BALB/c mice. *Mol Microbiol* **35**: 577-588.

27

1   Roop RM, Phillips RW, Hagius S, *et al.* (2001) Re-examination of the role of the

2   *Brucella melitensis* HtrA stress response protease in virulence in pregnant goats.

3   *Vet Microbiol* **82**: 91-95.

4   Rossier O, Dao J & Cianciotto NP (2008) The type II secretion system of *Legionella*

5   *pneumophila* elaborates two aminopeptidases, as well as a metalloprotease that

6   contributes to differential infection among protozoan hosts. *Appl Environ*

7   *Microbiol* **74**: 753-761.

8   Roy CR & Tilney LG (2002) The road less travelled: transport of *Legionella* to the

9   endoplasmic reticulum. *JCB* **158**: 415-419.

10   Sauer JD, Shannon JG, Howe D, Hayes SF, Swanson MS & Heinzen RA (2005)

11   Specificity of *Legionella pneumophila* and *Coxiella burnetii* vacuoles and

12   versatility of *Legionella pneumophila* revealed by coinfection. *Infect Immun* **73**:

13   4494-4504.

14   Seshadri R, Paulsen IT, Eisen JA, *et al.* (2003) Complete genome sequence of the Q-

15   fever pathogen *Coxiella burnetii*. *PNAS* **100**: 5455-5460.

16   Skorko-Glonek J, Laskowska E, Sobiecka-Szkatula A & Lipinska B (2007)

17   Characterization of the chaperone-like activity of HtrA (DegP) protein from

18   *Escherichia coli* under the conditions of heat shock. *Arch Biochem Biophys* **464**:

19   80-89.

20   Skorko-Glonek J, Wawrzynow A, Krzewski K, Kurpierz K & Lipinska B (1995) Site-

21   directed mutagenesis of the HtrA (DegP) serine-protease, whose proteolytic

22   activity is indispensable for *Escherichia coli* survival at elevated-temperatures.

23   *Gene* **163**: 47-52.

1 Striebel F, Imkamp F, Sutter M, Steiner M, Mamedov A & Weber-Ban E (2009)

2 Bacterial ubiquitin-like modifier pup is deamidated and conjugated to substrates

3 by distinct but homologous enzymes. *Nature Struct Mol Biol* **16**: 647-651

4 Takaya A, Kubota K, Isogai E & Yamamoto T (2005) Degradation of the HilC and

5 HilD regulator proteins by ATP-dependent Lon protease leads to

6 downregulation of *Salmonella* pathogenicity island 1 gene expression. *Mol*

7 *Microbiol* **55**: 839-852.

8 Torrado E, Frago AG, Castro A, *et al.* (2007) Evidence for an Intramacrophage

9 Growth Phase of *Mycobacterium ulcerans*. *Infect Immun* **75**: 977-987.

10 Vandal OH, Pierini LM, Schnappinger D, Nathan CF & Ehrt S (2008) A membrane

11 protein preserves intrabacterial pH in intraphagosomal *Mycobacterium*

12 *tuberculosis*. *Nature Med* **14**: 849-854.

13 Vranckx L, De Buck E, Anne J & Lammertyn E (2007) *Legionella pneumophila*

14 exhibits plasminogen activator activity. *Microbiol* **153**: 3757-3765.

15 Wang. T, Li H, Gang L, *et al.* (2009) Structural Insights on the *Mycobacterium*

16 *tuberculosis* Proteasomal ATPase Mpa. *Structure* **17**: 1377-1358.

17 White MJ, He H, Penoske RM, Twining SS & Zahrt TC (2010) PepD participates in

18 the Mycobacterial stress response mediated through MprAB and SigE. *J*

19 *Bacteriol* **EPUB**: doi:10.1128.

20 Zhong G, Fan T & Li L (1999) *Chlamydia* inhibits interferon gamma inducible major

21 histocompatibility complex class II expression by degradation of upstream

22 stimulatory factor. *Int J Exp Med* **189**: 1931-1937.

23 Zhong G, Fan T & Ji H (2000) Degradation of transcription factor RFX5 during the

24 inhibition of both constitutive and inteerferon gamma inducible major

29

1   histocompatibility complex class I expression in *Chlamydia* infected cells. *Exp*

2   *Med* **191**: 1525-0534.

3   Zhou H, Monack DM, Kayagaki N, Wertz I, Yin J & Dixit VM (2005) *Yersinia*

4   virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappa B activation.

5   *J Exp Med* **202**: 1327-1332.

