Analysis of the Maturation of *Rhodococcus equi*-containing Vacuoles in Macrophages

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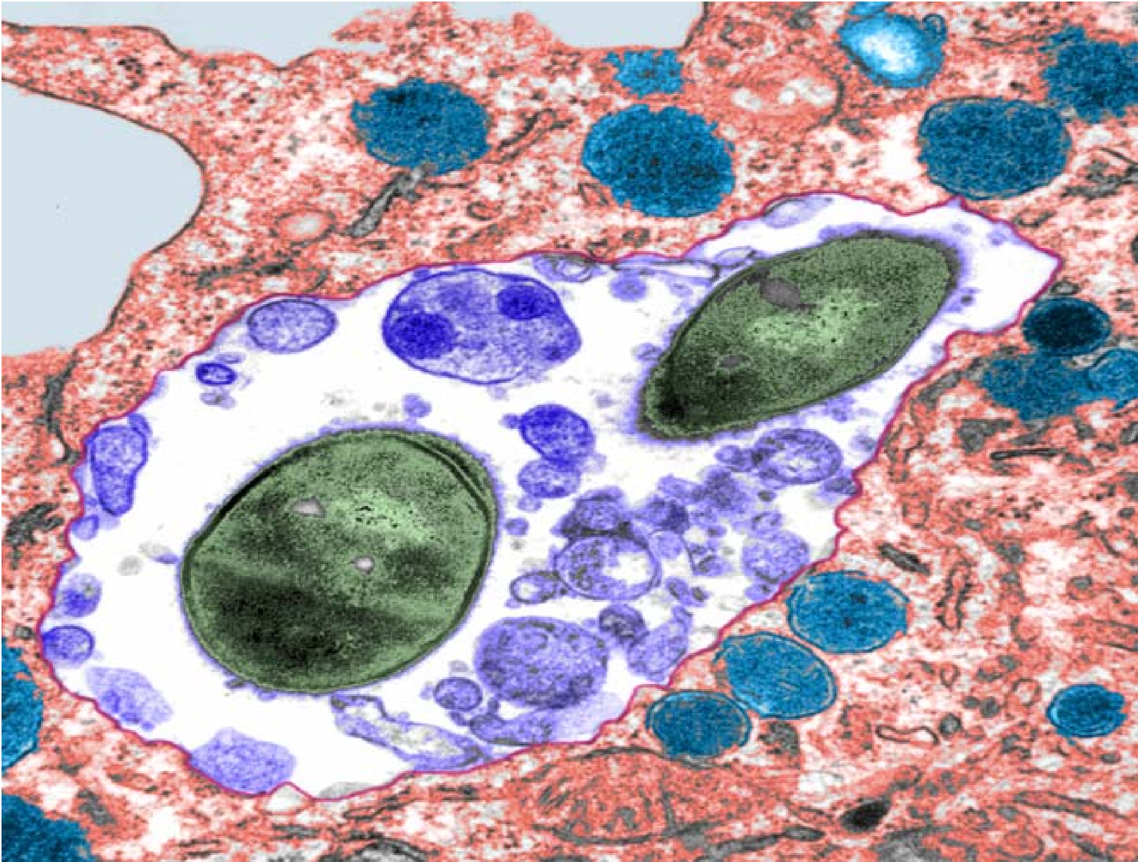

Vacuoles containing *Rhodococcus equi* in J774E macrophages as viewed by transmission electron microscopy

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Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und nur unter Zuhilfenahme der von mir angegebenen Hilfsmittel angefertigt habe.

Diese Dissertation hat weder in gleicher noch in ähnlicher Form bereits einem anderen Prüfungsverfahren vorgelegen.

Ich habe bislang noch keine akademischen Grade erworben oder versucht zu erwerben.

Würzburg, im März 2005

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Summary

Rhodococcus equi is a Gram-positive intracellular pathogen which can cause severe bronchopneumonia in foals. In recent years, the role of this bacterium as human pathogen has been noted, as *R.equi* infections in humans have increased in frequency. This increase is associated with the rise in immunosupressed individuals, specially AIDS patients, where infection leads to symptoms and pathology similar to those seen in foals with a high mortality rate. Due to its capability to survive and multiply in murine and equine macrophages, *R.equi* has been classified as a facultative intracellular bacterium. *R.equi* is found frequently in macrophages in alveolar infiltrate from infected animals. The pathogenicity of *R.equi* depends on its ability to exist and multiply inside macrophages and has been associated with the presence of virulence plasmids. It has been observed that, inside foal alveolar macrophages, *R.equi*-containing vacuoles (RCVs) do not mature into phagolysosomes. However, most of the intracellular events during *R.equi* infection have not been investigated in detail.
The aim of this study was to elucidate the intracellular compartmentation of *R.equi* and the mechanism by which the bacteria avoid destruction in host macrophages. The importance of the virulence-associated plasmids of *R.equi* for the establishment of RCVs was also evaluated. Furthermore, the intracellular fate of viable and non-viable *R.equi* was compared in order to study whether viability of *R.equi* influenciates the establishment of RCVs.

In this study, the RCV was characterized by using a variety of endocytic markers to follow the path of the bacteria through murine macrophages. Transmission electron microscopy-based analysis showed that *R.equi* was found equally frequently in phagosomes with loosely or tightly apposed membranes, and RCV often contains numerous membranous vesicles. Laser scanning microscopy of infected macrophages showed that the majority of phagosomes containing *R.equi* acquired transiently the early endosomal markers Rab5, PtIns3P, and EEA-1, suggesting initially undisturbed phagosome maturation. Although the RCV acquired some late endosomal markers, such as Rab7, LAMP-1, and LAMP-2, they did not acquire vATPase, did not interact with pre-labeled lysosomes, and failed to acidify. These data clearly suggest that the RCV is a compartment which has left the early endosomal stage but fails to acquire a typical late endocytic composition. *R.equi* could be localized in vacuoles that resemble multivesicular body compartments (MVB), which are transport intermediates between early and late endosomes and display internal vesicles very similar to the ones observed within RCVs.

Analysis of several *R.equi* strains containing either VapA- or VapB-expressing plasmids or neither demonstrated that the possession of the virulence-associated plasmids does not affect phagosome trafficking over a two hour period of infection. The finding that non-viable *R.equi* was still able to inhibit phagosome maturation (although not to the same extent as viable *R.equi* did) suggests that heat-insensitive factors, such as cell periphery lipids, may play a major role in inhibition of phagosome maturation, although heat-sensitive factors may also be involved.

Zusammenfassung

Rhodococcus equi ist ein Gram-positives, fakultativ intrazelulläres Bakterium, das unter anderem die Ursache von Bronchopneumonien bei Fohlen ist. Menschen und andere Säugetiere können ebenfalls von Infektionen mit *R. equi* betroffen sein. In den letzten Jahren ist die Häufigkeit klinischer Infektionen mit *R. equi* bei Menschen gestiegen. Die wachsende Anzahl an immunosupprimierten Patienten (hauptsächlich AIDS-Patienten) liegt dieser Zunahme an Infektionen zugrunde. Die Symptomatologie und Pathologie der Infektion mit *R. equi* ist bei AIDS-Patienten und Fohlen ähnlich. Die Sterblichkeitsrate ist in beiden Fällen hoch.
Die Fähigkeit der Rhodokokken, innerhalb von Makrophagen zu überleben und sich zu vermehren, ist mit dem Vorhandensein von Virulenzplasmiden (virulence-associated plasmids) verbunden.
Innerhalb des Makrophagen befinden sich die Rhodokokken in einem Phagosom, das nicht mit Lysosomen fusioniert. Die genaue Kompartimentierung der *Rhodococcus equi*-enthaltenden Phagosomen in Makrophagen war bisher unbekannt und wurde deshalb in der vorliegenden Promotionsarbeit untersucht. Mit Hilfe mehrerer endozytischer Marker wurde das *R. equi*-enthaltende Kompartiment charakterisiert. Mögliche Unterschiede zwischen der Kompartimentierung von *R. equi*(+)- und *R. equi*(-)-enthaltenden Phagosomen ist ebenfalls Thema dieser Promotionsarbeit. Weiterhin wurde die Etablierung des phagosomalen Kompartiments für jeweils lebende und tote Rhodokokken verglichen.
Transmissionselektronenmikroskopische Analysen haben gezeigt, dass die Phagosomenmembran *Rhodococcus equi*-enthaltender Phagosomen sowohl locker als auch eng anliegend sein kann (50%). Darüber hinaus wurden häufig zahlreiche, membranöse Vesikel in *R. equi*-enthaltenden Phagosomen gefunden. Diese Phagosomen zeigen somit Ähnlichkeiten zu *Multivesicular Bodies*. *Multivesicular Bodies* sind intermediäre Kompartimente zwischen frühen und späten Endosomen und zeigen ebenfalls eine Vielzahl von internen Vesikeln.
Untersuchungen am konfokalen Lasermikroskop ergaben, dass die Mehrheit der *R. equi*-enthaltenden Phagosomen die früh endosomalen Marker Rab5, PtIns3P und EEA-1 transient akquirieren. Dieser Befund deutet auf eine ungestörte phagosomale Reifung im frühen Stadium hin. Trotz der beobachteten Akquisition der spät endosomalen Marker Rab7, LAMP-1 und LAMP-2 konnte keine Akquisition der vATPase, keine Interaktion mit vormarkierten Lysosomen und keine Ansäuerung von *R.equi*-enthaltenden Phagosomen nachgewiesen werden.
Diese Ergebnisse weisen darauf hin, dass *R. equi*-enthaltende Phagosomen das früh endosomale Stadium abschließen, aber einen typisch spät endosomale Zustand nicht erreichen.
Die Analyse unterschiedlicher *R. equi*-Stämme, die entweder *vapA*- oder *vapB*-exprimierende Virulenzplasmide enthalten, hat gezeigt, dass die Anwesenheit von Virulenzplasmiden die phagosomale Reifung über eine Infektionsperiode von zwei Stunden nicht beeinflusst.
Getötete Rhodokokken waren in der Lage, die phagosomale Reifung zu inhibieren, aber in geringerem Ausmaß als lebende Rhodokokken. Das weist darauf hin, dass hitze-insensitive Faktoren (wie zum Beispiel Lipide der Zellwand) zur Inhibierung der phagosomalen Reifung entscheidend sind, obwohl dazu auch hitze-sensitive Faktoren (wie Proteine) relevant sein können.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ATCC	American Type Culture Collection
BHI	Brain-Heart Infusion Media
BMMs	Bone marrow macrophages
Cath D	Cathepsin D
CLSM	Confocal laser scanning microscope
d H_2O	distilled water
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EEA-1	Early endosome antigen 1
ER	Endoplasmic reticulum
FCS	Fetal calb serum
GA	Glutaraldehyde
HS	Horse serum
IL	Interleukin
KDa	Kilodalton
LAMP	Lysosome-Associated Membrane Protein
LB	Lennox broth
LBPA	Lyso-*bis*phosphatidic acid
LPS	Lypopolysaccharide
MHC	Major Histocompatibility Complex
MOI	Multiplicity of infection
M6PR	Mannose-6-phosphate receptor
NSF	N-Ethylmaleimidine-sensitive Fusion Protein
NHS	N-Hydroxysuccinimidyl
OD	Optical density
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde

PtdIns3P	Phosphatidylinositol 3-phosphate
RPM	Revolutions per minute
SNAP	Soluble NSF Attachment Protein
SNARE	Soluble NSF Attachment Protein Receptor
TACO	Tryptophane Aspartate Containing Coat Protein
TfR	Transferrin receptor
TGN	Trans-Golgi Network
TLR	Toll-like receptor
VAMP	Vesicle-associated Membrane Protein
Vap	Virulence-associated protein
VATPase	Vesicular proton ATPase

1- Introduction

Our bodies, and those of other animals, are subject to a continuous onslaught from the outside world by an immense variety of pathogenic microorganisms and other foreign invaders. Many of these can cause diseases if they enter the body. Normally, the skin and the surface of mucous membranes provide the first barrier to foreign agents from the outside world. If this barrier is breached, other mechanisms of the immune system are activated and their response generally leads to the destruction and removal of the foreign particles.

In order to simplify our understanding of immune mechanisms, the immune system has been divided into two major branches, innate and acquired immunity.

- Innate or non-specific immunity refers to the basic resistance to disease that an organism posseses. It does not require a prior contact phase with the potential pathogen, but instead relies on preformed cells and mediators. It comprises four types of defensive barriers: anatomic barriers, which are the first line of defence against infection (e.g. the skin and the surface of mucous membranes), physiologic barriers (e.g. temperature, pH, oxygen tension, and various soluble factors such as complement), endocytic-phagocytic barriers, and the barriers created by the inflammatory response, which are complex series of interactions involving a variety of chemical mediators.
- Acquired or specific immunity is the part of the immune system which is capable to specifically recognize and eliminate foreign molecules. Acquired immunity displays specificity, diversity, memory and self/nonself recognition [Kuby 1994].

Both branches of the Immune System work together to efficiently eliminate foreign particles, and the immune responses produced by potential pathogens have usually components of both branches. For example, cells of the phagocytic system (e.g. macrophages) are also involved in the activation of specific immune response. The specific immune branch will, in turn, produce some soluble factors that are able to activate or deactivate phagocytic cells.

1.1- Macrophages

An important innate defence mechanism is the ingestion of pathogens and particles through a process called phagocytosis. It provides a specialized mechanism for regulated ingestion and intracellular destruction of microbial pathogens as well as of apoptotic host cells and debris. In addition, phagocytosis initiates antigen processing and presentation, processes that are important for the development of cellular immune responses [Ramachandra *et al*, 1999; Ernst, 2000]. Most phagocytosis is accomplished by professional phagocytes which include blood monocytes, neutrophils, and macrophages.

During haematopoiesis in the bone marrow, granulocyte-monocyte progenitors differentiate into promonocytes. These cells then leave the bone marrow and enter the blood, where they further differentiate into mature monocytes. Monocytes circulate in the blood stream for approximately 8 hours, and then they migrate into tissues and differentiate into macrophages [Kuby 1994]. The differentiation of monocytes into macrophages changes the cells: macrophages are five- to ten-fold larger than monocytes, their phagocytic ability increases, they produce higher levels of lytic enzymes, and are able to secrete different soluble factors [Kuby 1994].

Macrophages are phagocytic cells capable of ingesting and digesting antigens such as whole microorganisms, injured or dead host cells, and cellular debris. In addition to the functions of killing potential pathogens and presenting antigens to other cells of the immune system, macrophages are also able to produce soluble factors, which have an important role in triggering an efficient immune response. For example, macrophages are able to produce and secrete IL-1 and IL-6 which promote differentiation of B cells into plasma cells, and also induce synthesis of acute phase proteins. Furthermore, other cytokines secreted by macrophages, such TNF-β, are able to induce the secretion of other cytokines [Kuby 1994]. Since macrophages play an important role in immunity, they are distributed throughout the body. Some macrophages are permanent residents in tissues (fixed macrophages) and they have different names to reflect their tissue location (e.g. Kupffer cells in liver or alveolar macrophages in the lungs). The other macrophages are called free macrophages because they wander through the body, searching for antigens.

1.2- Phagocytosis

In general, phagocytosis is a membrane-directed process driven by the host cell´s cortical cytoskeleton resulting in internalization of particles the size of bacteria and yeasts (0.5-5.0 μm in diameter) [Ernst 2000].

1.2.1- Receptors involved in phagocytosis

The ability of professional phagocytes to identify a pathogen is extremely important because it influences the fate of the invading pathogen, the following antigen presentation, and also the type of cytokines that will be secreted by the phagocytes. Thus, professional phagocytes express a repertoire of receptors that enable them to recognize pathogens and foreign molecules, and also to discriminate between the different invading particles. Phagocytes possess two major classes of receptors for sing potential pathogens: the first set of receptors recognize components of the pathogen, such as cell wall components in bacteria; the second components of the immune response, like complement components or antibodies which have bound to the surface of the pathogen in a process called opsonisation.

1.2.1.1- Non-opsonic receptors

Macrophages are able to recognize and bind characteristic conserved microbial molecular patterns (“pathogen associated molecular patterns” or PAMP), such as LPS, peptidoglycan, or bacterial CpG-rich DNA. Signalling triggered by receptors that recognize these microbial patterns, termed “pattern-recognition receptors” (PRR), increases the antimicrobial activities [Janeway 1992; Rosenberger & Finlay 2003].

Toll-like receptors (TLR´s) are present on mammalian cells, mostly cells involved in immune responses. They mediate the recognition of different microbial structures that are not present in eukaryotes, activating the transcription factor NF-$\kappa\beta$ (Nuclear Factor $\kappa\beta$) and stimulating the production of inflammatory cytokines [Rosenberger & Finlay, 2003]. The 10 different members of the TLR family recognize distinct microbial structures. For example, TLR4 binds to LPS in the cell surface, recognizing Gram-negative bacteria [Wilson *et al*. 2002], and TLR2 mediates the response to various surface molecules of Gram-positive bacteria [Underhill *et al*. 1999]. In addition, the members of TLR family can cooperate with each other to expand the repertoire of ligands that they recognize [Ozinsky 2000, Underhill & Ozinsky 2002].

Scavenger receptors are membrane glycoproteins that recognize bacterial cell surface molecules including LPS or lipoteichoic acid, thus identifying Gram-negative or Gram-positive bacteria [Krieger *et al*. 1993, Dunne *et al*. 1994]. It is not yet known whether these receptors can by themselves activate the cytoskeleton and, as a result, internalize bacteria or, alternatively, act to bind bacteria but phagocytosis is performed by other receptors.

Mannose receptors are 175 kDa transmembrane proteins which recognize complex carbohydrates, such as mannose, fructose, glucose and galactose on the surface of the pathogen [Ezekowitz *et al*. 1990]. These receptors are able to recognize not only bacterial pathogens, but also fungal pathogens such as *Pneumocystis carinii*. The recognition of the pathogen through mannose receptors leads to secretion of proinflammatory cytokines like TNF-α [Stein & Gordon 1991], IL-1β or IL-6 [Yamamoto *et al*. 1997].

1.2.1.2- Opsonic receptors

Phagocytes possess a variety of receptors that recognize the Fcγ region of immunoglobulin molecules forming immune complexes with the foreign particles. The three types of Fcγ receptors involved in phagocytosis (FcγRI, FcγRII, and FcγRIII) differ with respect of their patterns of expression on phagocytes and also with their affinity for the different IgG subclasses coated on the surface of the foreign particles [Ernst, 2000]. Binding of the IgG-coated particle with the Fc receptors activates the oxidative burst and also the arachidonic acid cascade [Klein 1991].

Each of the phagocytic cells expresses complement receptors (CR1, CR3, and CR4) that recognize and bind complement-opsonised particles. Complement receptors act to bind bacteria to the surface of the macrophage, but other signals are also needed in order to promote phagocytosis [Pommier *et al*. 1983].

A particular target, such as a pathogenic bacterium, displays numerous and diverse ligands on its surface and it is likely to be recognized by multiple receptors simultaneously. Thus, the different receptors expressed by the phagocytes could all be involved in the binding and phagocytosis of particular pathogens, working together to produce an efficient antimicrobial response.

1.2.2- Phagolysosome biogenesis

Binding of a foreign particle to the receptors on the surface of the phagocyte triggers a reorganization of the plasma membrane and the cortical cytoskeletal elements, conditions that lead to particle engulfment and formation of a new compartment, called phagosome [Alberts *et al.* 1994]. The environment within the phagosome gradually changes to generate degradative conditions: initially, the composition of the phagosomes is similar to that of the plasma membrane. But, soon after their formation, phagosomes undergo a serie of changes, modifying their polypeptides, phosphorylated proteins and phospholipid composition, and, as a result, acquiring sequentially characteristics of early endosomes, late endosomes, and finally of lysosomes [Pitt *et al*. 1992b; Desjardins *et al*. 1994a]. This process is called phagolysosome biogenesis [Desjardins, 1995]. During phagolysosome biogenesis, phagosomes move on microtubules [Desajardins *et al*. 1994b] or use the actin cytoskeleton [Al-Haddad *et al*. 2001], and intersect with the biosynthetic and endocytic pathways [Pitt *et al*. 1992a; Desjardins *et al*. 1997; Garin *et al*. 2001]. The interactions between phagosomes and the different organelles of the endocytic pathway are thought to occur through multiple transient fusion events, a process called “kiss and run” [Storrie & Desjardins 1996]. According to this process, when a phagosome makes contact with an endosome of the same maturing age, the membranes of both organelles recognize each other, and fuse in a local and transient manner, connecting their membranes through small parts of their surface [Desjardins, 1995]. Through this “local fusion”, soluble contents of the organelles are exchanged. Thereafter, the membranes of the organelles separate and another cycle of fusion can begin [Duclos *et al*. 2000]. Aging of phagocytic organelles is accompanied by changes in their ability to recognize or fuse with endocytic organelles, probably because they no longer possess the machinery required for these fusions [Desjardins, 1995; Desjardins *et al*. 1997]. Recent evidence indicates that, aside from the contribution of the plasma membrane and endocytic organelles to the phagosome composition, the endoplasmic reticulum (ER) also contributes to the phagosome membrane within macrophages, but not within neutrophils [Gagnon *et al*. 2002]. ER is directly recruited to the plasma membrane to form the phagosomes, and it is also recruited at various stages during phagolysosome biogenesis [Gagnon *et al*. 2002].

The first step of phagolysosome biogenesis involves the recycling of different plasma membrane molecules from the phagosomal membrane, such as transferrin receptor, Fcγ receptor, mannose

receptor or α-adaptin [Muller *et al*. 1980, Pitt *et al*. 1992b]. Rab4, Rab11, Rab5, members of a family of more than 60 small GTPase regulatory proteins, are thought to have roles in regulating this recycling step [Sonnichsen *et al*. 2000, Garin *et al*. 2001]. The newly formed phagosome acquires then characteristics of early sorting endosomes through interactions with these endocytic organelles, a process thought to be in some measure controlled by the small GTPase Rab5 [Desjardins *et al*. 1994b]. This GTPase is already found in the phagosomal membrane by the time of the phagosome formation [Desjardins *et al*. 1994b]. In the phagosome membrane, Rab5 regulates the binding and recruitment of a large number of effector proteins, which are called tethering and docking factors, and act cooperatively to control the fusion process [Pfeffer, 1999]. Aside from the role of Rab5 in promoting fusion of nascent phagosomes with early endosomes, this small GTPase is also implicated in the movement of the phagosomes on microtubules [Zerial & McBride, 2001]. For homotypic fusion, Rab5 interacts with different effector proteins, such as the tethering factors Rabaptin 5, Rabex-5 and EEA-1 [Christoforidis *et al*, 1999a]. Rabaptin-5 forms complexes with Rabex-5, which are able to activate Rab5 [Horiuchi *et al*. 1997]. In its activate state, Rab5 recruits to the membrane a phosphatidylinositol-3-OH-kinase, that specifically generates phosphatidylinositol 3-phosphate (PtdIns3P) [Schu *et al*. 1993, Christoforidis *et al*. 1999b]. The presence of PtdIns3P and activated Rab5 is essential for the localization of other two effector proteins: Rabenosyn-5 and EEA-1, which both bind to PtdIns3P through their FYVE finger domain and provide complementary regulatory functions [Christoforidis *et al*. 1999b, Nielsen *et al*. 2000]. This cascade of molecule-molecule interactions is required to bring the opposing membranes of the endocytic organelles into close proximity and to ensure that cis-SNARE partners, who are localized on both opposing membranes, are able to interact with each other, forming trans-SNARE complexes that are responsible for the membrane fusion [Pfeffer 1999, Woodman 2000]. After fusion, SNARE complexes are rapidly disassembled, and the SNAREs are recycled for another round of fusion [Sollner *et al*. 1993].

Aside from the interactions of the newly formed phagosome with early endosomes, early phagosomes also interact with vesicles coming from trans-Golgi Network (TGN). By this way early phagosomes acquire proteins such as cathepsin H [Claus *et al*. 1998] or vATPase [Bucci at al. 2000]. The vacuolar type H^+ ATPases (vATPases) are thought to be the main determinants of phagosomal acidification [Hackam *et al*. 1997]. Main characteristics of early phagosomes are their internal pH of 6.0-6.5 [Schaible *et al*. 1999], and the association of the following molecules with the phagosomal membrane:

Rab5 [Desjardins *et al*. 1994b], EEA-1 [Mu *et al*. 1995], NSF, α/β SNAP, transferrin [Alvarez-Dominguez *et al*. 1997], cathepsin H [Claus *et al*. 1998], annexin I [Harricane *et al*. 1996], and PtdIns3P [Vieira *et al*. 2001, Brumell & Grinstein 2003]. Although this first step of the phagolysosome biogenesis is thought to occur in the first 5 to 10 minutes after phagosome formation, its duration varies between different phagocytosed particles. Furthermore, the signals triggered by the different receptors that recognize the particle in the plasma membrane influence the duration of the different aging steps in the phagolysosome biogenesis.

Early phagosomes gradually loose Rab5, and acquire another small GTPase, Rab7, which is found in late endosomes but not in lysosomes [Desjardins *et al*. 1997]. Originally, Rab7 was thought to regulate transport from early to late endosomes [Feng *et al*. 1995], but recent work suggest instead a role in late endosome to lysosome traffic [Bucci *et al*. 2000]. Rab7 enables the phagosome to interact with late endosomes, and these interactions lead to changes in the phagosome. Late phagosomes are no longer able to interact with early endosomes, probably because they no longer possess the machinery required for fusions with these endosomes, thus giving directionality to the maturation process. Recent evidence indicates that Hrs (Hepatocyte growth factor-regulated tyrosine kinase substrate), an adaptor molecule involved in protein sorting, associates with phagosomes prior to their fusion with late endosomes and lysosomes [Vieira *et al*. 2004]. Hrs plays a central role in the formation of multivesicular bodies in the endocytic pathway [Raiborg & Stenmark 2002], and was shown to associate with phagosomes in a PtdIns3P-dependent manner [Vieira *et al*. 2004]. As suggested for endosomes [Raiborg & Stenmark 2002], interaction of Hrs with ubiquitylated substrates of phagosomes may drive the inward budding of vesicles by a process involving ESCRT (endosomal sorting complex required for transport) protein complexes. Accordingly, late phagosomes stain with antibodies to lyso-*bis*phosphatidic acid (LBPA), which typically accumulates on internal membranes of late endosomes [Kobayashi *et al*. 1998, Brumell & Grinstein 2004]. Late phagosomes also interact with vesicles coming from trans-Golgi Network (TGN). This way, late phagosomes acquire molecules like LAMP [Karlsson & Carlsson, 1998], mannose-6-phosphate receptor (M6PR), and cathepsins [Schaible *et al*. 1999] Main characteristics of late phagosomes are their internal pH of approximately 5.3 [Grabe & Oster 2001], and the association of the following molecules with the phagosome membrane: Rab7, M6PR, LAMP-1, and LAMP-2 [Rabinowitz *et al*. 1992], LBPA [Fratti *et al*. 2001],

Cathepsin D, Cathepsin L, and vATPase [Haas, 1998]. This maturation step is thought to occur 15-20 minutes after the formation of the phagosome.

The last maturation stadium of the phagolysosome biogenesis involves, as indicated by its etymology, the interaction of the late phagosomes with lysosomes. This interactions lead to formation of a phagolysosome. Lysosomes are membranous bags which possess an acidic pH of about 5, and contain about 40 different types of hydrolytic enzymes, including proteases, nucleases, glycosidases, lipases, phospholipases, phosphatases and sulfatases [Alberts *et al.* 1994]. Maturing phagosomes gradually loose some molecules, such as the small GTPase Rab7, and M6PR [Storrie & Desjardins 1996], making phagolysosomes unable to interact with early or late endosomes. Characteristics of phagolysosomes are their internal pH of 4.0-5.0 [Geisow *et al.*1981], and the association of the folllowing molecules to their membrane: LAMP-1, LAMP-2, vATPase [Desjardins, 1995], and hydrolytic enzymes [Griffiths 1996]. The acidification within the phagolysosome is a critical component of the microbicidal response: the acidic pH is directly toxic to many microorganisms and, also, enhances the efficiency of other bactericidal mechanisms. For example, lytic enzymes secreted into the phagolysosomal lumen function optimally at acidic pH [Haas & Goebel, 1992], and dismutation of O_2^- within the phagosome is maximal at pH of 4,8 [Fridovich 1978]. After destruction of potential pathogen proteins within phagolysosomes, the resulting peptides interact with the class II MHC molecules. This interaction is thought to occur within phagosomes [Lang *et al*. 1994], although it was also proposed that partially processed and fully degraded proteins can be transferred to late endosomal compartments, where they can bind to the class II MHC molecules [Tjelle *et al*. 1998]. MHC class II molecules are delivered to the maturing phagosomes through vesicles coming directly from TGN, or through phagosomal interactions with class II-positive endosomes, because these molecules can be detected in both early and late endosomes [Peters *et al*. 1991; Rabinowitz *et al*. 1992]. Peptide-loaded MHC class II molecules travel then to the cell surface, where they are presented to $CD4^+$ T cells, which are able to recognize MHC class II-peptide complexes, activating the acquired branch of the immune system [Kuby 1994]. Recent evidence shows that phagosomes are also able to process exogenous peptides for MHC class I presentation, and are then presented to $CD8^+$ T cells, a function linked to phagocytosis mediated by the endoplasmic reticulum [Houde *et al*. 2003]. Probably by this way, antigens from some intracellular pathogens, such as mycobacteria or *Brucella abortus,* are able to elicit an MHC class I-dependent $CD8^+$ T cell response. Phagocytic cells

also present other types of degrading products. For example, lipid antigens like mycolic acid and lipopeptides from mycobacteria are recognized by CD1 molecules and, after transported to the plasma membrane, presented to $CD8^+$ cells [Watts 2004].

A schematic model of typical phagosome maturation is showed in Figure 1.

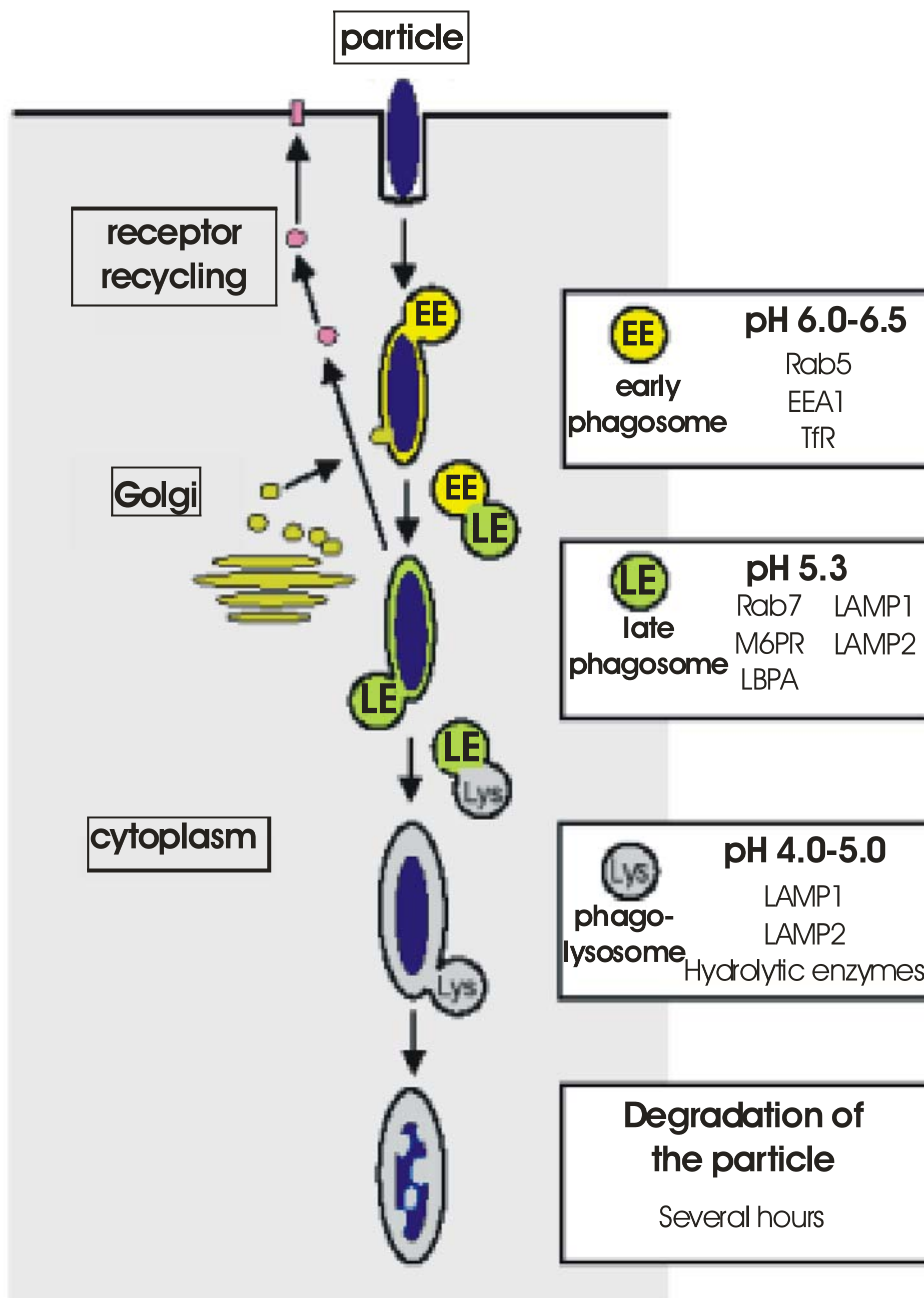

Figure 1: Model of normal phagosome maturation.
Internalized particles, such as non-pathogenic bacteria are enclosed in a membrane-bound compartment, the phagosome, which matures by sequential interaction with the endocytic compartments. This schema shows representative main characteristics of the different phagosome maturation stages.
EE-early endosome; LE-late endosome; Lys-lysosome.

1.3- Survival strategies of pathogens

A major mechanism for the destruction of potential pathogens that have invaded our bodies is killing by phagocytes. Interactions between pathogens and phagocytes are crucial for the development of an infection and survival of the microorganisms. Pathogens have recognized that interaction with phagocytes can be either an opportunity for or an obstacle to their own survival. Inside the body, obligate extracellular pathogens have developed diverse mechanisms to evade the immune responses triggered by phagocytes and therefore to assure their survival, such as avoiding their uptake by professional phagocytes. Another type of pathogens, the so-called intracellular pathogens, take advantage of the constant environmental conditions and supply of nutrients that are found inside host phagocytes, and have developed mechanisms to survive within these host cells. Intracellular pathogens can be grouped in four different classes based on the different strategies used to ensure survival inside phagocytes:

- Pathogens those are able to arrest the normal phagosome maturation,
- those which reside in a non-phagosomal organelle,
- those that escape from the phagosome, and
- those which resist the antimicrobial environment of the mature phagolysosome.

In the following sections, the mechanisms used by different pathogens to evade phagocytic function will be described. In section 1.3.1, mechanisms employed by some extracellular pathogens to avoid phagocytosis will be illustrated. In sections 1.3.2-1.3.4, different strategies used by intracellular pathogens will be described.

1.3.1- Evasion of phagocytosis

In order to evade killing by phagocytes, a variety of pathogens have evolved mechanisms to avoid interactions with these cells. Many bacteria express an outer coating of polysaccharide and/or protein called capsule that can protect the organism from attack by phagocytes. Thus, the type III capsular polysaccharide of *Streptococcus agalactiae* can prevent the deposition of C3b on the bacterial surface, and this way bacteria can evade their recognition and internalization through complement receptors on the surface of the phagocytes [Wilson *et al*. 2002]. Other bacteria, such as enteropathogenic *Escherichia coli* (EPEC), use type III secretion systems to translocate proteins with antiphagocytic properties that are able to inhibit phagocytosis [Goosney *et al*. 1999]. Type III secretion

systems have been described as “molecular syringes” and are thought to insert bacterial proteins directly into eukaryotic cells or translocate them into the extracellular environment [Hueck 1998].

Another mechanism evolved by pathogens to avoid interactions with phagocytic cells is to promote their internalization into non-professional phagocytes, such as epithelial cells and fibroblasts [Pieters 2001], and thereby protect themselves from the immune system. Using type III secretion systems, some gram-negative bacteria induce adhesion and internalization into non-phagocytic cells [Galan & Collmer 1999]. This way, *Salmonella enterica* serovar Typhimurium gain access inside ephitelial cells, thus enabling bacteria to trespass the immune barriers found within the intestinal tract and to establish an infection [Jones *et al*. 1994]. *Salmonella* organisms initiate ruffling of the host cell membrane at the site of the contact. This ruffling occurs as a result of rearrangement of the host cell cytoskeleton, initiated by action of different protein effectors translocated by the type III secretion systems of the bacteria [Galan & Zhou 2000].

1.3.2- Maturation arrest

In order to survive and replicate within phagocytes, intracellular pathogens must find ways to avoid the harsh environment of phagolysosomes. One mechanisms developed by some pathogens is to arrest the process of phagolysosome biogenesis. As a result, these pathogens reside in compartments which posses some characteristics of endocytic compartments, and offer the parasites an environment with the nutrients and conditions needed for their survival and replication. The maturation arrest can occur at both an early phagosome-like stadium, as in the case of *Mycobacterium tuberculosis* or *Ehrlichia chaffensis*, or at a late phagosome-like stage, exemplified by *Brucella abortus*, *Leishmania donovani,* or *Salmonella enterica* serovar Typhimurium.

1.3.2.1- *Mycobacteria*

Bacteria of the genus *Mycobacterium* can cause severe diseases, such as tuberculosis (cause by *M. tuberculosis*) or leprosy (caused by *M. leprae*). Members of this genus are characterized by their atypically lipid-rich cell wall, and their ability to catabolize fatty acids, conditions that seem to reflect preadaptations to a parasitic existence [Russell, 2001]. Several *Mycobacterium* species are able to inhibit phagosome maturation, surviving inside host macrophages. These species include: *M.tuberculosis* [Clemens & Horwitz 1995], *M. avium* [Chastellier *et al*. 1995, Oh & Straubinger 1996],

M. bovis [Lowrie *et al*. 1979], *M. marinum* [Barker *et al*. 1997], *M.leprae* [Sibley *et al*. 1987], and *M.microti* [Hart *et al*. 1987].

Pathogenic mycobacteria gain access to the macrophages through ligation of many phagocytic receptors, which include FcRs [Armstrong & Hart 1975], mannose receptors, fibronectin, scavenger receptors, and complement receptors [Ernst 1998, Scott *et al*. 2003]. However, complement receptors are believed to be the main route of entry of mycobacteria into macrophages [Ernst 1998, Scott *et al*. 2003]. There is no concrete evidence that pathogenic mycobacteria selectively use specific receptors to confer an intracellular survival advantage [Ernst 1998, Russell 2001], although the internalization of these bacteria via Fc receptors is thought to deliver the bacteria into lysosomes, thus impairing their survival [Armstrong & Hart 1975, Scott *et al*. 2003].

After phagocytosis, *Mycobacterium* is retained within a phagocytic vacuole until the host cell dies through apoptosis or necrosis [Russell, 2001]. Important characteristics of the *Mycobacterium*-containing vacuole are its incomplete lumenal acidification and the absence of mature lysosomal hydrolases [Sturgill-Koszycki *et al*. 1994, Russell 2001, Vergne *et al*. 2004a]. The pH of phagosomes containing pathogenic mycobacteria is 6.2-6.3 [Sturgill-Koszycki *et al*. 1994]. This limited acidification is due to the exclusion of the vATPase complex from mycobacterial phagosomes [Sturgill-Koszycki *et al*. 1994]. Despite the block in its phagolysosome biogenesis, the *Mycobacterium*-containing phagosome is a dynamic organelle, able to communicate with the plasma membrane: it contains transferrin receptor, major histocompatibility complex (MHC) class I and II molecules [Clemens & Horwitz 1995], glycosphingolipid constituents coming from the cell plasmalemn [Russell *et al*. 1996], and receives externally added transferrin [Clemens & Horwitz 1996] and cholera toxin B-subunit [Russell *et al*. 1996]. Studies have found that the small GTP-binding protein Rab5 (early endocytic) was rapidly acquired and retained by phagosomes containing pathogenic mycobacteria, while Rab7 (late endosomal) was not detected, confirming the early endocytic characteristics of mycobacterial phagosomes [Via *et al*. 1997]. Moreover, *Mycobacterium*-containing phagosomes retain unusually high concentrations of transferrin receptors and Rab11 [Fratti *et al*. 2000], features that suggest that these vacuoles may be part of the early endosomal recycling pathway [Schaible *et al*. 1999]. Despite the early endosomal characteristics displayed by mycobacterial phagosomes and their inhability to fuse with lysosomes, they acquire some “late endosomal/lysosomal proteins” like LAMP-1 and the

immature intermediate form of cathepsin D [Xu et al. 1994, Clemens & Horwitz 1995, Sturgill-Koszycki *et al*. 1996]. Several studies have showed that these "lysosomal markers" present on mycobacterial phagosomes are acquired from the biosynthetic pathway of the host cell (*trans*-Golgi) rather than from lysosomes [Sturgill-Koszycki *et al*. 1996; Ullrich *et al*. 1999]. This type of phagosome established by mycobacteria is advantageous for these bacteria because, besides of been disconnected from the degradation pathway, supplies mycobacteria with iron from transferrin [Haas 1998]. Thus, phagosomes containing pathogenic mycobacteria are selectively fusion competent vesicles that possess characteristics of early endocytic organelles, acquire "late endosomal/lysosomal" proteins from the biosynthetic pathway, and do not mature into phagolysosomes.

It is unclear how *Mycobacterium* is able to inhibit the phagolysosome biogenesis, but different hypotheses have been proposed:

- It has been suggested that ammonia produced by some species of *Mycobacterium* is sufficient to inhibit the acidification of mycobacteria-containing phagosome and also phagosome-lysosome fusion [Gordon *et al*. 1980]. Since the production and maintenance of pH gradients through vesicular membranes is important for membrane fusion events [Clague *et al*. 1994, Haas *et al*. 1994], alcalinization of mycobacteria-containing phagosomes due to the production of ammonia is a plausible explanation. However, experiments using urease-defective *Mycobacterium* have shown no significant difference in the intracellular survival compared with wild-type pathogenic bacteria [Reyrat *et al*. 1996].
- A factor identical to the phagocytosis protein coronin [Maniak *et al*. 1995], also termed TACO (tryptophan-aspartate containing coat protein), has been proposed to be retained on the mycobacterial phagosome, preventing phagosomal maturation [Ferrari *et al*. 1999]. TACO is an actin-binding protein which is recruited to the site of bacterial entry during the initial stages of infection [Schüller *et al*. 2001] and is normally released prior to phagosome-lysosome fusion [Grogan *et al*. 1997; Morrissette *et al*. 1999]. However, a recent study could not confirm the role of TACO in mycobacterial phagosome maturation in macrophages [Schüller *et al*. 2001].
- A characteristic product of mycobacteria, called cord factor (trehalose dimycolate), has been reported to inhibit Ca^{2+}-induced fusion between liposomes *in vitro* [Spargo et al. 1991] and it has been proposed that this membrane glycolipid is able to inhibit membrane fusions *in vivo* [Spargo et al. 1991, De Chastellier *et al*. 1995, Fujiwara 1997]. Recent evidence also suggests that the

cord factor is able to inhibit phagosome acidification [Indrigo *et al*. 2003]. However, the mechanism of the cord factor action is not known.

- It has been established that Ca^{2+} affects phagosomal maturation [Malik *et al*. 2000, 2001]. However, the exact mechanisms remain to be established. Infection of macrophages with *M. tuberculosis* prevents Ca^{2+} fluxes and inhibits activation of the Ca^{2+}-binding protein calmodulin and one of its partners, the Ca^{2+}/calmodulin protein kinase (CaMKII) [Malik *et al*. 2000, 2001]. Because Ca^{2+} and calmodulin influence the recruitment of the Rab5 effector phosphatidilinositol-3 kinase (hVPS34) in macrophages [Vergne *et al*. 2003], it was proposed that the mycobacterial interference with Ca^{2+}/calmodulin/CaMKII cascade blocks the recruitment of the phosphatidylinositol-3 kinase (hVPS34), which implicates a drop in the production of PtdIns3P on the mycobacterial phagosome, and therefore preventing the acquisition of EEA-1, hydrolases and vATPase [Vergne *et al*. 2004a]. Recently, Vergne *et al*. [2003], provided evidence that mannose capped lipoarabinomannan (ManLAM), a heavily glycosilated phosphatidylinositol produced by mycobacteria, inhibits a cytosolic Ca^{2+} rise and the recruitment of EEA-1 to phagosomes [Fratti *et al*. 2001]. These effects of ManLAM, while explaining, at least in part, mycobacterial inhibition of Ca^{2+} rise and its effect on phagosomal maturation, also open the question of how mycobacteria maintain interactions with early endosomes. Recent evidence indicates that another glycolipid produced by *M. tuberculosis*, phosphatidylinositol mannoside (PIM), specifically stimulates early endosomal fusion [Vergne *et al*. 2004b], suggesting that PIM action increases the delivery of recycling endosomal markers and contributes to the avoidance of phagosomal acidification [Vergne *et al*. 2004a, 2004b].

1.3.2.2- *Brucella abortus*

Bacteria of the genus *Brucella* are the causative agents of brucellosis, a worldwide zoonosis that affects a broad range of mammals, including humans. The facultative intracellular pathogen *Brucella abortus* has been found to survive and replicate in membrane-bound compartments within professional and non-professional phagocytes [Enright 1990, Moreno & Gorvel 2004]. The intracellular trafficking of *Brucella* differs greatly depending upon the type of cell infected. Within macrophages, phagosomes containing virulent *Brucella* acquire transiently characteristics of early endosomes: transferrin receptor, rab5 and EEA-1 are present in the phagosome membrane [Chaves-Olarte *et al*. 2002, Celli *et al*. 2003]. However, the association of *Brucella* with the early endocytic network is only

transient, since no labeling is detected with these early endosomal markers at 30 minutes postinfection [Pizarro-Cerdá *et al*. 1998, Chaves-Olarte *et al*. 2002]. *Brucella*-containing phagosomes are then transformed into acidic compartments [Porte *et al*. 1999] that acquire the late endosomal/lysosomal LAMP-1, but not the luminal lysosomal hydrolase Cathepsin D [Moreno & Gorvel 2004], and do not mature into phagolysosomes [Frenchick *et al*. 1985; Naroeni *et al*. 2001]. Finally, vacuoles containing *Brucella* organisms fuse with cisterns of the endoplasmic reticulum and bacteria establish their replication niche within this compartment [Moreno & Gorvel 2004]. In contrast with the infection within epithelial cells, within macrophages, *Brucella*-containing phagosomes never acquire characteristics of authophagosomes [Arenas *et al*. 2000]. Despite recent progress, the mechanisms of *Brucella* interaction with macrophages remain unclear. *Brucella melitensis, B.suis, and B. abortus* possess a type IV secretion system encoded by the components of the *virB* operon [Boschiroli *et al*. 2002]. Type IV secretion systems are secretion pathways ancestrally related to bacterial conjugation systems that deliver effector molecules to eukaryotic target cells. Although the nature of the effector molecule(s) translocated by this system is presently unknown, evidence suggests that the VirB type IV secretion apparatus is involved in maintaining the interactions between the *Brucella*-containing vacuoles and the endoplasmic reticulum [Celli *et al*. 2003].

1.3.3- Establishment of a non-endocytic compartment

Another general strategy used by some intracellular pathogens to avoid killing by the host cell is to inhabit vacuoles completely isolated from the endocytic pathway, displaying no markers of endosomal or lysosomal compartments [Hackstadt, 2000]. These vacuoles do not fuse with endocytic vesicles [Sinai & Joiner, 1997], but interact with host organelles other than those which are part of the endocytic pathway, such as mitochondria, endoplasmic reticulum or golgi [Hackstadt, 2000]. This strategy is used by *Chlamydia trachomatis, Toxoplasma gondii, Afipia felis,* and *Legionella pneumophila*.

1.3.3.1- *Chlamydia*

Chlamydiae are obligate intracellular bacteria which are causative agents of different human diseases, such as infectious blindness and sexually transmitted disease. All Chlamydiae display an unique life cycle which alternates between the metabolically inert, infectious extracellular form termed “elementary body” or “infectious body”, and the intracellular, metabolically active, noninfectious form

called "reticulate body" [Ward 1988]. Following internalization by macrophages, *Chlamydia* resides within compartments referred to as inclusions [Schramm *et al*. 1996]. The membrane of chlamydial inclusions is relatively deficient in host proteins and contains some bacterial proteins, called "inclusion membrane proteins (Inc) [Taraska *et al*. 1996]. Inclusions do not interact with endocytic organelles, as shown by the absence of transferrin, vATPase (phagosomal pH>6), M6PR, LAMP-1, LAMP-2, cathepsin D, and acid phosphatase [Friis 1972, Wyrick & Brownridge 1978, Heinzen *et al*. 1996]. Instead, chlamydial inclusions traffic to the Golgi area, where they intersect a vesicular pathway that exports sphingomyelin from the Golgi apparatus to the plasma membrane [Scidmore *et al*. 1996]. Although the mechanism used by *Chlamydiae* to promote fusions with the exocytic system is not fully understood, the modification of the inclusion membrane by the parasite-specific proteins is thought to regulate these interactions [Haas 1998].

1.3.3.2- *Toxoplasma gondii*

Toxoplasma gondii is a protozoan parasite capable of entering both phagocytic and non-phagocytic cells by active penetration (also called invasion) [Morisaki *et al*. 1995]. Inside host cells, the nascent *Toxoplasma*-containing vacuole is essentially devoid of host plasma membrane proteins, which are excluded or rapidly removed from the vacuole [Suss-Toby *et al*. 1996, Sinai & Joiner, 1997]. After invasion, the parasitophorous vacuole membrane is rapidly modified by secreted proteins from parasite rhoptries and dense granules [Sinai & Joiner 1997]. These secreted proteins are incorporated into the tubovesicular network within the vacuole, the lumen, or the parasitophorous vacuole membrane [Beckers *et al*. 1994, Sibley *et al*. 1986, Sinai & Joiner 1997]. The precise function of these proteins is not known, but they are thought to have an important role in establishment of the replication competent vacuole and to participate in nutrient acquisition from the host cytoplasm [Hackstadt 2000]. *Toxoplasma*-containing vacuoles do not acidify [Sibley *et al*. 1985] nor fuse with endosomes or lysosomes: the vacuoles are devoid of transferrin receptor, M6PR, LAMP-1, and vATPase [Baca & Kumar 1994]. Furthermore, soon after its formation, the *Toxoplasma*-containing vacuole forms extensive associations with the host cell mitochondria and ER [Sinai & Joiner 1997]. It is likely that the removal of the cell host proteins from the parasitophorous vacuole excludes recognition signals for interactions with the endocytic pathway, and that the interactions with ER and mitochondria help to mask the parasitophorous vacuole from the endocytic pathway [Sinai & Joiner 1997].