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Fig 1. A schematic figure showing the highly specialised niche vacuoles of the intracellular vacuole resident bacteria. The vacuole properties are indicated in the boxes. The *Salmonella* vacuole is effectively a late endocytic-lysosomal vacuole which is typified by long branching structures termed *Salmonella* induced filaments (sif). These filaments align with cytoskeletal components and the vacuole is typically surrounded by exocytic golgi vesicles. The *Brucella* vacuole is an autophagosome located at endoplasmic reticulum export sites, with a number of autophagosomal and ER markers on the vacuole. The *Chlamydia* vacuole has no classic endocytic or lysosomal markers, instead it has host cell derived sphingomyelin thought be obtained by capturing exocytic vesicle trafficking. The *Mycobacterium* vacuole is a stalled early endosomal vacuole which, unlike the other bacteria containing vacuoles, does not traffic into a nuclear or ER localisation but stays located in the periphery of the cell. The *Legionella* and *Coxiella* vacuoles both locate to the ER and establish as ER compartments, however whilst the *Legionella* vacuole is a stalled late endocytic vacuole the *Coxiella* vacuole fuses with lysosomes and is a lysosomal vacuole.

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Table 1: Conserved bacterial proteases which have a pathogenic function for intracellular bacterial pathogens which reside in a vacuole.

Protease	Organism	Virulence function
Lon	*Salmonella*	regulation virulence factors, intracellular virulence
Proteasome	*Mycobacteria*	Regulation, protein degradation
ClpXP	*Salmonella*	regulation virulence factors, intracellular virulence
HtrA	*Salmonella*	animal model virulence and intracellular survival
	Legionella	
	Brucella	
	Mycobacterium	
	Chlamydia	
RseP	*Mycobacterium*	regulation of virulence factors
Tsp	*Chlamydia*	Host cell immune downregulation
	Brucella	intracellular survival, animal model virulence

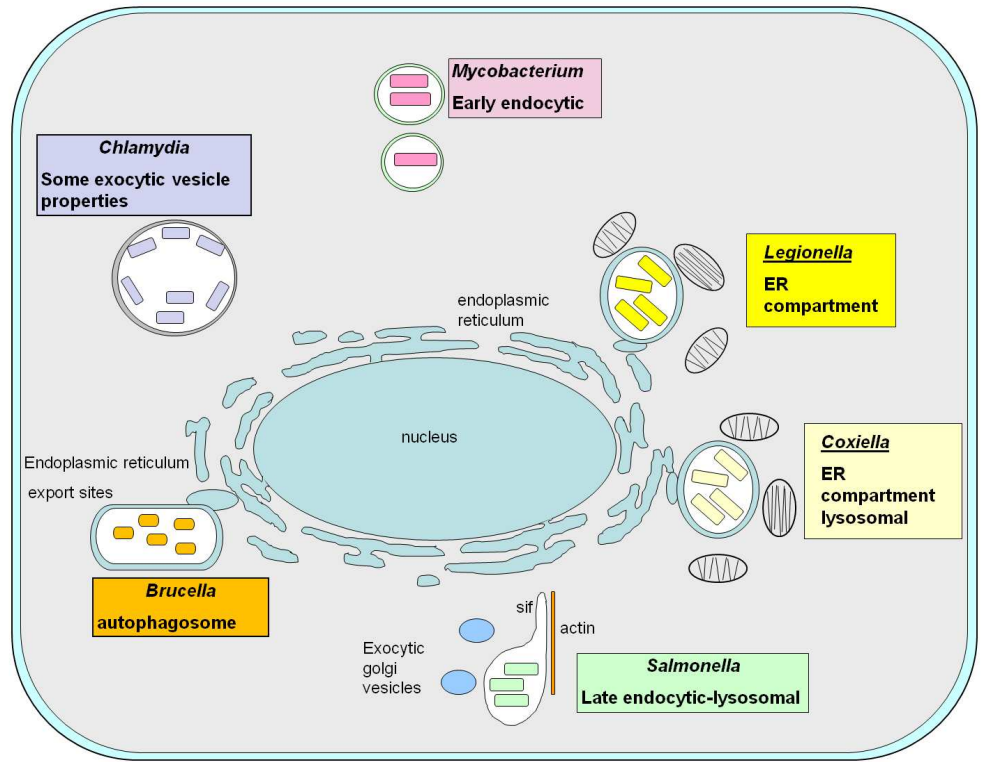**Table 2: Specialised Pathogenesis proteases with unique functions for the intracellular niche**

Protease	Organism	Substrate/Role
SseL	*Salmonella*	Ikκα to prevent NF-κβ activation
ChlaDub1	*Chlamydia*	Ikκα to prevent NF-κβ activation
CPAF	*Chlamydia*	Cytoskeleton for structural rearrangements, transcriptional regulators of immune activation
MspA/ProA	*Legionella*	IL-2, tissue destruction
Pla	*Legionella*	Plasminogen - activation
Mycosins	*Mycobacteria*	Unknown, bacterial cell wall maintenance? Host cell targets?
Rv2224c	*Mycobacteria*	GroEL cleavage and resistance to immune compounds
Rv3671c	*Mycobacteria*	Acid resistance

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