1.3.3.3- *Legionella pneumophila*

Legionella pneumophila, the etiologic agent of legionaire´s disease, is primary a parasite of fresh water protozoa, but in certain conditions can also infect humans, especially immunocompromised individuals [Steinert *et al.* 2002]. In humans, *L. pneumophila* is able to survive and multiplicate inside macrophages, in a manner similar to that observed in amoebas [Vogel & Isberg 1999]. That suggests that the strategies used by *Legionella* to survive inside macrophages have evolved from survival and growth in its proper host cell, the amoeba [Haas 1998]. *L. pneumophila* enters macrophages via "conventional" phagocytosis [Rechtnitzer & Blom 1989] or by a novel uptake process called coiling phagocytosis [Horwitz 1984]. This is a process in which a phagocyte pseudopod coils around the bacterium and "rolls" it up [Haas 1998].

After engulfment by the macrophage, the bacterium is found within a phagosome bounded by a single membrane [Horwitz 1984]. This phagosome avoids fusion with early endosomes [Clemens *et al*. 2000b], late endosomes [Clemens & Horwitz 1995, Roy *et al*. 1998], and lysosomes [Horwitz 1983b]. At early times after phagocytosis (5 minutes-2 hours post-infection), *Legionella*-containing phagosomes becomes intimately associated with ER-derived vesicles and mitochondria. Later, at 4-12 hours post-infection, these ER-derived vesicles fuse and form a large structure that surrounds the phagosome, which acquires ribosomes [Horwitz 1983a, Swanson & Isberg 1995]. Finally, at 6-10 hours post-infection, the bacterium begins to replicate in this rough ER-like compartment until the host cell dies [Joshi *et al*. 2001, Horwitz 1983a].

The mechanism by which *L.pneumophila* establishes its compartment is not fully understood. Screens for intracellular growth mutants of *Legionella* have identified genes that play an essential role in intracellular transport of *Legionella*-containing phagosomes. These genes are called *dot* (defective organelle trafficking) and *icm* (intracellular multiplication) [Segal *et al*. 1998, Vogel *et al*. 1998, Roy & Tilney 2002]. The *dot/icm* products comprise a secretion apparatus ancestrally related to the type IV secretion systems that is able to transfer proteins from the cytosol of *Legionella* to the host cytosol [Christie 2001, Nagai *et al*. 2002]. Several effectors proteins that are delivered to the host cells by the Dot/Icm secretion system of *Legionella* have been described [Roy & Tilney 2002, Conover *et al*. 2003, Chen *et al*. 2004]. However, the role of these effectors in the remodeling of the *Legionella*-containing vacuole is still unknown.

1.3.4- Escape from the phagosome

Another mechanism employed by some intracellular parasites to avoid delivery to the lysosome is to escape into the nutrient-rich cytoplasm by rupturing the phagosome. In the cytosolic environment, the parasite finds access to nutrients, protection from antimicrobial mechanisms, and a near neutral pH, conditions that ensure its survival and replication [Scott *et al*. 2003]. This strategy is used by *Trypanosoma cruzi* [Andrews 1994] and also by the bacteria *Listeria* [Smith *et al*. 1995], *Rickettsia* [Heinzen *et al*. 1999], and *Shigella* [High *et al*. 1992]. Once in the cytoplasm, these bacteria acquire motility by triggering the formation of actin tail comets [Dramsi & Cossart 1998]. The intracellular lifestyle of *Listeria monocytogenes* is detailed below.

1.3.4.1- *Listeria monocytogenes*

Listeria monocytogenes is a ubiquitous, Gram-positive bacterium with broad ecological niche and host range. It causes serious localized and general infections in humans and in other diverse vertebrates, including birds and mammals [Vásquez-Boland *et al*. 2001]. Within their hosts, *Listeria* is found inside macrophages, where it is able to survive and multiplicate [Vásquez-Boland *et al*. 2001]. In addition, *Listeria* organisms are invasive pathogens that can induce their own internalization in different types of non-phagocytic cells, such as hepatocytes [Vásquez-Boland *et al*. 2001]. Inside host cells, either phagocytes or non-professional phagocytes, the intracellular cycle of *L.monocytogenes* has common characteristics.

Listeria organisms are recognized and internalized by macrophages mainly via Fc- or complement-receptors, although non-opsonic receptor-ligand interactions are also involved in host cell recognition [Pierce *et al*. 1996]. The invasion to non-phagocytic cells is mediated by one or more bacterial proteins, named internalins [Braun & Cossart 2000]. After internalization, *L. monocytogenes* becomes engulfed within a phagocytic vacuole that interacts transient with early endosomes but does not mature into phagolysosome [Alvarez-Dominguez *et al*. 1997]. Thirty minutes after entry, bacteria begin to disrupt the phagosome membrane [Gaillard *et a*l. 1987], and within two hours, 50% of the intracellular bacteria are free in the cytoplasm [Tilney *et al*. 1989]. This membrane disruption step is mediated by the pore-forming, bacterial hemolysin listeriolysin O (LLO) in combination with bacterial phospholipases [Vásquez-Boland *et al*. 2001]. Soon after entry into the mammalian cytosol, *L.monocytogenes* induces the polimerization of host actin filaments that rearrange to form an actin tail

and uses the force produced by actin polimerization to move within the host cell, and later to move from cell to cell [Dramsi & Cossart 1998]. Actin-based intracytoplasmatic movement and cell to cell spread are mediated by the listerial surface protein ActA [Cameron *et al*. 2000].

1.3.5- Adaptation to the very acidic phagolysosome compartment

Another mechanism developed by some intracellular pathogens in order to assure their survival inside phagocytes is to adapt to the harsh environment within phagolysosomes. That is the case of *Leishmania mexicana* and *Coxiella burnetii*.

1.3.5.1- *Leishmania mexicana*

Within macrophages, the protozoan parasite *Leishmania mexicana* resides in a compartment called parasitophorous vacuole. This compartment is positive for the late endosomal/lysosomal markers LAMP-1 and LAMP-2 [Russell *et al*. 1992, Chen *et al*. 1985a], vATPase [Nelson 1987], lysosomal hydrolases [Prina *et al*. 1990], and possesses an internal pH of 4.7-5.2 [Antoine *et al*. 1990]. Interestingly, the parasitophorous vacuole is also positive for the late endocytic markers Rab7 and M6PR [Russell *et al*. 1992]. Thus, it is no clear whether these vacoules are typical lysosomal or rather late endosomal compartments [Haas 1998]. Because *Leishmania*-containing vacuoles remain in contact with the endocytic system, these parasites are able to acquire nutrients by fusions with vesicles from the endosomal continuum [Alexander and Vickerman 1975, Russell et al. 1992].

1.3.5.2- *Coxiella burnetii*

Coxiella burnetii, the agent of Q fever in man, a nearly world wide distributed zoonosis, is a Gram-negative, obligate intracellular pathogen. Inside host macrophages, *C. burnetii* resides in phagosomes that exhibit a delay in phagolysosomal fusion [Howe & Mallavia 2000], but that clearly mature into phagolysosomes [Tjelle *et al*. 2000]. These phagosomes, which are also called “spacious phagosomes” due to their large dimensions, are acidic (pH of 5.2), and positive for LAMP-1, LAMP-2, Rab7, Cathepsin D, lysosomal hydrolases, and vATPase [Heinzen *et al*. 1996, Mege *et al*. 1997, Beron *et al*. 2002]. To survive within the very antimicrobial environment inside the phagolysosomes, *Coxiella burnetii* produces enzymes that inhibit the production of toxic oxigen metabolites by the host cell [Baca *et al*. 1994]. Although it is assumed that molecules secreted by *Coxiella* organisms dictate the compositional and functional phenotype of the spacious phagosomes containing these bacteria,

there is no information as to the nature of the mechanisms by which they control the formation of these vacuoles. Recently, the genome sequence of *C.burnetii* revealed that this bacterium contains proteins homologous to the majority of the *Legionella pneumophila* Dot/Icm type IV secretion system proteins [Sexton & Vogel 2002]. Moreover, several *dot/icm* genes were expressed by *Coxiella* during host cell infection and this expression preceded the formation of the large replicative vacuoles [Zamboni *et al*. 2003]. These data suggest that this type IV secretion system may be involved in the modulation of vacuole biogenesis after uptake of *Coxiella* by eukaryotic host cells.

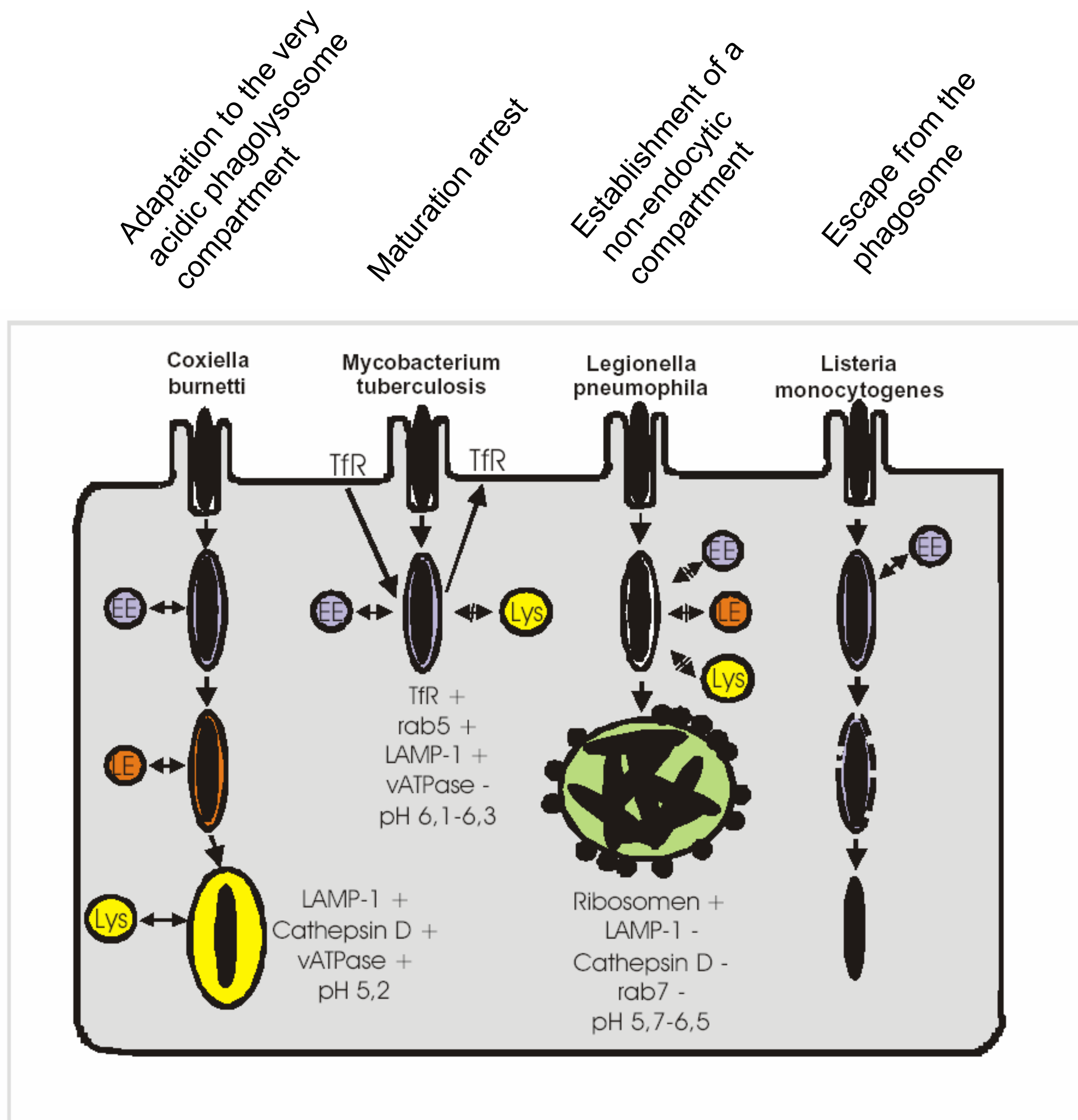

Figure 2: Strategies of intracellular pathogen survival.
Schematic representation of the survival mechanisms employed by different intracellular pathogens and that are described in section 1.3. In this schema are shown main characteristics of the bacteria-containing vacuoles, as well as interactions with the different endocytic compartments.
EE-early endosome; LE-late endosome; Lys-lysosome.

1.4- *Rhodococcus equi*

1.4.1- The genus *Rhodococcus*

The genus *Rhodococcus* is part of the phylogenetic group described as nocardioform actinomycetes, which contains the genera *Caseobacter, Corynebacterium, Mycobacterium, Nocardia*, and *Tsukamurella* [Goodfellow 1987, Collins *et al.* 1988]. Rhodococci are gram-positive, non-motile, non-spore forming, mycolate-containing, catalase-positive bacteria that are characterized by their rod-to-coccus morphologic variation during their growth cycle [Prescott 1991]. The genus name "*Rhodococcus"* was used for the first time by Zopf in 1891. Since its redefinition in 1977, some strains have been combined, transferred to existing genera or reclassified in new genera, and new species have been described [Goodfellow & Anderson 1977, Bell *et al*. 1998].

Bacteria included in the genus *Rhodococcus* are widely distributed in nature: rhodococci have been isolate from different sources such as soils, rocks, marine sediments, animal dung, guts of insects, and healthy and disease plants, animals, and humans [Goodfellow 1989, Ivshina *et al*. 1994]. Members of this genus are of interest for a variety of reasons. The ability of some members to degrade or transform a wide range of hazardous chemicals makes them useful in environmental and industrial biotechnology [Finnerty 1992, Warhust & Fewson 1994]. Other members are used as biosensors because of their ability to degrade compounds such as phenols and hydrocarbons [Riedel *et al*. 1993, Peter *et al*. 1996]. Furthermore, some species of *Rhodococcus* are well-recognized as pathogens of plants, wide range of animals, and even humans [Finnerty 1992, McNeil & Brown 1994]. The genus contains 12 established species classified on the basis of numerical, chemical and molecular taxonomy, namely *R. coprophilus, R. equi, R. fascians, R. erythropolis, R. globerulos, R. marinonascens, R. opacus, R. percolatus, R. rhodnii, R. rhodochrous, R. ruber* and *R. zopfii* [Bell *et al*. 1998].

1.4.2- *Rhodococcus equi*

R.equi was first isolated in 1923 as *Corynebacterium equi* from the lungs of foals with pygranulomatous pneumonia by Magnusson [Magnusson 1923]. Later, when the methods to classify the nocardioform actinomycetes were improved, this bacterium was reclassified as *Rhodococcus equi* [Goodfellow *et al*. 1982, Goodfellow 1987]. *R.equi* is a gram-positive, obligate aerobic, catalase-positive, oxidase-negative, and urease-positive coccobacillus [Prescott 1991]. It is non-motile, non-flagellated, and produces small numbers of pili [Yanagawa & Honda 1976]. The growth requirements of *R.equi* are simple: it grows over a wide range of temperature from 10 to 40°C on different non-selective media, such as BHI agar [Prescott 1991]. The optimal temperature of growth appears to be 30°C, although growth rate at 37°C is only marginally less than at 30°C [Hughes & Sulaiman 1987]. When cultivated for 24 hours at 37°C in nonselective media, rhodococcal colonies are 1 to 2 mm in diameter and are not distinctive. At 48 hours incubation the colonies vary in size from 2 to 4 mm and have developed their characteristic appearance: irregularly round, smooth, semitransparent, glistening, mucoid, teardrop colonies [Prescott 1991]. After 4 to 7 days of incubation, colonies may develop a shade of salmon pink, a pigmentation expected for all rhodococci members (red-pigmented cocci) [Prescott 1991]. *R.equi* possesses an antigenically variable, lamellar polysaccharide capsule [Woolcock & Mutimer 1978] that is the basis of the capsular serotyping system [Prescott 1981]. At least 27 different capsullar serotypes have been described, and among them, Prescott capsular serotype 1 is the most common worldwide [Prescott 1981]. There is no apparent relationship between serotype and strain virulence [Prescott 1991].

R.equi has been found and isolated worldwide. Beside the Antarctica, it is spread on all continents, and has been isolated from very different environments, including soil, water, and faeces from a variety of omnivores and herbivores [Ellenberger & Genetsky 1978 anja]. This bacterium is largely a soil organism, which multiplies preferentially in herbivore manure at summer temperatures in temperate climates, and therefore is widespread in grazing animals and their environment [Prescott 1987]. Of all the species comprised in the genus *Rhodococcus*, *R.equi* possesses the highest pathogenic potential for animals, including humans [Prescott 1991].

1.4.3- *R.equi* infections

R.equi has been recognized as a foal pathogen for more than 80 years [Prescott 1991]. Foals less than 6 moths of age are the only animals in which infection is common in otherwise healthy animals, although infection sometimes occurs in adult horses [Bell *et al*. 1998]. In the foal, *R.equi* infections are normally respiratory, producing suppurative bronchopneumonia, which is normally chronic and produces abscesses in the lung and in bronchial and mesenteric lymph nodes [Prescott 1991]. Untreated lesions can progress and cause dead by asphyxiation. In addition, lung infection can disseminate to the gut lining (where it produces ulcerative colitis), to other organs, and to the joints. Moreover, ingestion or introduction of the bacteria into cuts can cause intestinal or wound injuries [Bell *et al*. 1998]. Disease due to *R.equi* is rare in adult horses. It manifests as a sporadically occurring illness with characteristics similar to that observed in foals [Prescott 1991]. *R.equi* can also cause disease (usually respiratory infections) in a wide range of other animals, such as pigs, cattle, sheep, deer, and cats. Besides submaxillary lymphadenitis in pigs, infections in animals other than foals are generally rare [Prescott 1991].

Under most circumstances, *R.equi* is not considered a human pathogen. However, in recent years, the role of this bacterium as human pathogen has been noted, as *R.equi* infections in humans have increased in frequency. This increase is associated with the rise in the number of immunosupressed individuals, specially AIDS patients [Bell *et al*. 1998]. In immunosupressed patients, *R.equi* infections occur mainly in the lungs, causing pneumonia and abscesses [McNeil & Brown 1994], and the symptoms are often fever, cough, and chest pain [Harvey & Sunstrum 1991]. The infection can disseminate to cause lesions in other organs and bacteremia [Prescott 1991, McNeil & Brown 1994]. The appearance of such lesions usually means that the infection is serious, often fatal, both in AIDS and non-AIDS patients [Harvey & Sunstrum 1991]. Even with accurate diagnostic and antibiotic treatment, mortality rates in AIDS patients are usually high [Bell *et al*. 1998]. Rarely, *R.equi* infection occurs in individuals with no apparent immunological abnormalities [Linares *et al*. 1997, Kedlaya *et al*. 2001]. The source of exposure for humans can not always be detected, and most of the individuals do not report exposure to herbivores [Drancourt *et al*. 1992]. However, as *R.equi* is mainly a soil inhabitant, exposure to contamined soil could be important on many cases of human disease [Hondalus 1997].

1.4.4- Interaction of *R.equi* with phagocytes

R.equi is a facultative intracellular pathogen that is able to survive and even to multiply inside cells of the monocyte-macrophage lineage [Hondalus 1997]. Infectivity is limited to this kind of cells, whereas neutrophils from foals are able to clear the ingested bacteria [Meijer & Prescott 2004]. Macrophage entry through non-Fc receptors is thought to be important for *R.equi* survival, since opsonisation of bacteria with antibodies before infection enhances bacterial killing by equine macrophages [Hietala & Ardans 1987]. *In vitro*, optimal binding of *R.equi* to macrophages requires complement and is mediated by the complement receptor CR3 (CD11b/CD18), also called Mac-1 [Hondalus & Mosser 1994]. Other receptors may contribute to the entry of *R.equi* to macrophages: the lipoarabinomannan of *R.equi* may bind to mannose receptors [Meijer & Prescott 2004], and Toll-like receptors are also likely to be involved in recognition and internalization of the bacteria. Most of the intracellular events during *R.equi* infection have not been investigated in detail. Using electron microscopy, two groups have observed that, once inside foal alveolar macrophages, *R.equi* is found within membrane-enclosed vacuoles that do not fuse with lysosomes, and they also noted a correlation between this absence of phagosome-lysosome fusion and intracellular persitence of bacteria [Hietala & Ardans 1987, Zink *et al*. 1987]. In addition to survive inside macrophages, *R.equi* is capable to multiplicate within the host cells´phagosomes [Hietala & Ardans 1987, Zink *et al*. 1987, Hondalus & Mosser 1994]. Following an initial lag phase of several hours, the intracellular doubling time of R.equi is about 6-8 h [Hondalus & Mosser 1994] and bacterial multiplication appears to be confined to membrane-surrounded vacuoles [Zink *et al*. 1987]. *R.equi* infection has proved to be toxic for the macrophages. *In vitro*, phagocyte degeneration is evident after a few hours of infection [Lührmann *et al*. 2004, Hondalus 1997, Hietala & Ardans 1987, Zink *et al*. 1987], and *Rhodococcus*-induced cell death was shown to be necrotic rather than apoptotic [Lührmann *et al*. 2004]. In addition, Lührmann *et al*. [2004] have found that *R.equi* must be alive in order to be cytotoxic, and that the cytotoxic factor is not a constitutively produced and released rhodococcal factor. Which molecular factors are involved in the killing of macrophages is as yet unknown, but it is hypothesized that at least two factors act in concert to produce cytotoxicity to macrophages: one factor is necessary for low levels of necrosis and is present in most *R.equi* bacteria, and a second factor, dependent on a virulence-associated plasmid of *R.equi*, is needed for full cytotoxic potential [Lührmann *et al*. 2004].

1.4.5- Virulence factors

Great variation in virulence of *R.equi* isolates has been identified using experimentally infected mice and foals: clinical isolates are more pathogenic than isolates coming from environmental sources [Bowles *et al*. 1987], and strains isolated from the soil of farms with endemic rhodococcal disease are likely to be more virulent than those isolated from farms with no history of rhodococcal pneumonia [Takai *et al*. 1991b]. Among possible virulence factors are capsular polysaccharide, the exoenzyme cholesterol oxidase, cell wall mycolic acids, and the products encoded or regulated by virulence-associated plasmids [Hondalus 1997].

- The polysaccharide capsule of *R.equi* might inhibit phagocytosis of the organism, thus contributing to its virulence [Prescott 1991]. There is great variety of capsular antigens among strains of *R.equi* (serotypes 1-27), and capsular serotype 1 is the most common [Prescott 1981]. However, the role of the capsular polysaccharide in rhodococcal virulence remains obscure: there is no apparent relationship between capsular serotype and the source of bacterial isolate [Prescott 1981], and there were identified both virulent and avirulent isolates of the same capsular serotype [Takai *et al*. 1991a].
- Cholesterol oxidase, also termed "equi factor", is an exoenzyme produced by *R.equi* that may confer the bacteria membranolytic activity, and thus may be involved in host tissue damage [Linder & Bernheimer 1997]. *In vitro*, the enzyme is able to destabilize erythrocytes by the generation of 4-cholesten-3-one (cholestenone) in the membrane. The access to the cholesterol target can be conferred by phospholipases C or D produced by another bacterium, which modify the sphingomyelin located in the outer layer of the lipid leaflet. The possible cooperative hemolytic partners of *R.equi* are: *Staphylococcus aureus, Corynebacterium pseudotuberculosis, Arcanobacterium haemolyticum* and *Listeria monocytogenes* [Linder & Bernheimer 1997]. The role of cholesterol oxidase in the pathogenicity of *R.equi* is still unclear, as both virulent and avirulent isolates produce this enzyme [Prescott *et al*. 1982]. However, this enzyme may have some effect on lysosomal and cellular membranes of the host cells, contributing to the cytoxicity observed both *in vivo* [Johnson *et al*. 1983] and *in vitro* [Zink *et al*. 1987, Hietala & Ardans 1987, Hondalus & Mosser 1994].
- The cell envelope of *R.equi*, which consists of mycolic acids linked to arabinogalactan polysaccharide and glycolipids, may influence virulence. When mice were infected with strains of

R.equi expressing longer carbon chain mycolic acids, they developed more granulomas and their mortality rates were higher than mice infected with strains of shorter carbon chain mycolic acids [Gotoh *et al*. 1991]. In addition, strains of *R.equi* isolated from abscessed lymph nodes from infected pigs tend to contain longer chain mycolic acids than strains isolated from normal lymph nodes or tonsils of healthy pigs [Gotoh *et al*. 1991].

- Undoubtedly, the interest in virulence mechanisms of *R.equi* has been centred by the discovery of the virulence-associated antigens and plasmids. It was observed that many equine isolates of *R.equi* expressed a 15-17 kDa protein which was absent in non-pathogenic *R.equi* [Chirino-Trejo & Prescott 1987]. Furthermore, all isolates that were virulent for mice were shown to express this protein [Takai *et al*. 1991a]. Takai *et al*. [1991a] showed that serum from foals infected with *R.equi* possesses high levels of antibodies directed against a highly immunogenic protein of this molecular weight, which was called VapA. Following the identification of VapA as a virulence associated protein, both Takai *et al*. [1991c] and Tkachuk-Saad & Prescott [1991] established that isolates which expressed VapA also possess a large plasmid of approximately 85 kb. Later, the *vapA* gene was cloned, and its localization to the plasmid confirmed [Takai *et al*. 1993, Tan *et al*. 1995]. The ability of *R.equi* to survive and replicate in macrophages is specific to plasmid carrying strains. Cured isolates are avirulent for foals and mice [Giguere *et al*. 1999a, Hondalus & Mosser 1994]. Isolates from the submaxillary lymph nodes of infected pigs usually possess a large plasmid, but this plasmid does not encode VapA, but a related protein of 20 kDa, called VapB [Meijer & Prescott 2004]. In mice, pig isolates are of intermediate virulence (in contrast with the full virulence from foal isolates containing the *vapA* gene), suggesting host specificity of strains containing different plasmids [Takai *et al.* 1996b]. All these studies indicate that the virulence plasmids are essential for *R.equi* virulence in foals, mice, and pigs, and thus encode one or more virulence factors. *R.equi* strains infecting humans display great diversity and exhibit a broad range of virulence: human isolates contain plasmid encoding either VapA or VapB or lack a virulence plasmid [Takai *et al*. 1994a]. This suggests that the pathogenesis of *R.equi* infection in humans may be different than in foals and pigs. Despite the discovery of the virulence-associated plasmids, analysis of their nucleotide sequence did not provide the expected insight into the virulence mechanisms of *R.equi.* The reason is that the majority of the putative virulence proteins encoded within the plasmid do not share any similarity with proteins from other organisms,

suggesting that *R.equi* may employ a novel virulence mechanism [Meijer & Prescott 2004]. Nucleotide sequence of the VapA-expressing plasmid resulted in the finding that it encodes six *vapA* homologues (*vapC, D, E, F, G, H*), but the function of these proteins is still unclear [Meijer & Prescott 2004]. A number of observations pointed VapA as a possible virulence determinant: VapA is an exported protein [Tan *et al*. 1995] and its presence on the cell surface would enable it to interact with the host cell. Moreover, expression of *vapA* is both temperature and pH regulated, occurring between 34-41°C [Takai *et al*. 1992] and at pH of 5 [Takai *et al*. 1996a, Benoit *et al*. 2001], conditions that resemble the virulence gene expression patterns of other pathogenic bacteria. Interestingly, when *vapA* was expressed in plasmid negative *R.equi*, it did not restore the ability to growth and multiply in mice or foals, suggesting that VapA alone is not sufficient for the virulence of *R.equi* [Giguere *et al*. 1999a]. However, this study did not eliminate the possibility that VapA may need other plasmid products in order to achieve virulence. A recent study, using deletion mutants of *R.equi*, demonstrated the role of VapA in the virulence of this bacterium: *R.equi* lacking the DNA region of the virulence plasmid encompassing *vapA* (*vapA* mutant) are unable of multiplicate in mice and are cleared rapidly in comparison to the wild-type strain. In addition, expression of VapA was able to restore virulence the *vapA* mutant [Jain *et al*. 2003]. However, the mechanisms of action of VapA are still not clear: it could be involved in the acquisition of nutrients essential for the intracellular survival of *R.equi* within macrophages, or may play a direct role in the inhibition of phagosome maturation.

1.4.6- Immunity

Immunity to *R.equi* in foals depends on both antibody and cell-mediated components of the immune response, but their exact contribution remains to be cleared.

Antibodies against *R.equi* are widely expressed in horses. Antibody may contribute to immunity by altering the route by which the bacteria enter the macrophage, and thus decreasing the ability of *R.equi* to inhibit phagosome maturation [Speert 1992]. *In vitro*, immune serum has been shown to promote killing of *R.equi* by equine macrophages [Hietala & Ardans 1987], and passively transferred anti-*R.equi* have a protective effect against *R.equi* pneumonia, reducing morbidity and mortality in endemically affected farms [Meijer & Prescott 2004]. However, plasma must be present prior to

challenge: administration of hyperimmune plasma to foals seven days after infection with *R.equi* does not alter the course of disease [Chaffin *et al*. 1991]. This suggests that antibody may play a role only in the initial interactions of *R.equi* with its host.

The facultative intracellular nature of *R.equi* suggests that cell-mediated immune mechanisms may play a major role in resistance and clearance on infection. The two major mechanisms by which T lymphocytes clear intracellular pathogens are direct cytotoxicity of the infected cell (usually mediated by MHC class I restricted $CD8^+$ T-cells) and secretion of cytokines. $CD4^+$ T-lymphocytes can be divided into Th1 and Th2 subsets based on the cytokine production: Th1 cells produce IFN-γ and interleukin-2 (IL-2), and Th2 cells produce IL-4, IL-5, and IL-10. The relative balance of the Th1 or Th2 response determines the outcome and the ability of the infected host to control infections [Scott 1991, Yamamura *et al*. 1991]. Kanaly *et al*. [1993] demostrated the role of $CD4^+$ T-lymphocytes in the clearance of *R.equi* infection, since MHC I transgenic mice deficient in $CD8^+$ T-cells cleared virulent bacteria from lungs whereas MHC II transgenic mice deficient in $CD4^+$ T-cells did not. Immunocompetent mice experimentally infected with virulent *R.equi* developed a Th1 cytokine response and cleared the infection. Instead, mice in which a Th2 cytokine response was induced, developed pulmonary granulomas, suggesting that Th1 response is protective in mice and that a non-protective Th2 response is involved in development of disease [Kanaly *et al*. 1995, 1996]. IFN-γ is the major macrophage-activating factor and is capable of upregulating microbial killing by macrophages [Mosmann & Coffman 1989], stimulate phagolysosome formation, and enhance expression of Fc receptors [Nathan *et al*. 1983]. The data obtained with experimentally infected mice suggest that, at least for mice, IFN-γ is essential for immune clearance of *R.equi* and that Th1 response is necessary to promote protective immunity [Hines *et al*. 1997]. Infection of foals with virulent *R.equi* was shown to produce an immunomodulatory effect which triggers an ineffective, Th2-like response, reducing the production of IFN-γ by $CD4^+$ lymphocytes [Giguere *et al*. 1999b]. However, the clearance of virulent *R.equi* from the lung of experimentally infected adult horses was associated with increased numbers of IFN-γ producing lymphocytes [Hines *et al*. 2003]. This difference of immune responses between foals and adult horses may reflect unique features of the immune system of foals, differences between non-immune and immune animals, or differences in experimental procedures [Meijer & Prescott 2004].

1.5- Objectives

The objective of this work is to study the precise compartmentation of the facultative intracellular bacterium *Rhodococcus equi* within murine macrophages. Establishing the intracellular compartmentation of *R.equi* gives insight to the mechanisms of survival of this pathogen as well as the physiopathology of *R.equi* infection and helps to understand the interactions between intracellular pathogens and the immune system. In addition, another objective is to investigate the influence of the virulence-associated plasmids of *R.equi* (VapA- and B-encoding plasmids) in the establishment of *R.equi*-containing vacuoles (RCVs). The intracellular fate of viable and non-viable *R.equi* is also compared in this work with the intention to study whether viability of *R.equi* has an influence in the establishment of RCVs.

2- Materials and methods

2.1- Chemicals

• Agar-Agar	Roth
• Ammonium chloride	Sigma
• BHI	Difco
• di-sodium hydrogen phosphate anhydrous	Roth
• Calcium chloride	Roth
• DMEM	Sigma
• Donkey serum	Sigma
• DMSO (water free)	Sigma
• FCS (fetal calb serum)	Gibco
• Ficoll-Paque Plus	Amersham Biosciences
• Fugene 6	Roche
• L-Glutamine	Sigma
• Glycerin	Roth
• Glucose	Roth
• Goat serum	Sigma
• HEPES	Roth
• HS (horse serum)	Sigma
• JetPEI-Man	Qbiogene
• LB	International Diagnostic group (IDG)
• Magnesium chloride	Roth
• Midiprep kit	Sigma
• Paraformaldehyde	Merck
• Potassium di-hydrogen phosphate	Roth
• Potassium chloride	Roth
• Pyruvic acid	Sigma

• RPMI	Gibco
• Saponin	Sigma
• Sodium di-hydrogen phosphate	Roth
• Sodium carbonate	Roth
• Sodium hydrogen carbonate	Roth
• Sodium chloride	Roth
• Sodium hydroxide	Roth
• Trypsin-EDTA	Gibco
• Tris base	Roth
• Zymosan	Sigma

2.2- Materials

• 1 ml crytubes	Nunc
• 1.5 ml tubes	Brand
• 12 ml conical tubes	TPP
• Cell culture plates	TPP
• Cover slides	Menzel
• Cover slips	Menzel
• Pipetting tips	Abimed
• Petri dishes	Greiner
• Sterile pipettes (25, 10, and 1 ml)	TPP

2.3- Equipment

• Autoclave	Webeco, Systec
• Balance	Denver Instrument
• Biological safety cabinets	Nalge Nunc International
• Centrifuge	Hettich, Eppendorf
• Confocal laser scanning microscope (LSM 510)	Zeiss
• Counting chamber	Brand
• Fluorescence microscope	Zeiss

• Heating block	Haep Labor Consult
• Incubator	Heracell, Binder
• Light microscope	Zeiss
• Magnetic shaker	Wenzel Laborbedarf
• Microliter pipetting aids	Abimed
• pH-meter	inoLab
• Refrigerator	Privileg
• Shaker incubator	Stuart Scientific
• Spectrophotometer	Thermo Spectronic
• Transmission electron microscope (CM 120)	Philips
• Vortex	Scientific Industries
• Water bath	Memmert

2.4- Solutions and buffers

50 mM NH_4Cl

NH_4Cl	1.1 g
d H_2O	400 ml

The solution is autoclaved

0.1 M $NaHCO_3$ pH 8.2

$NaHCO_3$	0.84% (w/v)

The solution is filtered under steril conditions

Paraformaldehyde 8%

Paraformaldehyde	8 g
d H_2O	ad 100 ml

The solution is dissolved at 70°C with 1 or 2 drops of 1N NAOH, filtered and stored at –20°C.

Paraformaldehyde 3%

Paraformaldehyde 8%	3.8 ml
d H_2O	6.2 ml

PBS (1X)

PBS (20X)	50 ml

Ad 1000 ml d H_2O

PBS (20X)

NaCl	160 g
KCl	4g
KH_2PO_4	4 g
Na_2HPO_4	18.35 g
Ad 1000 ml d H_20	

Permeabilization buffer

Saponin	0.1%
Donkey or goat serum	5%
In 1X PBS	

Ringer´s solution

NaCl	155 mM
KCl	5 mM
$CaCl_2$	2 mM
$MgCl_2$	1 mM
HEPES	10 mM
Glucose	10 mM
NaH_2PO_4	2 mM

The pH was adjusted to 7.3 with 10 N KOH and autoclaved.

10 mM Tris-HCl pH 7.6

Tris base	1.21 g
d H_2O	80 ml

The pH is adjusted to 7.6 with 10 N HCl and autoclaved.

20 mM Tris-HCl pH 8.0

Tris base	2.42 g
d H_2O	1000 ml

The solution is filtered under steril conditions

2.5- Cell culture Media

RPMI complete medium

RPMI 1640 with 2mM glutamine	500 ml
FCS (heat inactivated)	50 ml

Dulbecco´s Modified Eagle Medium (DMEM) complete medium

DMEM	500 ml

FCS (heat inactivated) 50 ml

2.6- Media and agar for bacterial culture

<u>Brain-Heart Infusion (BHI) Media</u>

BHI 37 g

Ad 1000 ml d H_20

<u>Brain-Heart Infusion (BHI) Agar plates</u>

BHI 37 g

Agar 15 g

Ad 1000 ml d H_20

<u>LB Media</u>

LB 20 g

Ad 1000 ml d H_20

<u>LB-kanamycin Agar plates</u>

LB 20 g

Agar 15 g

Kanamycin (stock 100mg/2ml d H_2O) 1 µl/ml

Ad 1000 ml d H_20

The media was autoclaved and 1µl/ml kanamycin was given in sterile conditions.

<u>LB-ampicillin Agar plates</u>

LB 20 g

Agar 15 g

Ampicillin (stock 100 g/l d H_2O) 100 µg/ml

Ad 1000 ml d H_20

The media was autoclaved and 100 µg/ml ampicillin was given in sterile conditions.

2.7- Antibiotics

Antibiotic	Stock solution	Final concentration	Firma
Gentamicin	50 mg/ml d H_2O	10-150 µg/ml	Gibco BRL
Ampicillin	100 g/l d H_2O	100 µg/ml	Sigma
Kanamycin	50 mg/ml d H_2O	0.05 mg/ml	Sigma
Hygromycin	30 mg/ml d H_2O	300 µg/ml	Sigma
Geneticin (G418)	50 mg/ml d H_2O	500 µg/ml	Gibco BRL
Bafilomycin A	100 µM	0.25 µM	Calbiochem

2.8- Antibodies

2.8.1- First antibodies

Antigen	Antibody type	Specie of origin	Description	Reference
Transferrin receptor	monoclonal	rat	ATCC TIB-219 U. Schaible, Berlin Original: Development Study Hybridoma Bank, Iowa. Dilution 1:50	[Lesley *et al*. 1984]
LAMP-1	monoclonal	rat	clone 1D4B U.Schaible, Berlin Original: Development Study Hybridoma Bank, Iowa. Dilution 1:200	[Chen *et al*. 1985b]
LAMP-2	monoclonal	rat	clone ABL-93 U.Schaible, Berlin Original: Development Study Hybridoma Bank, Iowa. Dilution 1:10	[Chen *et al*. 1985a]
EEA-1	polyclonal IgG	goat	N-19 Santa. Cruz Biotech, Heidelberg, Germany. Dilution 1:10	[Mu *et al.* 1995]
EEA-1	polyclonal	rabbit	G. Mills and M. Clague, Liverpool. Dilution 1:50	[Mills *et al*. 1998]
LBPA	monoclonal	mouse	Jean Gruenberg, Department of Biochemistry, University of Geneva, Switzerland. Dilution 1:50.	[Kobayashi *et al*. 1998]
VATPase (16K)	polyclonal	rabbit	M. Skinner, University of Guelph, Canada. Dilution 1:40	[Skinner & Wildemann 1999]
Rhodococcus equi serotype 1	polyclonal	rabbit	C. Lämmler, University of Giessen, Germany Dilution 1:50	[Bern & Lämmer 1994]

2.8.2- Second antibodies

Antigen	Specie of origin	Conjugated fluorochrome	Description	Firma
Rat	donkey	Cy2	Affinity-pure Dilution 1:50	Jackson Immuno Research
Rat	goat	Fluorescein	Dilution 1:400	Sigma
Rabbit	goat	Fluorescein	Dilution 1:400	Sigma
Rabbit	donkey	Cy2	Affinity-pure Dilution 1: 50	Jackson Immuno Research
Goat	donkey	Cy2	Affinity-pure Dilution 1:50	Jackson Immuno Research
Rabbit	donkey	Cy3	Affinity-pure Dilution 1: 50	Jackson Immuno Research
Rat	donkey	Cy3	Affinity-pure Dilution 1:50	Jackson Immuno Research
Mouse	donkey	Cy2	Affinity-pure Dilution 1:50	Jackson Immuno Research

2.9- Fluorescent dyes

Fluorescent dye	Stock	Working solutions	Firma
Calcein	10 mM in RPMI 1640 Filtrated in sterile conditions and stored at –20°C	280 µM in RPMI complete medium	Sigma
Dextran-texas red (lysine fixable) 10 000 Da	1 mg/ml RPMI 1640 Filtrated in sterile conditions and stored at –20°C	0.2 mg/ml RPMI complete medium	Molecular Probes
NHS-Rho	20 µg/µl DMSO Stored at –20°C	0.01 µg/µl – 0.2 µg/µl $NAHCO_3$	Molecular Probes
NHS-CF	20 µg/µl DMSO	0.2 µg/µl $NAHCO_3$	Molecular Probes
NHS-OG	100 µg/µl DMSO Stored at –20°C	0.5 µg/µl $NAHCO_3$	Molecular Probes
Transferrin-alexa fluor	5 mg/ml 1X PBS Alicuoted and stored at 5°C	50 µg/ml Ringer´s solution	Molecular Probes
LysoTracker Red DND-99	1 mM in DMSO	Dilution 1:10 000 in RPMI complete medium	Molecular Probes

NHS-Rho: 5-(and 6-)-carboxytetramethylrhodamine, succinimidyl ester
NHS-CF: 5-(and 6-)-carboxyfluorescein, succinimidyl ester
NHS-OG: Oregon green 488 carboxylic acid, succinimidyl ester
Transferrin-alexa fluor: Transferrin from human serum, Alexa Fluor 488 conjugate

2.10- Manufacturers

- Abimed, Langenfeld, Germany.
- American Type Culture Collection, Manassas, VA, USA.
- Amersham Pharmacia Biotech, Freiburg, Germany.
- Binder, Tuttlingen, Germany.
- Brand, Wertheim A.M., Germany.
- Calbiochem AG, California, USA.
- Carl Zeiss, Oberkochen, Germany.
- Denver Instruments, Göttingen, Germany.
- Dianova, Hamburg, Germany.
- Difco, Sparks, MD, USA.
- Eppendorf, Hamburg, Germany.
- Gibco Life Technologies, Karlsruhe, Germany.
- Greiner bio-one, Frickenhausen, Germany.
- Haep Labor Consult, Bovenden, Germany.
- Philips, Hamburg, Germany.
- Hettich, Tuttlingen, Germany.
- Heraeus, Hanau, Germany.
- inoLab, UK.
- Jackson ImmunoResearch, PA, USA.
- Menzel-Gläser, Gerhard Menzel Glasbearbeitungswerk, Braunschweig, Germany
- Merck, Darmstadt, Germany.
- Molecular Probes, Eugene, OR, USA.
- Nalge Nunc International, NY, USA.
- Nunc, Wiesbaden, Germany.
- Privileg, Quelle AG, Fürth, Germany.
- Qbiogene, Heidelberg, Germany.
- Roche Diagnostics, Mannheim, Germany.
- Roth, Karlsruhe, Germany.
- Santa Cruz Biotech, Heidelberg, Germany.
- Sigma Aldrich, Taufkirchen, Germany.
- Stuart Scientific, UK.
- Thermo Spectronic, NY, USA.
- Webeco, Bad Schwartau, Germany.
- Wenzel, Heidelberg, Germany.

2.11- Cell culture

2.11.1- Cellular lines

Cell line	Description	Source	Reference
J774E	Strongly mannose receptor-expressing murine monocyte-macrophage-like cell line	Philip Stahl (Washington University, St. Louis, USA)	[Fiani *et al*. 1998]
Raw 264.7	Murine monocyte-macrophage-like cells derived from Abelson murine leukemia virus	Hubert Hilbi (ETH Zürich, Switzerland).	[Raschke *et al*. 1978]
Raw 264.7 PX1 (p40phox)	Stabil transfected with a NT-GFP-tagged PX domain of the oxidase component $p40^{phox}$ (GFP-PX)	P.T Hawkins (The Babraham Institute, Cambridge, U.K).	[Ellson *et al*. 2001]
CHO- FcRγII	Chinese hamster ovary cells stabil transfected with FcRγII	Craig R. Roy (Yale University School of Medicine, New Heaven, USA).	[Joiner *et al*. 1990]
Murine bone marrow derived macrophages (BMMs)	Primary murine macrophages derived from bone marrow cells	Isolated from C57BL/6ByJ female mice, 6 weeks old	[Schaible *et al*. 1999]
L929	Murine fibrosarcoma cells	Strain collection of the Chair of Microbiology, University of Würzburg, Germany.	[Hutz *et al*. 1984]

2.11.2- Culture of J774E and Raw 264.7 cells

J774E and Raw 264.7 cells can be stored for years in DMSO-containing solution at -196°C in liquid nitrogen. For thawing the cells, 1 cryotube containing approx. 6.0×10^{6} cells was removed from the liquid nitrogen freezer and placed immediately into water at 37°C until the liquid started melting. The content of the cryotube was then placed into a conic tube containing 10 ml of RPMI complete medium and centrifuged at room temperature for 3 minutes at 1200 rpm. After removing the supernatant, the

cell pellet was suspended with 10 ml of RPMI complete medium, placed in a 90 mm polycarbonate culture dish and incubated at 37°C/5%CO_2. To eliminate rests of DMSO, the medium was changed after 2 hours, when most of the cells have attached to the surface of the culture dish. Cells were subcultured every 2 days, at a split ratio of 1:3. For that purpose, medium was removed from the culture dishes and 3 ml of fresh medium were added. The layer of cells was gently scraped using a rubber policeman and placed in new polycarbonate dishes containing RPMI complete medium.

2.11.3- Culture of L929

L929 cells were cultured in 25 cm^2 polycarbonate culture flasks containing DMEM complete medium at 37°C/7% CO_2. Cells were subcultured every 2 days, at a split ratio of 1:3. For that purpose, medium was removed from the flasks, cell monolayers were then washed twice with PBS to remove all traces of serum which contains trypsin inhibitors, and 2 ml of Trypsin-EDTA solution were given to each flask. After gently swirling, Trypsin-EDTA solution was withdrawn and flasks were incubated at 37°C until the cell layer was dispersed (approx. 3 minutes). Fresh DMEM complete medium was given to each flask and the suspension was mixed by pipetting up and down. Cell suspension was then placed in new polycarbonate flasks containing DMEM complete medium.

To obtain culture supernatant from the cell line L929, cells were cultured in 150 cm^2 polycarbonate culture flasks containing DMEM complete medium at 37°C/7% CO_2 until cells were approx. 80% confluent. Medium was then withdrawn, 40 ml of fresh DMEM complete medium was given to each flask, and flasks were incubated for 1.5 weeks at 37°C/7% $CO_{2.}$ Medium was then removed from the flasks, filtered under steril conditions, and alicuoted in 10 ml tubes. Alicuots of culture supernatant from L929 were stored at -20°C until needed.

2.11.4- Culture of CHO-FcRγII

CHO-FcRγII cells were cultured in 90 mm culture dishes containing DMEM complete medium at 37°C/7% CO_2. Cells were subcultured every 2 days, at a split ratio of 1:4 as follows: culture medium was removed from the dishes and cell monolayer was washed twice with PBS to remove all traces of serum which contains trypsin inhibitors. 2 ml of Trypsin-EDTA solution were then given to each dish, and, after gently swirling, Trypsin-EDTA solution was withdrawn and dishes were incubated at 37°C until the cell layer was dispersed (approx. 3 minutes). Fresh DMEM complete medium was given to

each dish and the suspension was mixed by pipetting up and down. Cell suspension was then placed in new polycarbonate dishes containing DMEM complete medium.

2.11.5- Isolation and culture of BMMs

Primary bone marrow-derived macrophages were prepared from 6 week old female C57BL/6ByJ mice. Mice were killed by cervical dislocation and bone marrow cells were flushed out of the femura and tibiae using a 27G needle and collected in bacterial Petri dishes with 3 ml PBS, then diluted with 10 ml of DMEM medium containing: 3.7 g/l Na_3CO_3 , 4.5 g/l glucose, 10 mM HEPES (pH 7.3), 10 mM glutamine, 1 mM pyruvate, 10% FCS (heat inactivated), 5% horse serum (heat inactivated), penicillin (0.1 mg/ml), streptomycin (1 U/ml), and 20 vol.-% spent culture supernatant from L929 murine fibrosarcoma cells (section 2.11.3). L929 culture supernatant contains monocyte growth factors, such as GMCSF and MCSF, which are important for the differentiation of the macrophages.The bone marrow cells were permitted to adhere, differentiate and multiply for 6 days at 37°C/7% CO_2. BMMs were recovered from the Petri dishes by replacing the medium with ice-cold PBS, and leaving the dishes at 4°C for 30 minutes. Cells were then removed by gentle scraping using a rubber policeman and placed in new polycarbonate dishes containing the medium described above. The BMMs were used directly for infection with bacteria or stored in liquid nitrogen until further use.

To store BMMs in liquid nitrogen, 4×10^6 - 8×10^6 cells were suspended in 1 ml DMEM medium containing 20% FCS (heat inactivated) and the cell suspension was placed in a cryo tube. Small drops of DMSO were then added into the tube until a final concentration of 10% (v/v) was reached. Cell suspension was frozen slowly: first, the tubes were placed 2 hours at -20°C, followed by 24 hours at -80°C. Finally, the tubes were stored at -196°C in liquid nitrogen.

2.12- Bacteria

2.12.1- Bacterial strains

Strain	Characteristics	Source	References
Rhodococcus equi ATCC 33701 (+)	Contains an 85-kb plasmid and produces a 15-17 kD antigen (VapA)	From JF Prescott (University of Guelph, Canada) Isolated from foals	[Takai *et al*. 1991c]
Rhodococcus equi ATCC 33701 (-)	Plasmid-cured VapA-negative isogen derivate of strains ATCC 33701 (+)	From JF Prescott (University of Guelph, Canada) Isolated from foals	[Takai *et al*. 1991c]
Rhodococcus equi 103 (+)	Contains an 85-kb plasmid and produces a 15-17 kD antigen (VapA). Its virulence associated plasmid has almost the same nucleic acid sequence as ATCC 33701 strain.	From JF Prescott (University of Guelph, Canada) Derived from a foal with *R.equi* pneumonia	[Giguere *et al*. 1999a]
Rhodococcus equi 103 (-)	Plasmid cured VapA-negative isogen derivative of strain 103(+).	Was obtained by serial passaging of *R.equi* 103(+) at 37°C for 2 months to facilitate loss of the virulence plasmid.	[Giguere *et al*. 1999a]
Rhodococcus equi A2(+)	Contains a 79kb plasmid and produces VapB.	From Takai Shinji, School of Veterinary Medicine and Animal Sciences, Kitasato University, Japan.	[Takai *et al*. 1995]
Rhodococcus equi A2(-)	Plasmid-cured VapB-negative isogen derivate of strains A2(+)	From Takai Shinji, School of Veterinary Medicine and Animal Sciences, Kitasato University, Japan.	[Takai *et al*. 1995]
Rhodococcus equi A11(+)	Contains a VapB-expressing plasmid.	From Takai Shinji, School of Veterinary Medicine and Animal Sciences, Kitasato University, Japan.	[Takai *et al*. 1995]

Strain	Characteristics	Source	References
Rhodococcus equi A11(-)	Plasmid-cured VapB-negative isogen derivate of strains A11(+).	From Takai Shinji, School of Veterinary Medicine and Animal Sciences, Kitasato University, Japan.	[Takai et al. 1995]
Escherichia coli DH5α (psc301 GFP)	Constitutive expressed GFP psc301	From Marco Polidori	[Cowley & Av-Gay 2001]
Rhodococcus equi ATCC 33701 (+) GFP	Constitutive expressed GFP psc301	From Marco Polidori	[Cowley & Av-Gay 2001]
Rhodococcus equi ATCC 103 (+) GFP	Constitutive expressed GFP psc301	From Marco Polidori	[Cowley & Av-Gay 2001]

2.12.2- Growth conditions

2.12.2.1- Growth of *Rhodococcus equi*

All *R.equi* strains were grown aerobically in brain heart infusion (BHI) broth at 37°C in a rotatory shaker at 190 rpm for 16 hours. *R.equi* to be used directly in experiments were grown at 37°C because temperatures of >32°C are necessary for expression of *VAP* genes [Takai *et al*. 1992]. *R.equi* 103/GFP and *R.equi* 33701/GFP were grown aerobically in BHI broth containing hygromycin B (300 µg/ml) in a rotatory shaker at 190 rpm for 16 hours. Rhodococcal cultures obtained were from early stationary phase (Optical density at 600 nm of approximately 3.0). Bacteria were then harvested by centrifugation at room temperature for 5 minutes at 8000 rpm and treated as described in the following sections. For *R.equi*/GFP, bacterial fluorescence was confirmed by fluorescence microscopy before the infection. All cultures that were not used directly in an experiement were incubated at 30°C, as increased temperatures can lead to a spontaneous loss of the VapA-expressing plasmid [Takai *et al*. 1994b].

2.12.2.2- Growth of *Escherichia coli*

E.coli was grown on BHI agar plates at 37°C. To be used directly in experiments, *E.coli* was grown aerobically in BHI broth at 37°C in a rotatory shaker at 190 rpm for 16 hours. *E.coli* psc301/GFP was grown aerobically in BHI broth containing hygromycin B (300 µg/ml) at 37°C in a rotatory shaker at 190 rpm for 16 hours. Bacteria were then harvested by centrifugation at room temperature for 5 minutes at 8000 rpm and treated as described in the following sections. For *E.coli* psc301/GFP, bacterial fluorescence was confirmed by fluorescence microscopy before the infection.

2.12.3- Measurement of bacterial numbers

Microbial cells scatter light striking them. Because bacteria in a population are of roughly constant size, the amount of light scattering from a bacterial culture is proportional to the concentration of bacterial cells present at low absorbance levels. To obtain the concentration of bacteria in a culture, 1 ml of the overnight culture was centrifuged at 8000 rpm for 5 minutes at room temperature, bacterial pellet was then resuspended with 1 ml PBS by vortexing and the optical density at 600 nm (OD_{600}) was determined using a spectrophotometer.

The life cell count was calculated assuming that:

1 OD_{600} =	1.5×10^8 bact/ml	For all the *Rhodococcus* species
1 OD_{600} =	2.0×10^8 bact/ml	For *Escherichia coli*

2.12.4- Treatment of bacteria before infection of macrophages

2.12.4.1- Fluorescent labeling of phagocytic probes

Bacteria surface or zymosan particle amines were directly surface labeled with succinimidyl esters of rhodamine or oregon green. For labeling, 2.6×10^7 zymosan particles were mixed with 1 µl of fluorescent dye (2 µg per µl water-free DMSO) and incubated for 30 minutes at 5°C in 200 µl of a sodium carbonate buffer (0,1 M, pH 8.2). $3,1 \times 10^7$ *R.equi* bacteria were mixed with 1,5 µl of dye (20 µg per µl water-free DMSO) and incubated for 30 minutes at 5°C in 200 µl of 0,1 M sodium carbonate buffer (pH 8.2). Particles were separated by a spin in a microfuge at 8.000 rpm for 5 min at ambient

temperature. 200 µl of 20 mM Tris-HCl (pH 8.0) were used to resuspend the pellet (modifier quenching), particles were extensively washed with PBS at ambient temperature and re-collected. Particles were finally suspended in 20 µl PBS and added to mammalian cells at the indicated MOIs or treated as described in the following sections.

2.12.4.2- Treatment with paraformaldehyde

1.0 x 10^8 *R.equi* bacteria (fluorescently labeled as described in section 2.12.4.1) were suspended in PBS containing 3% paraformaldehyde and incubated at room temperature for 30 minutes. The suspension was then centrifuged for 5 minutes at 8000 rpm and the bacterial pellet was washed one time with a solution of 50 mM NH_4Cl (to inactivate the formaldehyde groups) and three times with PBS. As reported by Lührmann *et al*. [2004], this condition killed 100% of the treated bacteria.

2.12.4.3- Treatment with heat

Bacterial pellets (containing 1.0-3.0 x 10^7 *R.equi* bacteria) in 1.5 ml microfuge tubes were placed in a heating block at 75°C for 15 minutes. Bacteria were washed 2 times with PBS and, when needed, labeled as described in section 2.12.4.1. As reported by Lührmann *et al*. [2004], this condition killed 100% of the treated bacteria.

2.12.4.4- Opsonization of phagocytic probes with antibodies

E.coli-GFP, *R.equi* and zymosan were opsonized as follows: bacterial or zymosan pellets were suspended with 20 µl of RPMI or DMEM complete medium containing specific antibodies against *E.coli* (1:10), *R.equi* (1:50), or zymosan (1:50). Suspensions were then incubated for 20 minutes at room temperature.

2.13- Analysis of RCVs in infected macrophages by transmission electron microscopy

Mammalian cells: J774E cells and BMMs
Phagocytic probes:

- *E.coli*
- *R. equi* 33701 (+) live
- *R. equi* 33701 (-) live
- *R. equi* 33701 (+) heat-killed

J774E cells or BMMs were grown in 60 mm diameter culture plates until they reached approximately 80% confluency at the growing conditions described in sections 2.11.2 and 2.11.5. Cells were infected with unlabeled bacteria at an MOI of 20 for 30 min at 37°C/7% CO_2 for BMMs and 37°C/5% CO_2 for J774E. After washing 3 times with PBS to remove non-internalised bacteria, infection was allowed to continue for 2 h for J774E and for up to 24 h for BMMs. When the infection was allowed to continue for 24 h, fresh culture medium was suplemented with 10 µg/ml gentamicin sulfate to prevent bacteria from multiplying in the media. The media was removed and the cells fixed *in situ* with a solution containing 2% formaldehyde and 0.25% glutaraldehyde in PBS overnight at 4°C. After fixation, the cells in culture plates were rinsed with distilled water and stained with a solution containing 1.5% potasium ferricyanide and 1% osmium tetroxide in distilled water at 4°C for 60 min, followed by extensive rinsing with distilled water. The samples were then incubated with 4% uranyl acetate in distilled water for 60 min at 4°C, followed by 3 min in distilled water. After incubating the dishes with 0.1% tannic acid in distilled water for 30 min at room temperature, cells were washed with distilled water, dehydrated using standard ethanol dilution series, scraped off the culture dishes with a rubber policeman and placed into a microfuge tube. The samples were embedded in Epon, the block was cut and thin sections were collected on nickel grids and examined in a Philips CM 120 transmission electron microscope.

2.14- Interaction of RCVs with fluid phase markers

Cell line: J774E

Phagocytic probes:

- Zymosan
- *R. equi* 33701 (+) live
- *R. equi* 33701 (-) live
- *R. equi* 33701 (+) heat-killed
- *R. equi* 33701 (+) formaldehyde-killed
- *R. equi* 103 (+) live
- *R. equi* 103 (-) live
- *R. equi* A2 (+) live
- *R. equi* A2 (-) live
- *R. equi* A11 (+) live
- *R. equi* A11 (-) live

2.14.1- Potential co-localization of lysosomal dextran-texas red and calcein with EEA-1, LAMP-1, and LAMP-2

To test whether only the late endocytic compartments of J774E cells were selectively prelabeled with dextran-texas red or calcein, immunofluorescence assays were performed which allowed analysis of co-localization of dextran-texas red or calcein with the late endosomal/lysosomal LAMP-1 and LAMP-2 and with the early endosomal EEA1.

J774E cells growing on cover slips in a well of a 24-well plate were incubated in 1 ml RPMI complete medium containing 0.2mg/ml dextran-texas red or 280 µM calcein at 37°C/5% CO_2 for 16 hours. After washing 3 times with PBS to remove excess fluid phase marker, the cover slips were incubated in label-free medium for 1 hour (for calcein) or 3 hours (for dextran-texas red) at 37°C/5% CO_2 to chase the marker into lysosomes. Cover slips were then washed 3 times with PBS and fixed in 3% formaldehyde in PBS for 30 minutes at room temperature. After washing 3 times with PBS, free aldehyde groups were quenched with 50 mM NH_4Cl for 15 minutes at room temperature. Glass slides were then washed 3 times with PBS, carefully removed from the 24-well plates and placed on a parafilm surface with 50 µl of the blocking and permeabilization buffer (0.1% saponin, 5% donkey serum in PBS) for 60 minutes at room temperature in a humid chamber. Next, the slides were serially incubated for 60 minutes at room temperature with primary and secondary antibodies each diluted in the blocking and permeabilization solution, mounted on glass microscopic slides using mounting medium and examined using CLSM, as described in section 2.14.2. Co-localization was considered when the signal coming from the antibodies totally covered the fluorescence coming from the dextran-texas red or calcein, appearing as bright yellow spots.

Primary antibodies were diluted in the blocking and permeabilization solution as follows:
Anti-LAMP1 1: 200, anti-EEA1 1: 50, and anti-LAMP2 1:10.
Secondary antibodies were diluted 1:400 in the blocking and permeabilization solution.

2.14.2- CLSM analysis

The labeled preparations were analysed using Confocal Laser Scanning Microscope LSM 510 equipped with a 63 x 1.4 oil immersion objective. Images were further processed with Abode

Photoshop software. When co-localization of RCVs with different endocytic markers was analysed, percentages of co-localization were obtained with a minimum of 50 phagosomes counted per experiment and sample.

2.14.3- Fluid phase labeling of lysosomes and analysis of interaction of lysosomal dextran-texas red and calcein with RCV

J774E cells on cover slips in a well of a 24 well plate were incubated in 1 ml medium containing 280 μM calcein (MW 622 Da) or 0,2 mg/ml dextran-texas red (MW 10,000 Da) at 37°C / 5% CO_2 for 16 hours. After washing three times with PBS to remove excess fluid phase marker, the cover slips were incubated in label-free medium for 1 h (for calcein) or 3 h (for dextran-texas red) at 37°C / 5% CO_2 to chase the marker into lysosomes. Cells were infected for 30 min with the pre-labeled bacteria or zymosan at MOI 20, whereas the MOI with heat-killed bacteria was 30. After washing 3 times with PBS to remove non-internalised bacteria, the cells were incubated for 2 hours (37°C/5% CO_2). Samples were washed 3 times with PBS and fixed in 3% formaldehyde in PBS for 30 minutes at room temperature. After washing 3 times with PBS, free aldehyde groups were quenched with 50 mM NH_4Cl for 15 minutes at room temperature. Glass slides were then washed 3 times with PBS, mounted on glass microscopic slides using mounting medium and examined using CLSM, as described in section 2.14.2. The presence of a fluorescent ring surrounding the bacteria was considered a positive signal. The sample was considered positive also when the fluorescent signal coming from the fluid phase markers totally coincided with the bacteria.

2.14.4- Addition of the fluid phase marker dextran-texas red after establishing an infection

J774E cells on cover slips in a well of a 24 well plate were infected for 30 min with the pre-labeled bacteria or zymosan at MOI 20, whereas the MOI with heat-killed bacteria was 30. After washing 3 times with PBS to remove non-internalised bacteria, the cells were incubated for 2 hours (37°C/5% CO_2). Cells were then washed 3 times with PBS and were incubated with 1 ml medium containing 0.5 mg/ml dextran-texas red at 37°C/5% CO_2 for 4 hours. Samples were washed 3 times with PBS and fixed in 3% formaldehyde in PBS for 30 minutes at room temperature. After washing 3 times with PBS, free aldehyde groups were quenched with 50 mM NH_4Cl for 15 minutes at room temperature. Glass

slides were then washed 3 times with PBS, mounted on glass microscopic slides using mounting medium and examined using CLSM, as described in section 2.14.2. The presence of a red ring surrounding the green labeled bacteria/zymosan was considered a positive signal.

2.15- Qualitative assessment of acidification of RCVs

Mammalian cells: J774E
Phagocytic probes:

- Zymosan
- *R.equi* 33701 (+)/GFP
- *R.equi* 103 (+)/GFP
- *R.equi* 103 (-)/GFP
- *E. coli* psc301/GFP (antibody opsonized).

2.15.1- Inhibition of phagosomal acidification by bafilomycin A

To determine whether only acidified compartments were labeled with LysoTracker, control experiments were performed using J774E cells infected with *E.coli* labeled with GFP or zymosan labeled with oregon green and the proton ATPase (vATPase) inhibitor bafilomycin A_1. For this purpose, J774E cells growing on cover slips in a well of a 24 well plate were infected for 30 min with the *E.coli*/GFP or zymosan at MOI 20. After washing 3 times with PBS to remove non-internalised bacteria, the cells were incubated for 1 hour (37°C/5% CO_2). RPMI complete medium containing 0.25 µM Bafilomycin A_1 was added to the infected cells, followed by incubation for 1 h at 37°C/5% CO_2. The medium was then removed and 1 ml of fresh medium containing LysoTracker (diluted 1: 10000) and 0.25 µM bafilomycin A was added to each well. Plates were then incubated for 30 min at 37°C/5% CO_2, washed 3 times with PBS, mounted on microscope slides using agarose (low melting point) and examined immediately using CLSM (section 2.14.2).

To mount the cover glasses for CLSM analysis, 1 g of agarose were disolved in 7 ml of PBS at 50°C. The solution was diluted 1:2 in PBS (at 50°C). 10 µl of the solution were placed on slides, and the cover glasses containing the cell monolayers were placed over the solution on the slides and examined immediately.

2.15.2- Co-localization of RCVs with LysoTracker

Acidification of phagosomes was assesed by use of the acidotropic dye LysoTracker Red DND-99. J774E cells cultivated on cover slides in 24-well tissue culture plates were infected for 30 minutes with GFP-expressing bacteria or with pre-labeled zymosan at MOI 20. After washing 3 times with PBS to remove non-internalised bacteria, the cells were incubated for 2 hours at 37°C/5% CO_2. The medium was then removed and the plates were incubated with 1 ml of RPMI complete medium containing LysoTracker (diluted 1:10.000) for 30 min at 37°C/5% CO_2, washed with PBS, mounted on microscope slides using agarose low melting point (section 2.15.1), and examined immediately using CLSM as described in section 2.14.2. An acidic pH in the phagosomes was revealed by red fluorescent labeling surrounding or totally covering the green bacteria inside the phagosomes.

2.16- Acquisition of endocytic markers by RCVs

Mammalian cells: J774E, BMMs
Phagocytic probes:

- Zymosan
- *R.equi* 33701 (+) live
- *R.equi* 33701 (-) live
- *R.equi* 33701 (+) heat-killed
- *E.coli* live

2.16.1- Kinetics of acquisition of endocytic markers by RCVs

J774E cells or BMMs were grown on cover slides in 24-well tissue culture plates at the growing conditions described in sections 2.11.2 and 2.11.5 until they reached approx. 80% confluency. For infection, prelabeled bacteria or zymosan were added at MOI 10 for J774E cells or at MOI 5 for BMMs, whereas the MOI with heat-killed bacteria was 20 for J774E and 10 for BMMs. Infection was made at 4°C and the plates were centrifuged at 1200 rpm for 30 minutes at 4°C. The centrifugation step allows the bacteria to make close contact with the mammalian cells and helps in synchronization of the infection. The medium was withdrawn and replaced for fresh warm medium at 37°C, being that set as 0 min. After incubating the cells at 37°C for 5, 15, 60, and 120 minutes (for EEA1), for 10, 30, 60, and 120 minutes (for LBPA), or for 10, and 180 minutes (for LAMP-1, LAMP-2, TfR and vATPase), infected cells were fixed for 30 minutes in 3% formaldehyde in PBS, followed by quenching the fixative with 50 mM NH_4Cl in PBS for 15 minutes at ambient temperature. Glass slides were then incubated with the

blocking and permeabilizing solution (0.1% saponin and 5% donkey serum in PBS) for 60 minutes. Next, the slides were serially incubated for 60 minutes at room temperature with primary and secondary antibodies each diluted in the blocking and permeabilizing solution and were mounted on glass microscopic slides using mounting medium. The labeled preparations were analysed using CLSM as described in section 2.14.2. In all cases, the presence of a green fluorescent ring surrounding the red labeled bacteria was considered a positive signal. Phagosomes were considered positive for LBPA (present on vesicles within organelles) when the green antibody fluorescence at least partially co-localised with red labeled bacteria. In addition, all immunolabelings were specific, as confirmed through testing whether bacteria outside host cells were negative for antibody stain.

Primary antibodies were diluted in the blocking and permeabilization solution as follows:
Anti-LAMP1 1: 250, anti-LAMP-2 1: 5, anti-EEA1 1: 50, anti-LBPA 1:50, anti-vATPase 1: 40, and anti-TfR 1: 50.
Secondary antibodies were diluted 1:50 in the blocking and permeabilization solution.

Before labeling the samples, the rabbit polyclonal antibodies against vATPase were preabsorved with *R.equi* as follows:
Approximately 10^8 *R.equi* bacteria were placed into 1.5 ml tube and harvested by centrifugation at room temperature for 5 minutes at 8 000 rpm. After removing the supernatant, antibodies against vATPase diluted in the blocking and permeabilization solution were given to the bacterial pellet, suspended by vortexing and incubated for 20 minutes at room temperature. The suspension was then centrifuged at room temperature for 5 minutes at 8000 rpm and the pellet was discharged. This procedure was repeated twice.

2.16.2- Experiments involving experimental infection for 24 hours

BMMs were grown on cover slides in 24-well tissue culture plates at the growing conditions described in sections 2.11.5 until they reached approx. 80% confluency. Cells were infected with unlabeled *R.equi* at MOI 4 for 1 hour at 37°C/7% CO_2. After washing 3 times with PBS to remove non-internalised bacteria, the medium was replaced with DMEM containing 150 µg gentamicin/ml and the slides were incubated for 1 h at 37°C/7% CO_2. The medium was then replaced with DMEM containing 10 µg gentamicin/ml and the cells were incubated for 24 hours at 37°C/7% CO_2. The samples were

washed 3 times with PBS, fixed, and processed for immunolabeling as described above (section 2.16.1). Bacteria were stained with polyclonal antibody specific for *R.equi* serotype 1 (dilution 1:50), or with 3 µl propidium iodide/ 500 µl PBS and 0.1% saponin for 15 minutes.

2.17- Exogenously administered transferrin as a marker for sorting and recycling endosomes

Mammalian cells: BMMs
Phagocytic probes:

- Zymosan
- *R.equi* 33701 (+) live
- *R.equi* 33701 (-) live
- *R.equi* 33701 (+) heat-killed

2.17.1- Co-localization of transferrin-alexa fluor with antibodies against transferrin receptor and LAMP-1

To analyse the behaviour of transferrin-alexa fluor, cover slides containing BMMs were carefully placed on a parafilm surface and incubated with a solution containing 50 µg/ml Alexa 488-fluor conjugated transferrin and 5 mg/ml unlabeled holotransferrin in Ringer´s solution for 20 minutes at 5°C, following by 10 minutes incubation at 37°C. Before use, the transferrin solution was briefly cleared by centrifugation in a microfuge to remove potentially present aggregates. The cover slides were then placed on a 24-well culture plate, washed 3 times with PBS, and fixed for 30 minutes in 3% formaldehyde in PBS, followed by quenching the fixative with 50 mM NH_4Cl in PBS for 15 minutes at ambient temperature. The samples were then mounted on glass microscopic slides using mounting medium and analysed using CLSM as described in section 2.14.2.

To study the location of transferrin-alexa fluor within BMMs, immunofluorescence assays were performed to visualized TfR and LAMP-1. For this purpose, cover slides containing BMMs were carefully placed on a parafilm surface and incubated with a solution containing 50 µg/ml of Alexa 488-fluor conjugated transferrin in Ringer´s solution for 20 min at 5°C, following by 10 minutes incubation at 37°C. Before use, the transferrin solution was briefly cleared by centrifugation in a microfuge to remove potentially present aggregates. The cover slides were then placed on a 24-well plate, washed 3 times with PBS, and fixed for 30 minutes in 3% formaldehyde in PBS, followed by quenching the

fixative with 50 mM NH_4Cl in PBS for 15 minutes at ambient temperature. Glass slides were then incubated with the blocking and permeabilizing solution (0.1% saponin and 5% donkey serum in PBS) for 60 minutes. Next, the slides were serially incubated for 60 minutes at room temperature with primary and secondary antibodies each diluted in the blocking and permeabilizing solution and were mounted on glass microscopic slides using mounting medium. The labeled preparations were analysed using CLSM as described in section 2.14.2. A sample was considered positive when the red signal coming from the antibodies totally covered the green fluorescence coming from transferrin-alexa fluor, appearing as bright yellow spots.

Primary antibodies were diluted in the blocking and permeabilization solution as follows:
Anti-LAMP1 1: 250 and anti-TfR 1: 50.
Secondary antibodies were diluted 1:50 in the blocking and permeabilization solution.

2.17.2- Access of RCV to exogenously administered transferrin

BMMs were grown on cover slides in 24-well tissue culture plates at the growing conditions described in sections 2.11.5 until they reached approx. 80% confluency. Cells were infected for 30 minutes with prelabeled bacteria or zymosan at MOI 20 (37°C/7% CO_2), whereas the MOI with heat-killed bacteria was 30. After washing 3 times with PBS to remove non-internalised bacteria, the cells were incubated for 2 hours at 37°C/7% CO_2. The cover slides containing BMMs were carefully placed on a parafilm surface and were then incubated with 50 μg Alexa 488-fluor conjugated transferrin in 1 ml Ringer´s solution for 20 min at 4°C, followed by 10 min incubation at 37°C. Before use, the transferrin solution was briefly cleared by centrifugation in a microfuge to remove potentially present aggregates. The cover slips were placed on the 24-well plate, washed 3 times with PBS and fixed in 3% formaldehyde in PBS for 30 minutes at room temperature. After washing 3 times with PBS, free aldehyde groups were quenched with 50 mM NH_4Cl for 15 minutes at room temperature. Glass slides were then washed 3 times with PBS, mounted on glass microscopic slides using mounting medium and examined using CLSM, as described in section 2.14.2. The presence of a green fluorescent ring surrounding the red labeled bacteria was considered a positive signal.

2.18- Compartment analysis of RCVs using EGFP fusion proteins

Mammalian cells: Raw 264.7 and CHO FcRγII

Phagocytic probes:

- Zymosan
- *R.equi* 33701 (+) live
- *R.equi* 33701 (-) live
- *R.equi* 33701 (+) heat-killed

2.18.1- Plasmids

EGFP-fusion protein	Description	Source	Reference
Rab4/EGFP	Ligated into the vector pCMV EGFP-C3 (Clontech), kan^r and neo^r.	Marino Zerial, Dresden, Germany	[Bucci *et al*. 2000, Sönnichsen *et al*. 2000]
Rab11/EGFP	Ligated into the vector pCMV EGFP-C3 (Clontech), kan^r and neo^r.	Marino Zerial, Dresden, Germany	[Bucci *et al*. 2000, Sönnichsen *et al*. 2000]
Rab7/EGFP	Ligated into the vector pCMV EGFP-C3 (Clontech), kan^r and neo^r.	Marino Zerial, Dresden, Germany	[Bucci *et al*. 2000, Sönnichsen *et al*. 2000]
Rab5/EGFP	Ligated into the vector pCMV EGFP-C3 (Clontech), kan^r and neo^r.	Marino Zerial, Dresden, Germany	[Bucci *et al*. 2000, Sönnichsen *et al*. 2000]
LAMP-1/EGFP	kan^r and neo^r.	Antoine Galmiche, Max Planck Institute for Infection Biology, Berlin, Germany	[Lebrand *et al*. 2002]
Transferrin receptor/EGFP	Ligated into the vector pJPA5, amp^r and neo^r.	Gary Banker, Oregon Health Science University, Portland, USA	[Silverman *et al.* 2001]

2.18.2- Transformation of competent *E.coli*

1 ml tubes containing aliquots of competent *E.coli* were thawed on ice and 2 µl of the DNA plasmid suspension were given to each tube. After incubating for 30 min at 4°C, the tubes were incubated 1 min at 42 °C, following by 2 minutes incubation at 4°C. 1 ml of LB-medium was given to each tube and the bacteria were incubated 1 hour at 37 °C in a rotatory shaker at 190 rpm. Bacteria were harvested by centrifugation at room temperature for 5 minutes at 8 000 rpm, 100 µl of the pellet were then plated onto LB agar plates with kanamycin (50 µg/ml) or ampicillin (100 µg/ml), and the plates were incubated overnight at 37°C. To isolate the plasmids, 1 colony was picked from the plates, placed in 30 ml of LB medium containing kanamycin (50 µg/ml) or ampicillin (100 µg/ml), and incubated overnight at 37 °C in a rotatory shaker at 190 rpm. The plasmids were then isolated as described in section 2.18.3. To store transformed bacteria, 1 colony was picked from the plates, placed in tubes with 5 ml of LB medium containing kanamycin (50 µg/ml) or ampicillin (100 µg/ml), and incubated overnight at 37°C rotatory shaker at 190 rpm. Glycerin (20%) was given to the bacterial suspension. The suspension was then aliquoted and kept at –80°C.

2.18.3- Isolation of the plasmids

The plasmids were isolated by using Midiprep kit (Sigma) as follows:

30 ml of an overnight bacterial culture of transformed E.coli (section 2.18.2) were harvested by centrifuging at 1780 g for 10 minutes at room temperature. All the media supernatant was removed and the bacterial pellet was completely suspended with 1.2 ml of Resuspension Solution by pipetting up and down. The resuspended bacterial cells were then lysed by adding 1.2 ml of lysis solution, following by immediately mixing of the contents by gentle inversion (6-8 times). The mixture was then incubated at room temperature until becomes clear and viscous. The lysis solution was neutralized by adding 0.8 ml of neutralization solution to the lysate. The contents were then immediately mixed thoroughly by gentle inversion and the cell debris was pelleted by centrifuging at 15000 g for 15 minutes at 2-8°C. To remove endotoxins, the cleared lysate was transfered into a 15 ml conical tube and 300 µl of the endotoxin removal solution were added to the lysate. After mixing thoroughly by inversion, the tube was chilled on ice until the solution became light blue and clear and the tube was then warmed in a 37°C water bath for 5 minutes (the solution turned cloudy). To separate the phases, the tube was then centrifuged at 1780 g in a swinging bucket rotor for 5 minutes at room temperature. The clear upper phase contains plasmid DNA and the blue lower phase contains endotoxins. The

clear upper phase containing the plasmid DNA was carefully transfered into a fresh 15 ml conical tube and the blue lower phase was discarded. When needed, the procedure to remove endotoxins was repited before the plasmid DNA was isolated. To isolate the plasmid DNA, 0.8 ml of the DNA binding solution were added to the endotoxin-free lysate and the contents were mixed thoroughly by inversion or vortexing. The lysate was then loaded into a GenElute Midiprep binding column seated in a collection tube, centrifuged in a swinging bucket rotor at 1780 g for 3 minutes, and the flow-through liquid was discarded. The column containing plasmid DNA was then washed by adding 2.0 ml of the optional wash solution, following by centrifuging in a swinging bucket rotor at 1780 g for 3 minutes. The flow-through liquid was then discarded and 3 ml of diluted Wash Solution were added to the column.The column was then centrifuged in a swinging bucket rotor at 1780 g for 5 minutes and the flow-through liquid was discarded. To elute the plasmid DNA, the column was transfered to a fresh collection tube and 1 ml of endotoxin free water was added to the column, following by centrifuging in a swinging bucket rotor at 1780 g for 3-5 minutes. The flow-through liquid contains the plasmid DNA. Recovery and purity of the plasmid DNA were determined by spectrophotometric analysis and the plasmids were aliquoted and stored at –20°C.

2.18.4- Transient transfection of Raw 264.7 cells

To obtain a confluent monolayer of metabolic active cells, a suspension of Raw 264.7 cells in RPMI complete medium were deposited into 24-well culture plates with 12 mm steril glas coverslips at a density of 2.0 X 10^5 cells/well, and incubated for 24 hours at 37°C/5% CO_2. Cells were transient transfected by using JetPEI-Man transfection reagent as follows:

1 µg of the isolated plasmid DNA (section 2.18.3) was placed into a tube containing 50 µl of 150 mM NaCl, and gently mixed. 3.2 µl of transfection reagent jetPEI-Man were placed into a tube containing 50 µl of 150 mM NaCl, and gently mixed. The two solutions were then mixed by adding 50 µl of the jetPEI-Man solution to the tube containing the plasmid DNA solution, and the mixture was incubated for 20 minutes at room temperature with the purpose of forming transfection reagent/plasmid DNA complexes. 100 µl of transfection reagent/plasmid DNA mixture were added dropwise to each well containing Raw 264.7 cells and the mixture was homogenized by gently swirling. After centrifuging the culture plates at 1200 rpm for 5 minutes to bring the complexes in contact with the cells, culture plates were incubated at 37°C/5% CO_2 for 24 hours. Transfection complexes were then removed from the

culture plates by washing 3 times with PBS, and cells were incubated with RPMI complete medium at 37°C/5% CO_2 for 24 hours.

2.18.5- Stabil transfection of Raw 264.7 cells

To obtain a confluent monolayer of metabolic active cells, a suspension of Raw 264.7 cells in RPMI complete medium were deposited into 24-well culture plates with 12 mm steril glas coverslips at a density of 2.0 X 10^5 cells/well, and incubated 24 hours at 37°C-5% CO_2. Cells were transient transfected as described in section 2.18.4. The transfected cells were incubated in RPMI complete medium containing 500 µg/ml geneticin for 1 week at 37°C/5% CO_2. The transfected cells were sorted 3 to 5 times by using FACSscan (Beckon Dickinson). Cells were incubated in RPMI complete medium containing 500 µg/ml geneticin and subcultured every 2 days, at a split ratio of 1:3.

2.18.6- Transient transfection of CHO FcRγII cells

To obtain a confluent monolayer of metabolic active cells, a suspension of CHO FcRγII cells in DMEM complete medium were deposited into 24-well culture plates with 12 mm steril glas coverslips at a density of 1.0 X 10^5 cells/well, and incubated 24 hours at 37°C/7% CO_2. 3 µl of fugene 6 were placed into a small sterile tube containing serum-free DMEM, to a total volume of 100 µl. The solution was gently mixed. After adding 1 µg of the isolated plasmid DNA (sections 2.18.3), the solution was gently mixed and incubated for 20 minutes at room temperature to allow the transfection reagent/plasmid DNA complexes to form. 100 µl of the transfection reagent/DNA mixture was then added dropwise to each well containing CHO FcRγII cells, and the plates were incubated at 37°C, 7% CO_2 for 24 hours. After removing the complexes by washing 3 times with 1x PBS, 1 ml of DMEM complete medium was given to each well and the plates were incubated for 24 hours at 37°C/7% CO_2.

2.18.7- Co-localization of EGFP-fusion proteins with endocytic markers

To study the distribution EGFP-labeled proteins within transfected cells, immunofluorescence assays were performed to visualize the endocytic markers TfR, LAMP-1, and EEA-1.

Monolayers of Raw 264.7 cells growing on 12 mm cover glasses in 24-well plates were transient transfected as described in section 2.18.4. Cells were washed 3 times with PBS, fixed for 30 minutes in 3% formaldehyde in PBS, followed by quenching the fixative with 50 mM NH_4Cl in PBS for 15 minutes at ambient temperature. Glass slides were then incubated with the blocking and permeabilizing solution (0.1% saponin and 5% donkey serum in PBS) for 60 minutes. The slides were serially incubated for 60 minutes at room temperature with primary and secondary antibodies each diluted in the blocking and permeabilizing solution and were mounted on glass microscopic slides using mounting medium. The labeled preparations were analysed using CLSM as described in section 2.14.2. A sample was considered positive when the red signal coming from the antibodies totally covered the green fluorescence coming from the EGFP-fusion proteins, appearing as bright yellow spots.

Primary antibodies were diluted in the blocking and permeabilization buffer as follows:
Anti-LAMP1 1: 200, anti-EEA1 1: 50, and anti-TfR 1:50.
Secondary antibodies were diluted in the blocking and permeabilization buffer 1: 50.

2.18.8- Acquisition of EGFP-fusion proteins by RCVs

Mammalian cells: Raw 264.7 cells, CHO FcRγII cells.
Phagocytic probes:

- Zymosan
- *R.equi* 33701 (+) live
- *R.equi* 33701 (-) live
- *R.equi* 33701 (+) heat-killed

Monolayers of Raw 264.7 or CHO FcRγII cells growing on 12 mm cover glasses in 24-well plates were transient transfected as described in sections 2.18.4 and 2.18.6. The transfected cells were infected with pre-labeled bacteria at MOI 5 (for Raw 264.7 cells) of MOI 7 (for CHO FcRγII cells) for 30 min at 4°C with centrifugation of 160 x g. After infection, media were removed and macrophages incubated in fresh media at 37°C/5% CO_2 (for Raw 264.7 cells) or at 37°C/7% CO_2 (for CHO FcRγII cells) for up to 120 min. Cells were washed 3 times with PBS, fixed for 30 minutes in 3% formaldehyde in PBS, followed by quenching the fixative with 50 mM NH_4Cl in PBS for 15 minutes at ambient temperature. Glass slides were then washed 3 times with PBS, mounted on glass microscopic slides using

mounting medium and examined using CLSM, as described in section 2.14.2. The presence of a green fluorescent ring surrounding the red labeled bacteria was considered a positive signal.

Before infecting CHO FcRγII cells, *R.equi* and zymosan were opsonized with antibodies as follows: Pre-labeled *R.equi* was suspended in DMEM complete medium containing rabbit polyclonal antibodies against *R.equi* (dilution 1:500) and incubated 15 minutes at room temperature. Pre-labeled zymosan was incubated with antibodies against yeast diluted 1:500 in DMEM complete medium for 15 minutes at room temperature.

2.18.9- Kinetics of acquisition of PtdIns3P by RCVs

Mammalian cells: Raw 264.7 PX1 (p40phox).
Phagocytic probes:

- Zymosan
- *R.equi* 33701 (+) live
- *R.equi* 33701 (-) live
- *R.equi* 33701 (+) heat-killed

Raw 264.7 PX1 cells on cover slips in a well of a 24 well plate were infected for 30 min with the pre-labeled bacteria or zymosan at MOI 10 for 30 min at 4°C with centrifugation of 160 x g, whereas the MOI with heat-killed bacteria was 20. After infection, media were removed and macrophages incubated in fresh media at 37°C/5% CO_2 for up to 120 minutes. Cells were washed 3 times with PBS, fixed for 30 minutes in 3% formaldehyde in PBS, followed by quenching the fixative with 50 mM NH_4Cl in PBS for 15 minutes at ambient temperature. Glass slides were then washed 3 times with PBS, mounted on glass microscopic slides using mounting medium and examined using CLSM, as described in section 2.14.2. The presence of a green fluorescent ring surrounding the red labeled bacteria was considered a positive signal.

3- Results

Interactions between infectious agents and phagocytic cells are crucial for the development of an infection. The fate of the intracellular pathogens relies on their ability to establish an intracellular niche where they can obtain nutrients and also hide from other components of the immune system. In this work, the intracellular location of the facultative intracellular bacterium *Rhodococcus equi* was analysed using different microscopical methods. Determining the intracellular compartmentation of *R.equi* may help to understand the mechanisms of survival of this pathogen as well as the physiopathology of *R.equi* infection and also gives insight to the interactions between immune system and intracellular pathogens.

In the first part of this study, ultrastructural events after macrophage ingestion of *R.equi* were studied, using transmission electron microscopy. Aim of this section was to observe the morphology of *R.equi*-containing vacuoles (RCVs) over time and to address whether *R.equi* could eventually escape from its phagosome. In addition, potential association of RCVs with other intracellular organelles was also analysed. Because of its high resolution, transmission electron microscopy can reveal the phagosome membrane and possible association of the bacteria-containing phagosomes with intracellular organelles can be determined following the continuity of the phagosome membrane that surrounds the ingested bacterium. Moreover, electron microscopy allows the study of morphological changes in the phagosomes enclosing ingested bacterium, and also possible damage in the infected host cell.

In the second part of this study, the precise compartmentation of *R.equi* within murine macrophages was analysed using co-localization fluorescence assays. For this purpose, acquisition of different endocytic markers by RCVs was observed using soluble tracers which label specific compartments of the endocytic pathway, immune detection of endocytic marker molecules, and also non-immune detection with Enhanced Green Fluorescent Protein (EGFP) fusion proteins.

3.1- Analysis of RCVs in infected macrophages by transmission electron microscopy

Previous studies have shown that, within primary foal alveolar macrophages, *R.equi* persisted and multiply within membrane-surrounded compartments, with the phagosomal membrane widely separated from the bacteria [Zink *et al*. 1987, Hietala & Ardans 1987]. In order to analyze the development of RCVs over time, transmission electron microscopy of infected J774E cells and primary BMMs was performed. J774E, a strongly mannose receptor-expressing mouse macrophage cell line, was chosen as a model to elucidate *R.equi*-macrophage interactions because this cell line possesses characteristics typical of macrophages [Ralph *et al*. 1975] and, as the cell line J774.A1, has been used to study intracellular survival of pathogenic bacteria [McDonough *et al*. 1993, Via *et al*. 1997, Via *et al*. 1998, Lührmann *et al.* 2001]. As reported by Lührmann *et al* (2004), virulent *R.equi* is toxic for J774E macrophages, leading to cell detachment from the culture dishes and death of the macrophages by necrosis after 6-28 hours. This feature makes J774E cells unsuitable for assays that require long periods of infection (e.g. 24 hours). Because of this, RCVs were also analyzed in primary BMMs which have been shown to be a good model to study the interactions between *R.equi* and its host cells [Darrah *et al*. 2000, Jain *et al*. 2003], and allow rhodococal infection for 24 hours (Tobias Sydor, personal communication).

As described in section 2.13, J774E or BMMs were infected with unlabeled bacteria for 30 minutes and, after washing to remove non-internalized bacteria, infection was allowed to continue for 2 hours for J774E and for up to 24 hours for BMMs. The samples were then fixed and processed for electron microscopy. Ultrathin sections were cut and examined using transmission electron microscopy.

At 2 hours postinfection, all *R.equi* organisms ingested by J774E cells and BMMs were found in membrane-surrounded compartments which contain one or two bacteria. Approximately 50% each of the RCVs possessed either tightly apposed membranes or loosely fitting membranes that contained numerous internal vesicles, with vesicles being either electron-dense or electron-translucent (Figure 3A). This result is in agreement with previous data using alveolar macrophages, where the majority of ingested *R.equi* was observed within “loose phagosomes” with the phagosomal membrane widely separated from the bacteria [Zink *et al*. 1987]. In contrast to that observed with viable *R.equi*, the majority of phagosomes containing non-viable *R.equi* or non-pathogenic *Escherichia coli* exhibited

tightly apposed membranes. The different phagosome phenotypes observed between *R.equi* (“loose phagosomes”) and the non-pathogenic *E.coli* (tightly apposed membranes), which normally undergoes an undisturbed phagosome maturation; suggest that the “loose phagosomes” with vesicular material may reflect a different compartmentation of RCVs.

At 24 hours postinfection in BMMs, the majority of *R.equi* was still retained within phagosomes, although some bacteria were found free in the cytoplasm of damaged macrophages. Most *R.equi* was morphologically intact and resided in very spacious, multilobed vacuoles with uneven shape and many internal membranes and vesicles, similar to the vesicles observed in multivesicular bodies (Figure 3B). In contrast with that observed at 2 hours postinfection, 24 hours RCVs were communal vacuoles, usually containing more than two bacteria. Clear signs of bacterial multiplication were never observed, not even after 24 hours postinfection.

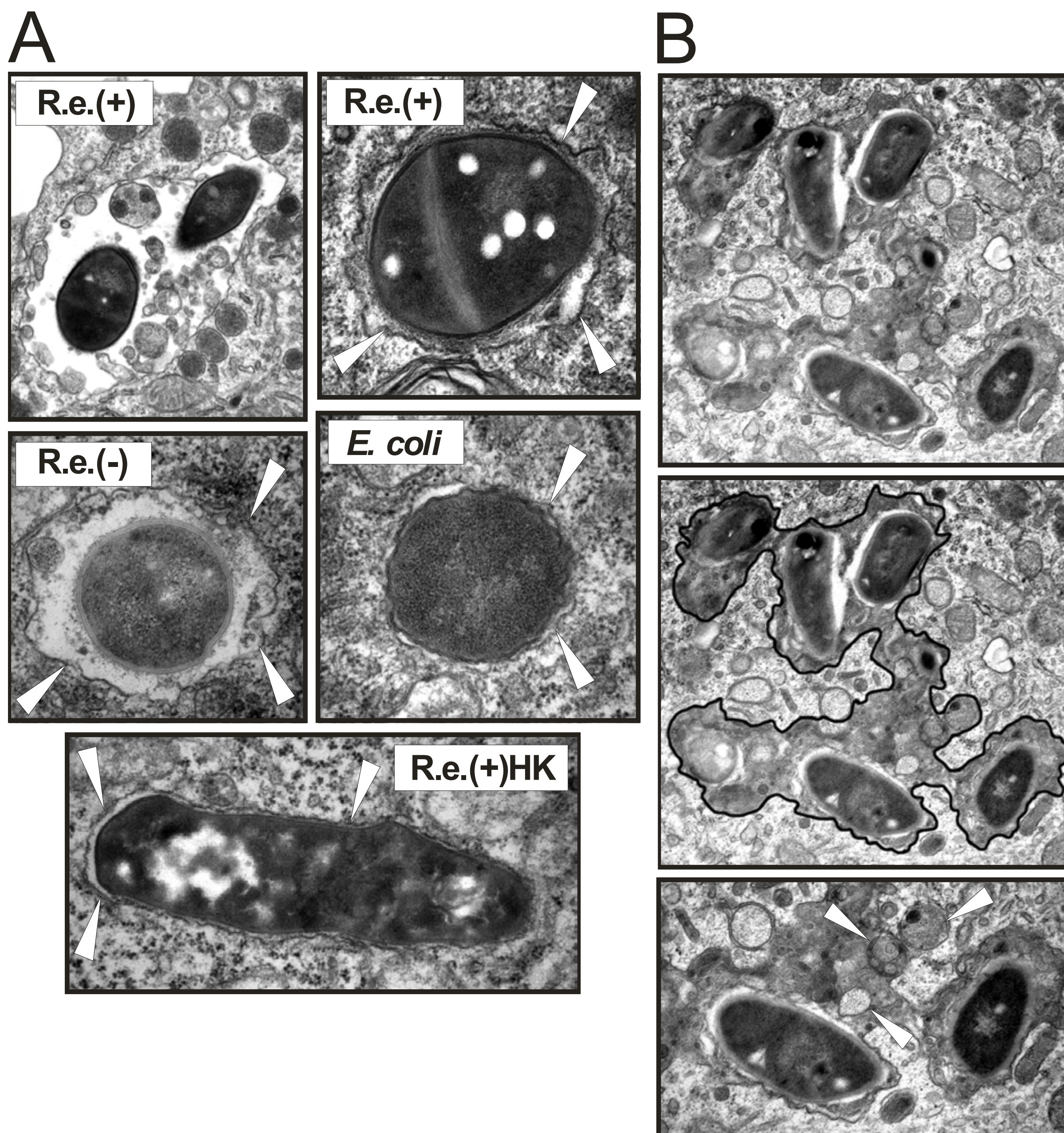

Figure 3: Transmission electron microscopy of *R. equi*-infected cells at various times after ingestion of the bacteria. A) J774E cells were infected with the indicated phagocytic probes for 30 min followed by a 2 h chase in the absence of added particles, then fixed and prepared for electron microscopy. Open arrows point at phagosome limiting membranes. (B) BMMs infected with *R. equi*(+) for 30 min, followed by a 24 h chase and sample preparation as above. Top, communal vacuole, middle: as in top but with computer-assisted marking of the vacuole limited membrane, bottom: detail of top frame with open arrows indicating some of the internal membrane vesicles present in the sample. Phagocytic probes: live *R.equi* 33701+ [R.e.(+)], live *R.equi* 33701- [R.e.(-)], heat-killed *R.equi* 33701+ [R.e.(+)HK], *Escherichia coli* [*E.coli*].

3.2- Interaction of RCVs with fluid endocytic markers

Endosomal compartments of host cells can be marked using fluid phase molecules such as dextran or calcein. These fluid phase tracers are ingested by pinocytosis and transported through the endocytic pathway, where they label different stages of the endocytic pathway. Which endosomal compartment is labeled using these tracers depends on the pulse/chase time periods. Thus, labeling of lysosomes requires at least a 2 h pulse, followed by 2-4 h chase period, whereas long time pulse without chase labels the full early to late endosomal continuum [Schaible & Kaufmann 2002].

Many intracellular pathogens are able to inhibit maturation of their phagosomes and establish vacuoles that interact with the endocytic pathway, conditions that enable the bacteria to take up nutrients for survival and multiplication. Other intracellular pathogens, in contrast, establish vacuoles that are disconnected from the endocytic pathway, but usually maintain interactions with other intracellular organelles, which provide these parasites with nutrients. In order to study whether *R.equi* is able to block maturation of its phagosome and establish vacuoles that maintain communication with the endocytic pathway, the interaction of RCVs with the fluid phase endocytic markers calcein and dextran-texas red was examined in the following sections, using two different approaches. In the first approach, fluid phase markers (dextran-texas red or calcein) were used to label the lysosomes of J774E macrophages, following by infection with prelabeled bacteria. This approach was intended to answer whether the phagosomes containing *R.equi* mature into phagolysosomes. In the second approach, J774E macrophages were first infected with the prelabeled bacteria, and the endocytic marker dextran-texas red was then used to label the whole endocytic pathway. This assay was performed to test whether RCVs are able to interact with the whole endocytic pathway.

The hydrophilic membrane impermeant polysaccharide dextran-texas red (MW 10.000 Da) was chosen as an endocytic marker because, once taken up by fluid phase endocytosis, is transported through the endocytic pathway and delivered into lysosomes [Geisow *et al*. 1984, Swanson 1989, Wiater *et al*. 1998]. Its biologically uncommon α-1.6-polyglucose linkages are resistant to cleavage by most endogenous cellular lysosomal glycosidases, making it an ideal long term tracer for live cells. Moreover, lysine residues are incorporated into the dextran molecule, which makes it useful for applications that require fixation with aldehydes. The second fluid phase marker, calcein (MW 622 Da), is a polyanionic membrane-impermeant fluorescein derivative commonly used to study the

interactions of late endocytic compartments with phagosomes [Oh & Straubinger, 1996; Kuehnel *et al*. 2001].

Phagosomes containing baker´s yeast cell wall fragments (zymosan) were used as control phagosomes because they mature normally [Oh & Straubinger 1996, Wang & Goren 1987, Düzgünes *et al*. 1993, Lührmann *et al*. 2001].

3.2.1- Dextran-texas red and calcein as lysosomal markers

3.2.1.1- Potential co-localization of dextran-texas red and calcein with LAMP-1, LAMP-2, and EEA-1

To study the interactions of RCVs with lysosomal compartments of host macrophages, pulse/chase assays with fluid phase markers were performed which allow the fluorescent labeling of the late endocytic compartments of the macrophages. To confirm the late endocytic localization of the dyes I analysed the co-localization of dextran-texas red and calcein with the late endosomal/lysosomal markers LAMP-1 and LAMP-2 and with the early endosomal marker EEA1. J774E cells growing on coverslips were incubated overnight in the presence of dextran-texas red or calcein. After washing with PBS to remove non-engulfed dye, the markers were chased for 1 hour (in the case of calcein) or 3 hours (for dextran-texas red) into the late endocytic compartments of the macrophage. The samples were fixed and immunofluorescence assays were performed to visualize the endocytic markers LAMP-1, LAMP-2, or EEA-1 (section 2.14.1). The samples were mounted on coverslides and examined using CLSM, as described in section 2.14.2.

Approximately 95% of compartments containing calcein co-localized with the late endocytic/lysosomal marker LAMP-1, while co-localization with early endosomal EEA-1 was rare (approximately 5%, Figure 4). Therefore, after 1 hour chase, calcein resided in late endocytic organelles, which express LAMP-1 but not EEA-1. Approximately 95% of the dextran-containing vesicles co-localized with LAMP-1, and only approximately 5% with EEA-1. Interestingly, the percentage of co-localization of dextran-containing vesicles was higher with LAMP-1 (95%) than with LAMP-2 (75%), suggesting a difference in the localization of these markers within J774E macrophages. Therefore, after 3 hours chase, dextran-texas red was located within late endocytic compartments, which are positive for LAMP-1 and

LAMP-2 but not for EEA-1. Thus, the pulse/chase protocols achieved in this section allow the selective fluorescent labeling of the late endocytic compartments of host macrophages.

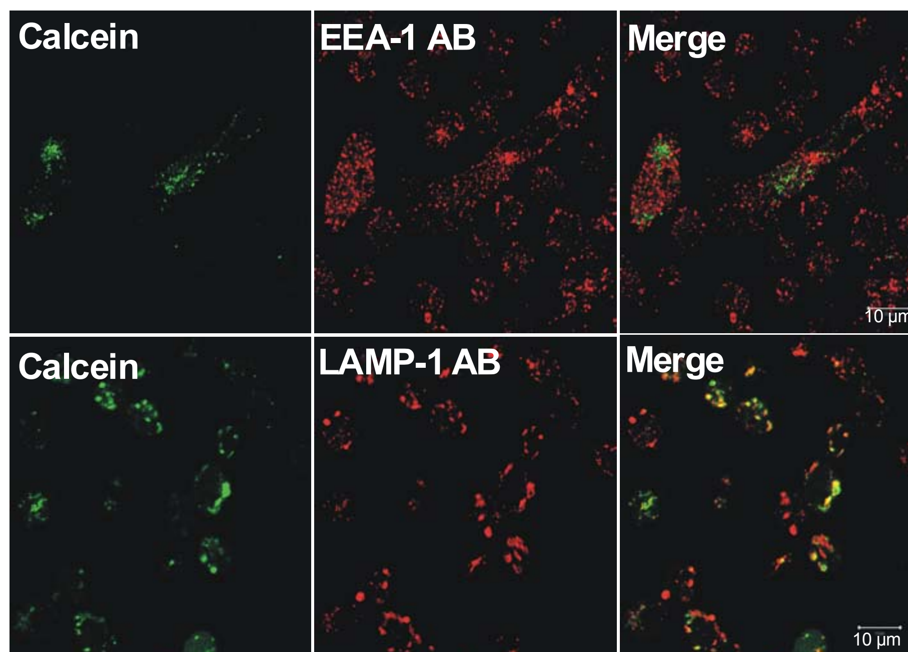

Figure 4: Lysosomal calcein co-localizes with LAMP-1 but not with EEA-1.
J774E macrophages were pre-incubated with calcein, followed by a 1 h chase in the absence of the dye, leading to fluorophore accumulation in lysosomes. Samples were fixed and immunofluorescence assays were performed to visualize the endocytic markers LAMP-1 or EEA-1. Samples were analysed using CLSM. Shown are single channels and the computer-assessed superimpositions (right panels). Bars indicate 10 μm.

3.2.1.2- Acquisition of the lysosomal dextran-texas red and calcein by RCV

To study potential interactions of RCVs with lysosomes, J774E lysosomes were labeled with dextran-texas red or calcein as above (3.2.1.1). Cells were then infected with prelabeled bacteria or zymosan for 30 minutes, followed by a 2 hours chase. This time is sufficient for phagosomes to mature into phagolysosomes [Haas 1998, Lührmann *et al*. 2001]. The samples were fixed, mounted on coverslides and examined using CLSM, as described in section 2.14.2.

The results obtained using dextran-texas red as a marker for lysosomes suggest that *R.equi* has the capability to change phagolysosome formation (Figure 5). Only 16% of the vacuoles containing viable plasmid-bearing *R.equi* (VapA +), but 92% of the zymosan-containing phagosomes, co-localized with lysosomal dextran-texas red. This high level of co-localization obtained with phagosomes enclosing zymosan was the expected for phagosomes which undergo undisturbed phagolysosome formation.

In addition, the results also suggest that the ability of *R.equi* to inhibit phagosome-lysosome fusion can be hampered if the bacteria loose their viability. Phagosomes containing non-viable plasmid bearing *R.equi* co-localized more frequently with the lysosomal marker (42% for heat-killed bacteria and 35% for formaldehyde-killed bacteria) as compared to the phagosomes containing viable plasmid bearing *R.equi* (16%). Interestingly, the inhibition of phagosome-lysosome fusion was not completely abolished when the ingested bacteria were non-viable.

The results in Figure 5 also suggest that the possession of the VapA-expressing plasmid has some influence on maturation of *R.equi*-containing phagosomes. Phagosomes containing viable plasmid-bearing *R.equi* (VapA +) co-localized less frequently with the lysosomal marker (16%) than did phagosomes containing the plasmid cured isogenic bacteria, *R.equi* VapA- (44%). This suggests that, the possession of the virulence-associated VapA-expressing plasmid is advantageous for *R.equi,* because bacteria that possess this plasmid can block the phagosome maturation more efficiently than bacteria that do not possess the plasmid. However, its possession is not strictly required for this block in phagolysosome biogenesis, because plasmid-cured bacteria are still able to block their phagosome maturation.

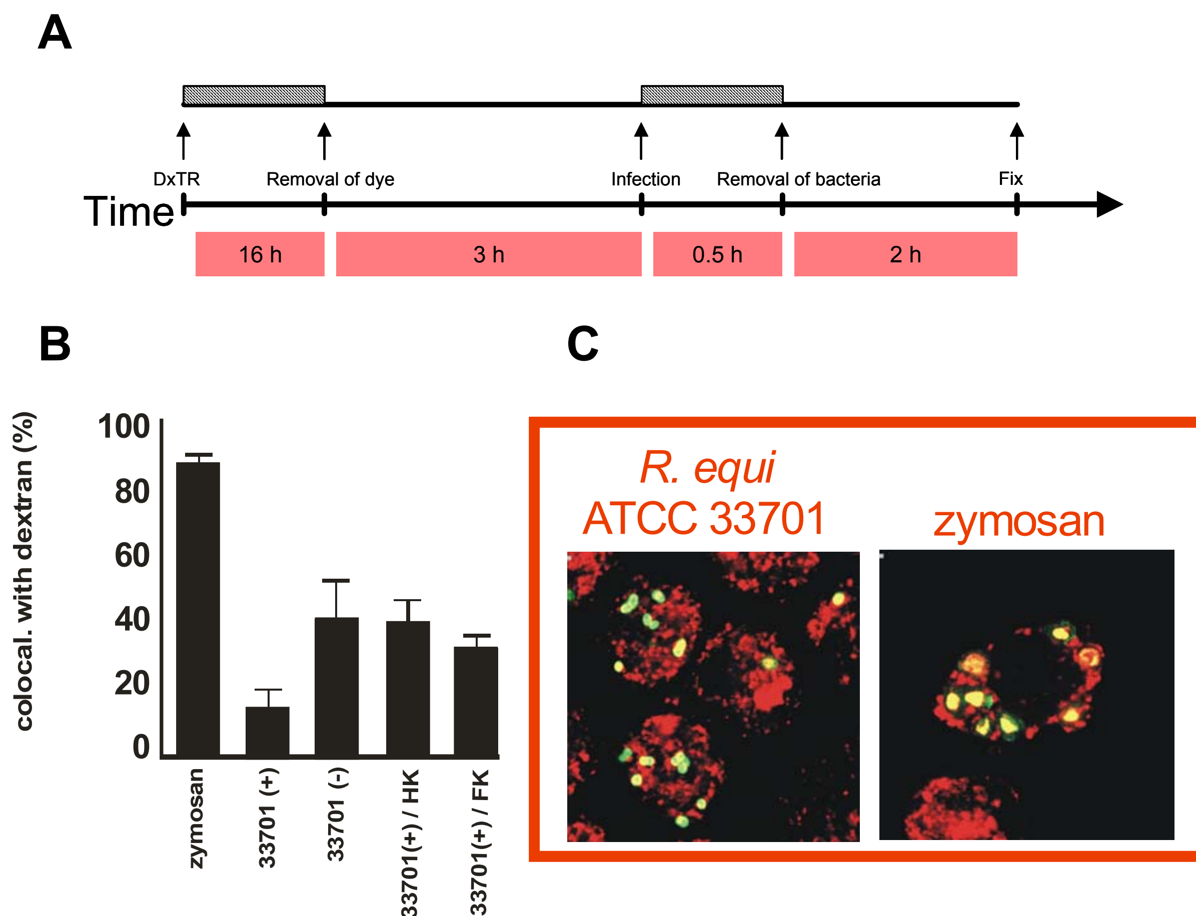

Figure 5: RCVs do not acquire dextran-texas red pre-loaded into macrophage lysosomes.
(A) Pulse-chase protocol for dextran-texas red-labeling of lysosomes in J774E. Co-localization between the dye and phagocytic probes was quantified using CLSM and expressed in % of co-localization of particles with the fluorophores. (B) Co-localization percentages of phagosomes containing zymosan, live *R. equi* 33701 + [33701(+)], live *R. equi* 33701 - [33701(-)], heat-killed *R. equi* 33701 + [33701(+)/HK], or formaldehyde-killed *R. equi* 33701 + [33701(+)/FK]. Data shown are the means and standard deviations of 3 independent experiments with a minimum of 50 phagosomes counted per experiment and sample. (C). CLSM micrographs showing the phagocytic probes zymosan and *R.equi* 33701(+). Co-localization of phagocytic probes (green fluorescence) with dextran-texas red (red fluorescence) is indicated by yellow to orange fluorescence.

The results obtained using calcein as a marker for lysosomes are very similar with those obtained using dextran-texas red (Figure 6), which also suggest that *R.equi* has the ability to block the maturation of its phagosome into phagolysosomes. Only 9% of vacuoles containing viable plasmid bearing *R.equi* (VapA +) co-localized with calcein-labeled organelles. In contrast, 67% of the zymosan-containing phagosomes acquired calcein, as expected for phagosomes which mature into phagolysosomes.

The results using calcein also indicate that bacterial viability influences the ability of *R.equi* to inhibit phagosome-lysosome fusion. 36% of phagosomes enclosing heat-killed *R.equi* co-localized with calcein, and only 9% of phagosomes containing viable bacteria. Although the inhibition of phagosome-lysosome fusion was clearly hampered if bacteria loose their viability, the majority of phagosomes containing non-viable *R.equi* were still able to inhibit phagosome-lysosome, suggesting that the main mechanism responsible for the block in phagosome maturation may be heat-insensitive factor(s).

The results obtained with dextran-texas red suggested that the possession of the VapA-expressing plasmid in *R.equi* influences to some extent the ability of the bacteria to inhibit the phagolysosome formation. However, the observed differences (Figure 5) between the plasmid-bearing *R.equi* strain 33701(33701 VapA+) and its isogenic plasmid-cured partner (33701 VapA-) could be also due to chromosomal mutations that occurred only in the plasmid cured 33701- strain. In order to address this possibility and also to study the role of the VapB-expressing plasmid in the phagosome biogenesis, the acquisition of lysosomal calcein by phagosomes enclosing *R.equi* containing either VapA- or VapB-expressing plasmids was tested. For this purpose, four different strains of *R.equi* were used:

- Two strains containing the VapA-expressing plasmid: ATCC 33701 (33701+) and 103 (103+), and their isogenic, plasmid cured partners 33701- and 103-. The virulence-associated plasmid of 103 strain has almost the same nucleic acid sequence as that of ATCC 33701 strain [Takai *et a*l. 2000b].
- Two strains containing the VapB-expressing plasmid: A2 (A2+) and A11 (A11+), and their isogenic, plasmid cured partners A2- and A11-.

All of the *R.equi* strains tested (plasmid-bearing bacteria and also their isogenic plasmid cured partners) possess the ability to change the normal course of maturation of their phagosome (Figure 6). None of the *R.equi* strains tested co-localized more than 30% with lysosomal calcein, but 68% of phagosomes containing zymosan.

The results obtained using the *R.equi* strain 33701 (bearing the VapA-expressing plasmid) suggest that the possession of this plasmid in *R.equi* influences in some extent the ability of *R.equi* to block phagolysosome biogenesis: only 8% of the phagosomes containing 33701+ co-localized with calcein. In contrast, 19% of the phagosomes containing its cured derivative (33701-) co-localized with the late endocytic marker. However, *R.equi* 103 (+) was not as efficient at interfering with phagolysosome formation as *R.equi* 33701(+) was, and phagosomes containing *R.equi* 103(-) did not fuse significantly more frequently with lysosomes (28% +/- 6.6) than 103 (+) did (20% +/- 1.7). Interestingly, there was no significant difference in phagosome maturation between strains of *R.equi* bearing the VapB-expressing plasmid and their plasmid-cured partners (all strains tested co-localized with calcein between 19% and 23%, Figure 6). These results suggest that the main mechanism responsible for the block in phagolysosome maturation is unlikely to be regulated by plasmid genes in *R.equi*.

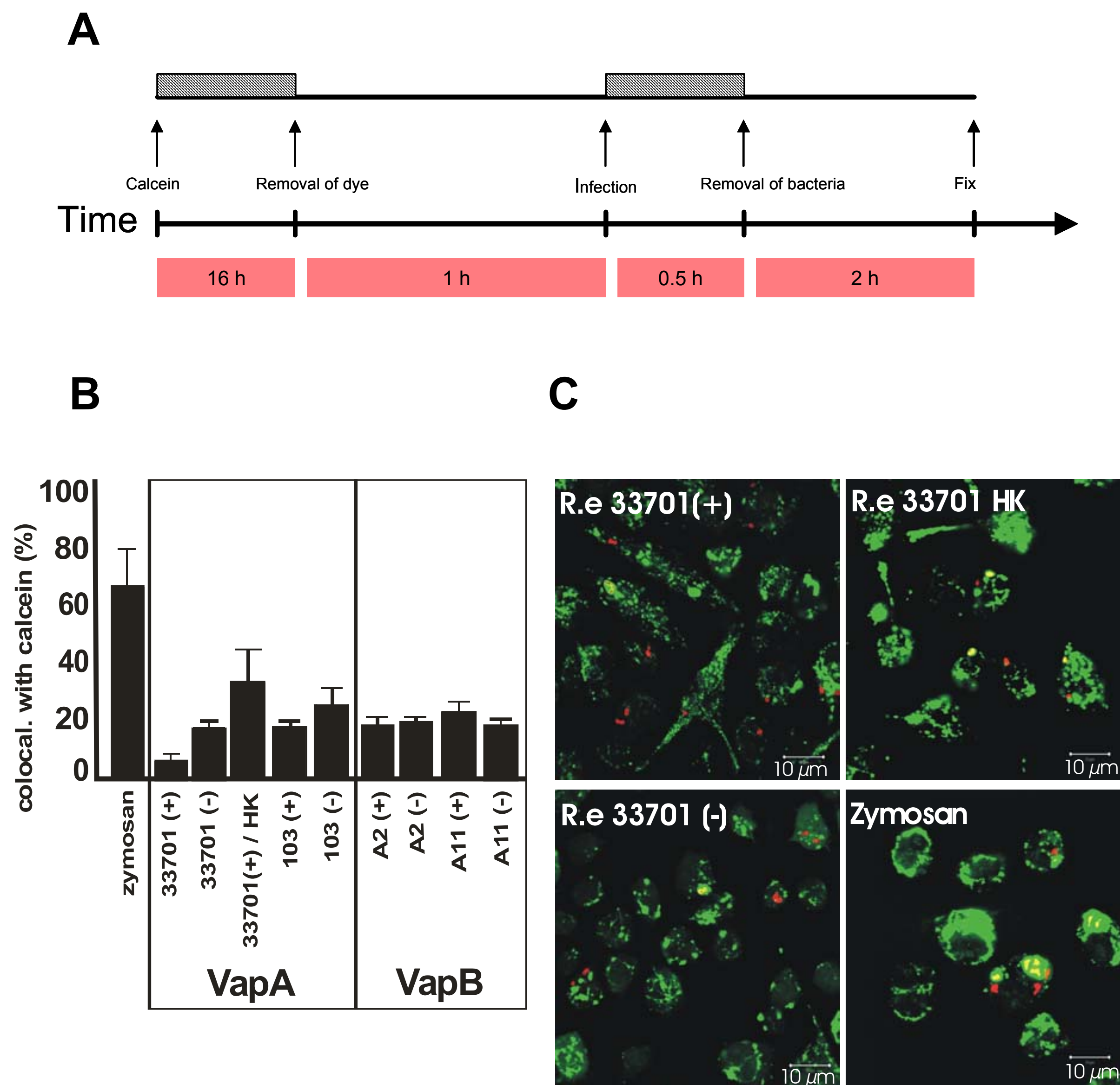

Figure 6: RCVs do not acquire calcein pre-loaded into macrophage lysosomes.
(A) Pulse-chase protocol for calcein-labeling of lysosomes in J774E. (B) Co-localization percentages of phagosomes containing zymosan, live *R. equi* 33701 + [33701(+)], live *R. equi* 33701 - [33701(-)], heat-killed *R. equi* 33701 + [33701(+)/HK], live *R. equi* 103 + [103(+)], live *R. equi* 103 - [103(-)], live *R. equi* A2 + [A2(+)], live *R. equi* A2 - [A2(-)], live *R. equi* A11 + [A11(+)], or live *R. equi* A11 - [A11(-)]. Data shown are the means and standard deviations of 3-6 independent experiments with a minimum of 50 phagosomes counted per experiment and sample. (C). CLSM micrographs with the phagocytic probes, live *R.equi* 33701(+) [*R.e* 33701(+)], heat-killed *R.equi* 33701(+) [*R.e* 33701(+) HK], live *R.equi* 33701(-) [*R.e* 33701(-)], or zymosan. Co-localization of phagocytic probes (red fluorescence) with calcein (green fluorescence) is indicated by yellow to orange fluorescence. Bars indicate 10 µm.

3.2.2- Addition of the fluid-phase marker dextran-texas red after establishing an infection

In order to analyse whether *R. equi* is able to restrict fusion of its phagosome with all compartments of the endocytic pathway, J774E cells were first infected with labeled bacteria, and endocytic compartments were then labeled with dextran-texas red, followed by quantitation of the fraction of RCVs that had acquired dextran-texas red. Following the labeling protocol outlined in Figure 7A and section 2.14.4, dextran-texas red should be labeling all compartments of the endocytic system. Co-localization of the green bacteria or zymosan with the red-labeled dextran was taken as a sign of interaction of the bacteria-containing phagosomes with the endocytic pathway.

At 2 hours postinfection, *R.equi* resides in phagosomes which maintain interaction with the endocytic pathway (Figure 7): 62%+/-11 of RCVs containing viable *R.equi*(+) co-localized with the endocytic marker dextran-texas red, a percentage similar than that obtained with control phagosomes containing zymosan (70%). Interestingly, RCVs containing viable and also non-viable, plasmid cured *R.equi*(-) co-localized less frequently with dextran-texas red than RCVs containing the viable plasmid-bearing *R.equi*(+) did [43% for the viable *R.equi*(-), 47% for the heat-killed *R.equi*(-) bacteria, and 62% for the viable *R.equi*(+)]. Moreover, RCVs containing non viable *R.equi*(+) co-localized less frequently with dextran-texas red than RCVs containing viable plasmid-bearing *R.equi* (31% for the heat-killed bacteria, 45% for the formaldehyde-killed *R.equi*, and 62% for the viable *R.equi*+). These findings suggest that vacuoles containing viable *R.equi* interact more avidily with the endocytic pathway than RCVs containing non-viable bacteria, thus acquiring more marker.

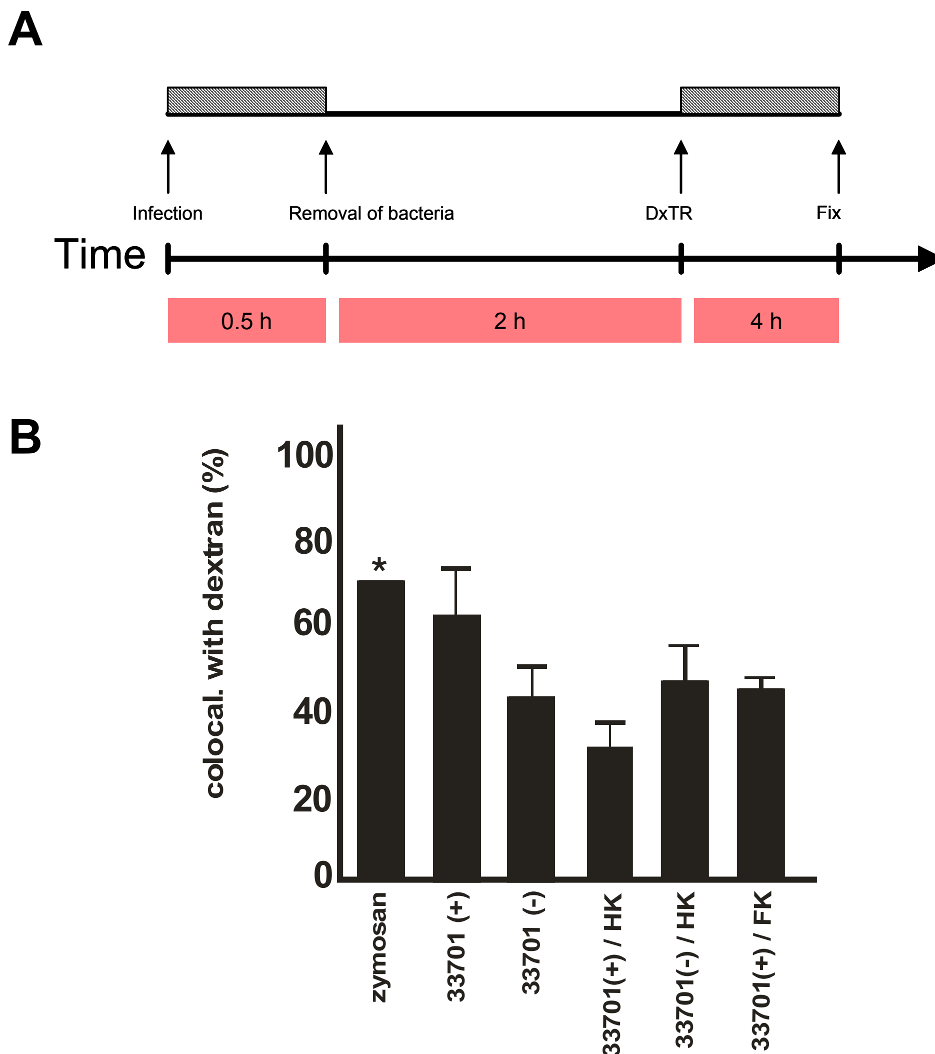

Figure 7: RCVs are accessible to dextran-texas red added after establishment of an infection. (A) Scheme for labeling of the J774E endocytic pathway with dextran-texas red. Co-localization between the dye and phagocytic probes was quantified using CLSM and expressed in % of co-localization of particles with the fluorophore. (B) Co-localization percentages of zymosan, live *R. equi* 33701+ [33701(+)], live *R. equi* 33701- [33701(-)], heat-killed *R. equi* 33701+ [33701(+)/HK], heat-killed *R. equi* 33701- [33701(-)/HK], or formaldehyde-killed *R. equi* 33701+ [33701(+)/FK]. Data shown are the means and standard deviations of 3 independent experiments with a minimum of 50 phagosomes counted per experiment and sample. (*) Data shown with zymosan are the mean of 2 independent experiments with a minimum of 50 phagosomes counted per experiment and sample.

3.3- Qualitative assessment of acidification of RCVs

The results obtained with the fluid-phase endocytic markers calcein and dextran-texas red (section 3.2) suggest that *R.equi* is able to block the maturation of its phagosome into phagolysosome, and resides in a vacuole which maintains interaction with the endocytic pathway. This block in phagosome maturation may delay and attenuate acidification of RCVs, a condition that would possibly help the bacteria to survive within phagosomes. To address this possibility, acidification of *R.equi*-containing phagosomes was monitored by using an acidotropic probe, LysoTracker Red DND-99. The acidotropic LysoTracker probe consists of a red fluorophore linked to a weak base that is only partially protonated at neutral pH and that freely permeates cell membranes. Once protonated, it remains trapped in acidified organelles, revealing compartments with acidic pH by red fluorescence, including acidic phagosomes.

Two *R.equi* (VapA+) strains expressing Green Fluorescence Protein (GFP) were used: *R.equi* strains ATCC 33701 and 103 harbouring the GFP plasmid pSC301 (103+/GFP and 33701+/GFP). Antibody-opsonized *Escherichia coli* containing the GFP plasmid pSC301 (*E.coli*/GFP) were used as positive control. Studies have shown that antibody-opsonized *E.coli* are efficiently killed within macrophages and that phagosomes containing these bacteria are acidic [Baorto *et al*. 1997].

3.3.1- Inhibition of phagosomal acidification by Bafilomycin A

Phagosome acidification is attributed principally to the activity of vacuolar type H^+ ATPases (vATPases), which accumulate in the phagosomal membrane as the phagosome matures [Hackam *et al*. 1997]. In order to determine whether only acidified compartments were labeled with LysoTracker, J774E cells were incubated with the vATPase inhibitor bafilomycin A, thereby blocking the acidification of endocytic compartments [Bowman *et al*. 1988; Schneider *et al*. 2000; Ibrahim-Granet *et al*. 2003]. The cells were infected with E.coli/GFP or fluorescently labeled zymosan for 30 minutes and chase for 1 hour. Medium was withdrawn and bafilomycin A was added to the monolayers for 1 hour. LysoTracker in presence of bafilomycin A was added, and the cells were incubated for another hour. Samples were examined immediately using CLSM (sections 2.14.2 and 2.15.1).

The results obtained confirmed that the red fluorescence emitted by LysoTracker was present only in the acidified compartments of host macrophages (Figure 8), as cells treated with bafilomycin A did not emit any red fluorescence. Consequently, neither phagosomes containing *E.coli*/GFP nor zymosan-containing phagosomes acquired LysoTracker in bafilomycin A treated cells. In some of the zymosan-containing phagosomes (approx. 2%), a very weak red signal was observed, suggesting that a few lysosomal compartments could remain acid after treatment with bafilomycin A and may have interacted with some zymosan-containing phagosomes.

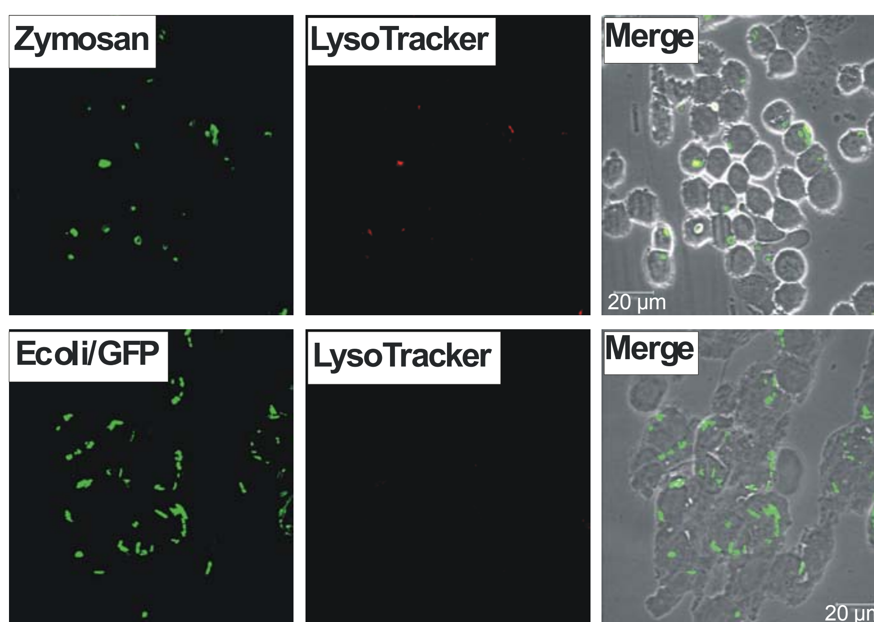

Figure 8: Inhibition of phagosomal acidification by bafilomycin A.
J774E were infected with *E.coli*/GFP or prelabeled zymosan for 30 minutes, followed by 1 h chase in the absence of added particles. Fresh medium containing bafilomycin A was given to cells, and after 1 hour incubation, medium was replaced for fresh medium containing LysoTracker in presence of bafilomycin A, followed by a 1 hour incubation. Samples were then washed, mounted on slides, and examined immediately using CLSM. Acidification of phagosomes was analysed using laser-scanning microscopy. Shown are single channels and the computer-assessed superimpositions (right panels), including the phase contrast views. Bars indicate 20 μm.

3.3.2- Co-localization of RCVs with LysoTracker

To assess phagosomal acidification, J774E cells were infected with GFP-expressing bacteria or with fluorescently labeled zymosan, chased for 2 hours, and incubated for 30 minutes in presence of LysoTracker (section 2.15.2). Samples were examined immediately using CLSM (section 2.14.2). Acidic pH in the phagosomes was revealed by red fluorescent labeling surrounding or totally covering the green bacteria inside the phagosomes, appearing in the latter case as bright yellow spots.

The results obtained suggest that, at 2 hours postinfection, the majority of *R.equi* reside in poorly or non-acidic phagosomes within murine macrophages (Figure 9). Only 13% of the phagosomes containing *R.equi* 33701+/GFP and 17% of the phagosomes containing the strain 103+/GFP acquired the acidotropic LysoTracker, compared with 92% of the phagosomes containing zymosan, indicating that most of the zymosan resides in acidified phagosomes. The latter is the expected for phagolysosomes, which posses lower pH (phagolysosomal pH is typically between 4.5 and 5.0) compared with early phagosomes, which are normally weakly acidic (pH 6.0-6.5) [Geisow *et al*.1981, Porte *et al*. 1999, Schaible *et al*. 1999]. In addition, the majority of phagosomes containing *E.coli*/GFP acquired LysoTracker (69%), as expected for these bacteria [Baorto *et al*. 1997].

Interestingly, the percentage of RCVs labeled by LysoTracker was similar to the percentage of co-localization of RCVs with lysosomal marker calcein (section 3.2.1.2). 13% of vacuoles containing *R.equi* strain 33701 (VapA +) co-localized with LysoTracker and 9% of vacuoles enclosing these bacteria co-localized with calcein. In the same way, 17% of phagosomes containing *R.equi* strain 103 (VapA +) co-localized with LysoTracker and 20% with calcein. Thus, the results indicate that *R.equi* resides in vacuoles that do not mature into phagolysosomes, as shown the poor acidification of the phagosome and limited acquisition of lysosomal calcein. In spite of the ability of *R.equi* to inhibit phagolysosome formation, a small percentage of RCVs mature into phagolysosomes, thus co-localizing with calcein and with the acidotropic probe LysoTracker. When acidification of RCVs containing *R.equi* 103(+) or 103(-) were compared, a significant difference was observed between the 2 strain partners: 17% (+/-5.8) of RCVs containing *R.equi* 103(+) acquired LysoTracker, compared with 34% (+/-0.6) of RCVs containing *R.equi* 103(-) that acquired the marker. This finding suggests that factor(s) encoded by the virulence-associated plasmid may contribute to the establishment of RCV.

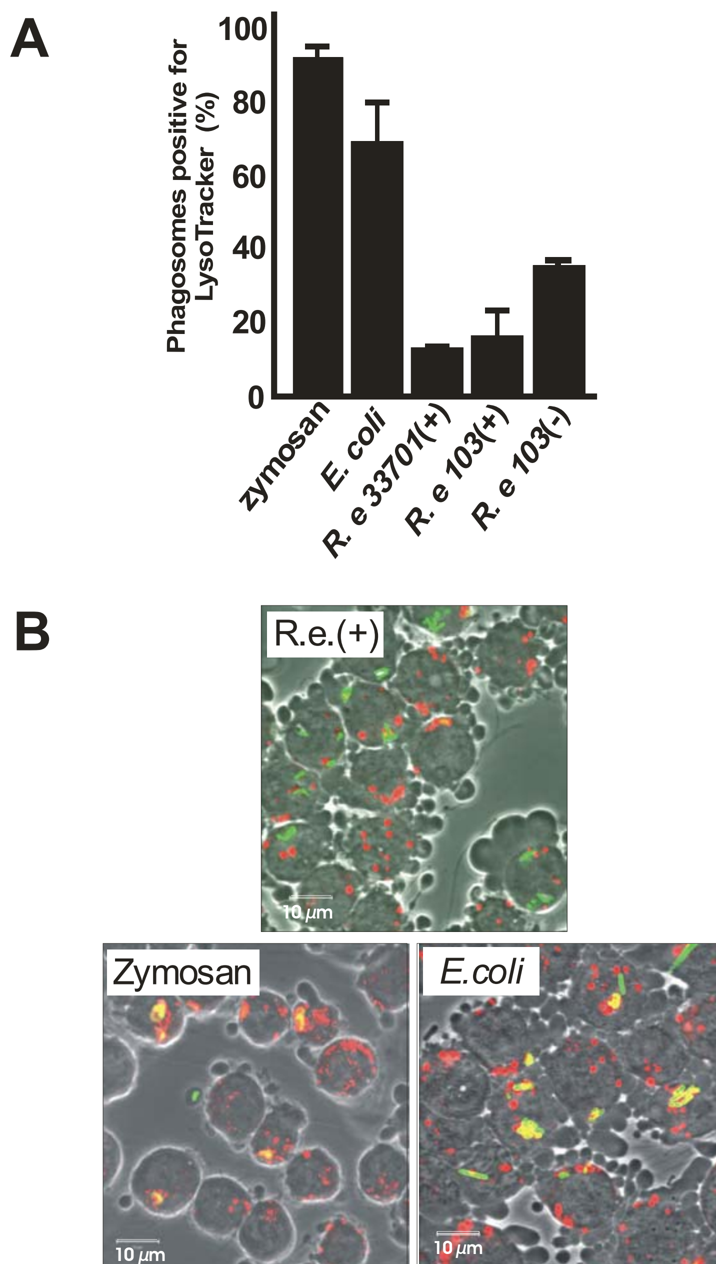

Figure 9: RCVs are not acidic.
J774E macrophages were incubated with either zymosan, *Escherichia coli* [*E.coli*], *R.equi* 33701+ [*R.e* 33701(+)], *R.equi* (103+) [*R.e* 103(+)], or *R.equi* (103-) [*R.e* 103(-)] for 30 min, followed by a 2 h chase. Medium was replaced for fresh medium containing Lysotracker, followed by 30 minutes incubation. Samples were fixed and prepared for fluorescence microscopy. Co-localization frequencies between the red LysoTracker dye and green phagocytic probes were quantified using CLSM (A). Data are the means and standard deviations of 3 experiments with a minimum of 50 phagosomes counted per experiment and sample. (B) Typical computer-assessed superimpositions including the phase contrast views are shown using either *R.equi* 33701+ [*R.e.*(+)], zymosan, or *E. coli* as phagocytic probes. The 'blebbing' phenotype of the macrophages shown was a result of the embedding in low melting point agarose.

3.4- Acquisition of endocytic markers by RCVs

Phagosome maturation has been shown to be very similar to endosome maturation: during phagolysosome biogenesis, phagosomes intersect with the endocytic pathway and fuse sequentially with early endosomes, late endosomes, and lysosomes [Pitt *et al*. 1992, Desjardins *et al*. 1994a, Desjardins *et al*. 1994b, Desjardins *et al*. 1997, Jahraus *et al*. 1998]. These interactions allow the phagosomal acquisition of membrane and luminal components, and the removal of others, conditions that confer new properties to the maturing phagosomes [Garin *et al*. 2001]. Phagosome maturation can be followed experimentally by analysing the presence of various molecule markers of subcellular compartments, such as EEA-1, Rab5, and transferrin receptor (all for early endosomes), Rab7, LAMP-1, LAMP-2, and vATPase (all for late endosomes/lysosomes), and LBPA (for late endosomes). Phagosomes containing intracellular pathogens often develop into non-canonical compartments which still possess characteristics of the endocytic pathway, such as phagosomes containing *M.tuberculosis* that possesses characteristics of early endosomes. Hence, acquisition or removal of different endocytic molecules by maturing phagosomes can be used as a tool to analyse the characteristics display by phagosomes containing intracellular pathogens as well as possible interactions with the different endocytic compartments.

In order to analyse the precise compartmentation of *R.equi* in murine macrophages, the occurrence of various endocytic marker molecules in RCVs was assessed by immunofluorescence microscopy analyses with infected J774E cells and BMMs. Unfortunately, *R.equi* stained weakly with most antibodies that were tested, regardless whether they were monoclonal or affinity-purified or contained in complete serum. Using affinity-purified secondary antibodies, different antibodies preparations were detected which did not react with *R.equi* bacteria and therefore used in this section (antibodies against EEA-1, TfR, LBPA, LAMP-1, LAMP-2, and vATPase).

Kinetics of acquisition of the endocytic markers by RCVs within the first 3 hours of infection was examined using J774E and BMMs as host cells. Because *R.equi* infection is toxic for murine macrophages [Lührmann *et al*. 2004], analysis of endocytic markers at 24 hours of infection was only performed using less sensitive BMMs (Tobias Sydor, personal communication). To examine the acquisition of the markers within the first 3 hours of *R.equi* infection, J774E cells or BMMs growing on coverslips were infected with prelabeled bacteria or zymosan. Infection was made at 4°C and the

plates were centrifuged for 30 minutes at 4°C. The centrifugation step allows the bacteria to make close contact with the mammalian cells and helps in the synchronization of the infection. Cold medium was then withdrawn and replaced for fresh warm medium at 37°C, being that set as 0 min. After incubating the cells at 37°C for up to 180 minutes, samples were fixed and endocytic markers were labeled using antibodies directed against them (section 2.16.1). To examine the presence of endocytic markers at 24 hours of *R.equi* infection, BMMs growing on coverslips were infected with unlabeled *R.equi* for 30 minutes at 37°C. After washing to remove non-ingested bacteria, samples were incubated for 24 hours in medium containing gentamicin. Gentamicin was added to the medium to prevent bacteria for multiplying in the cell culture medium. After washing with PBS, samples were fixed and antibodies were used to label the different endocytic markers and also *R.equi* (section 2.16.2). Samples were then mounted on slides and analysed using CLSM, as described in section 2.14.2. In all cases, the presence of a green fluorescent ring surrounding the red labeled bacteria was considered a positive signal. Phagosomes were considered positive for LBPA (present on vesicles within organelles) when the green antibody fluorescence at least partially co-localized with red labeled bacteria. In addition, specificity of the immunolabeling was confirmed by the fact that bacteria lying outside the cells were negative with all the above mentioned antibodies.

3.4.1- Kinetics of acquisition of EEA-1 by RCVs

Early Endosome Antigen 1 (EEA-1) is a membrane-associated protein localized in early endosomes [Mu *et al*. 1995] which has been shown to be a tethering protein implicated in the docking of incoming endocytic vesicles [Christoforidis *et al*. 1999a]. It is recruited to the membranes of early endosomes by PI-(3)-kinase products and Rab5 and it is believed to provide directionality to Rab5-dependent membrane trafficking [Simonsen *et al*. 1998]. Several studies have shown that EEA1 is only transiently acquired by model phagosomes with a peak of acquisition in the first minutes after phagocytosis [Pizarro-Cerdá *et al*. 1998, Fratti *et al*. 2000, Lührmann *et al*. 2001].

The data obtained indicate that, in BMMs, RCVs acquire EEA-1 transiently, with a peak of acquisition of the marker in the first minutes after internalization of the bacteria (Figure 10). In all samples studied (*R.equi*(+), *R.equi*(-), and *R.equi* HK), between 21% and 27% of RCVs co-localized with EEA-1 at 5 min postinfection. At 15 minutes postinfection, the percentages of co-localization had decreased, and at 60 minutes postinfection, less than 10% of RCVs co-localized with the early endocytic marker. The

low levels of co-localization of all the *R.equi* tested with EEA-1 (no more than 27% at any given time) suggest either a rapid and transient interaction with early endosomal compartments (expressing EEA-1) in BMMs or that most of the bacteria avoid interactions with compartments positive for EEA-1. Since most of RCVs interact with the endocytic pathway (section 3.2.2), the first hypothesis is the more plausible. Interestingly, the percentage of RCVs containing *R.equi*(+) that co-localized with EEA-1 at 120 minutes (17%) was higher than the percentage at 60 minutes (5%), suggesting an accumulation over time. Immunofluorescence analysis at 24 hours of infection showed, however, that RCVs lack EEA-1 (Figure 11). Thus, at least in BMMs, RCVs acquire EEA-1 transiently, and at 24 hours of infection, RCVs are completely devoid of this marker.

The general pattern of EEA-1 acquisition detected when J774E were used as host cells are in agreement with those obtained using BMMs: RCVs acquire EEA-1 transiently. Approximately 40% of RCVs co-localized with EEA-1 at 15 minutes postinfection, and only 10% at 120 minutes. The percentages of co-localization of RCVs with the marker were higher in J774E when compared with BMMs, possibly due to differences in the kinetics of acquisition of this marker between host cells.

Thus, the results obtained using J774E and BMMs as host cells suggest that RCVs acquire EEA-1 only transiently, with a peak of acquisition in the first minutes after phagocytosis. Kinetics of association of this marker was similar between *R.equi*(+), *R.equi*(-) and heat-killed *R.equi*, suggesting that the transient acquisition of EEA-1 is independent from the viability of *R.equi* or the possession of the virulence-associated plasmid. This pattern of transient accumulation of EEA-1 resembles that observed using model phagosomes [Pizarro-Cerdá *et al*. 1998, Fratti *et al*. 2000].

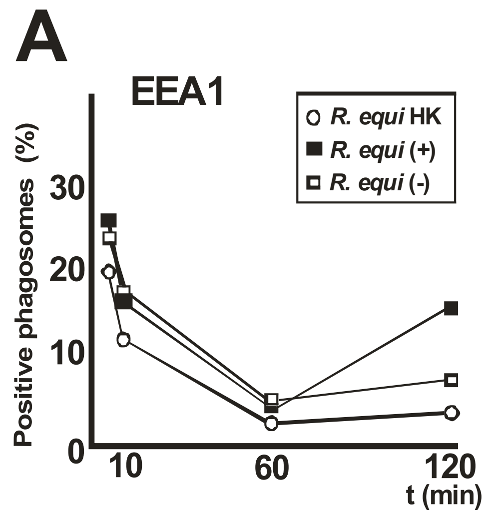

Figure 10A: Kinetics of acquisition of EEA-1 by RCVs.
BMMs were infected with prelabeled bacteria for up to 120 minutes. Samples were then fixed and immunofluorescence assays were performed to visualize the endocytic marker EEA-1. Co-localization frequencies were quantified using CLSM and expressed in % of co-localization of particles with the marker. (A) Co-localization percentages of the following phagocytic probes: live *R.equi* 33701+ [*R.equi*(+)], live *R.equi* 33701- [*R.equi*(-)], and heat-killed *R.equi* 33701+ [*R.equi*(+)HK]. Data are the means of 3 experiments with a minimum of 50 phagosomes counted per experiment and sample. Standard deviations from the mean were omitted for clarity, but never exceeded 7%.

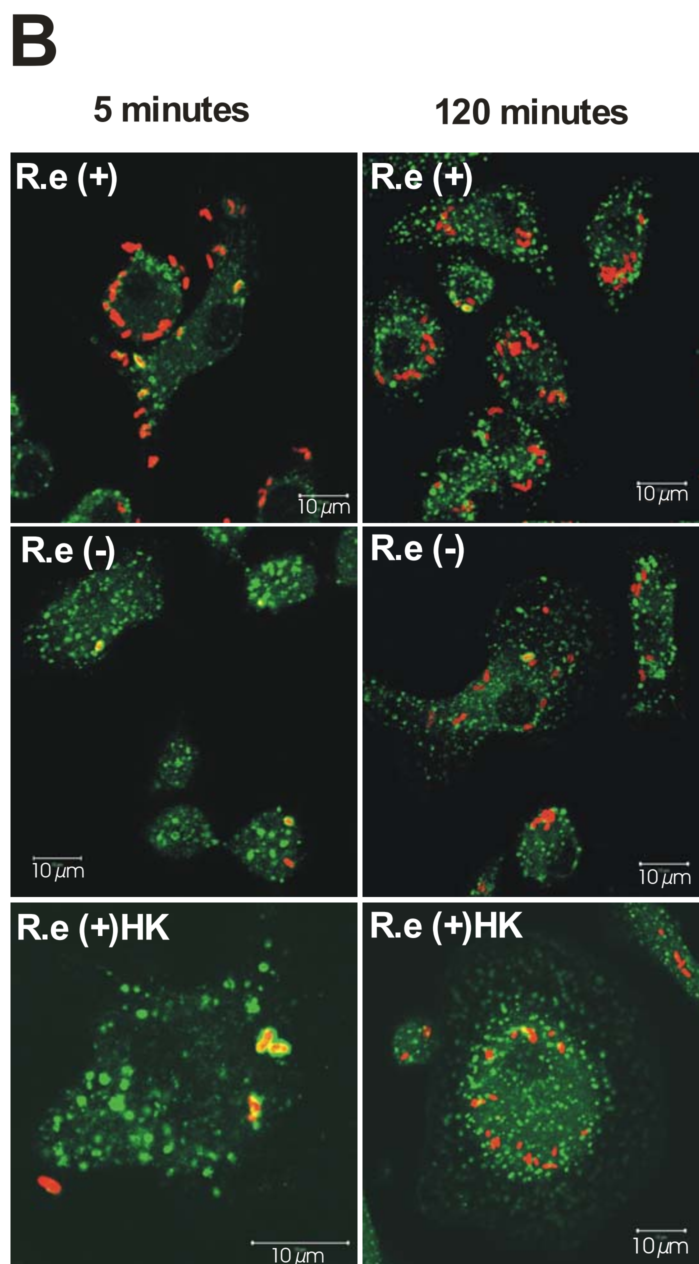

Figure 10B: Kinetics of acquisition of EEA-1 by RCVs.
CLSM micrographs of samples stained with antibodies against EEA1, phagocytic probes are the same as in (A). Bars indicate 10 µm.

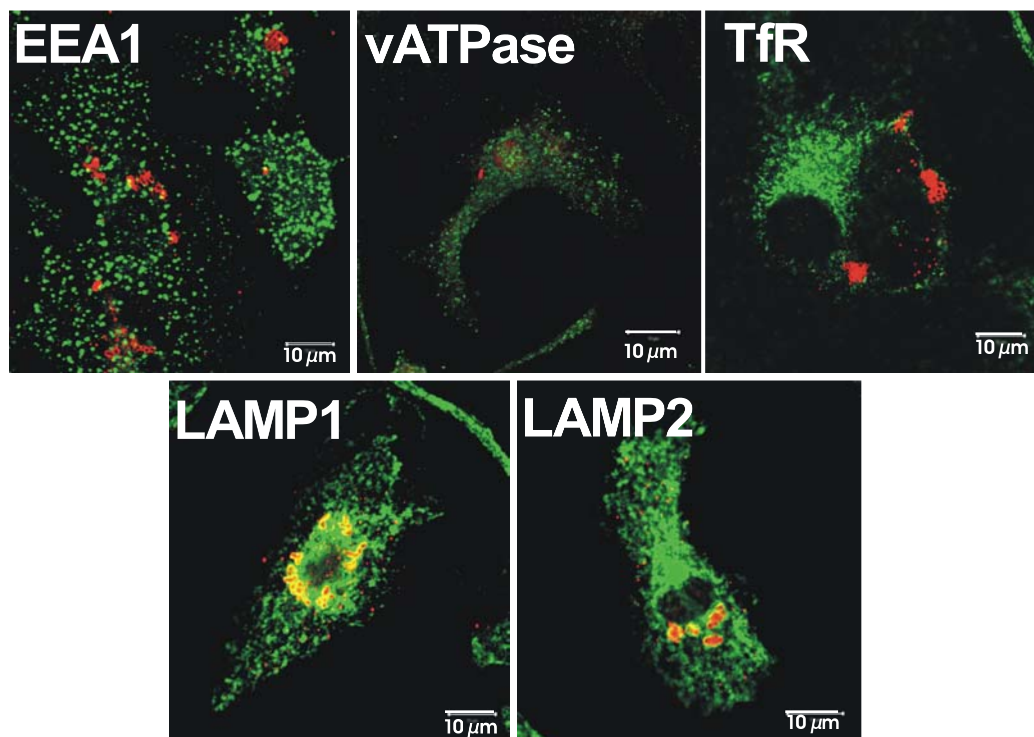

Figure 11: Immunofluorescence analysis of BMMs infected with *R. equi* 33701(+) for 24 h. CLSM micrographs of samples stained with antibodies to EEA1, vATPase proteolipid, TfR, LAMP1, or LAMP2. Two to five independent experiments were done with similar results. Bars indicate 10 μm.

3.4.2- Acquisition of transferrin receptor by RCVs

Transferrin receptors (TfR) are synthesized in the endoplasmic reticulum and transported via the Golgi complex to the cell surface. In the cell surface, they bind holotransferrin (iron-saturated transferrin), and then receptor-ligand complexes are internalized into vesicles that interact with early endosomes. Acidification of the endosome to pH 6.5-6.0 causes release of iron from the transferrin, resulting in formation of apotransferrin (iron-unsaturated transferrin), which is together with transferrin rapidly recycled to the plasma membrane [Dautry-Varsat *et al*. 1983, Sönnichsen *et al*. 2000]. Thus, transferrin receptors in complex with transferrin do not traffic through the late endosomes/lysosomes. These characteristics make TfR a good marker for sorting and recycling endosomes. In order to study whether RCVs retain transferrin receptor (TfR), the occurrence of this marker in RCVs was assessed, using BMMs and J774E as host cells.

At 10 minutes postinfection, approximately 5% of RCVs co-localized with TfR, in a manner similar to that observed in control phagosomes containing zymosan (Figure 12). At 2 hours postinfection, *R.equi* was found predominantly in vacuoles that lacked TfR. No difference was observed between all the *R.equi* studied (*R.equi* (+), *R.equi*(-), and *R.equi* HK) and control phagosomes containing zymosan. Moreover, at 24 hours of infection, RCVs were void of TfR (Figure 11). Therefore, the results suggest that, in murine macrophages, *R.equi* resides in vacuoles that do not communicate with recycling endosomes, thus lacking transferrin receptor.

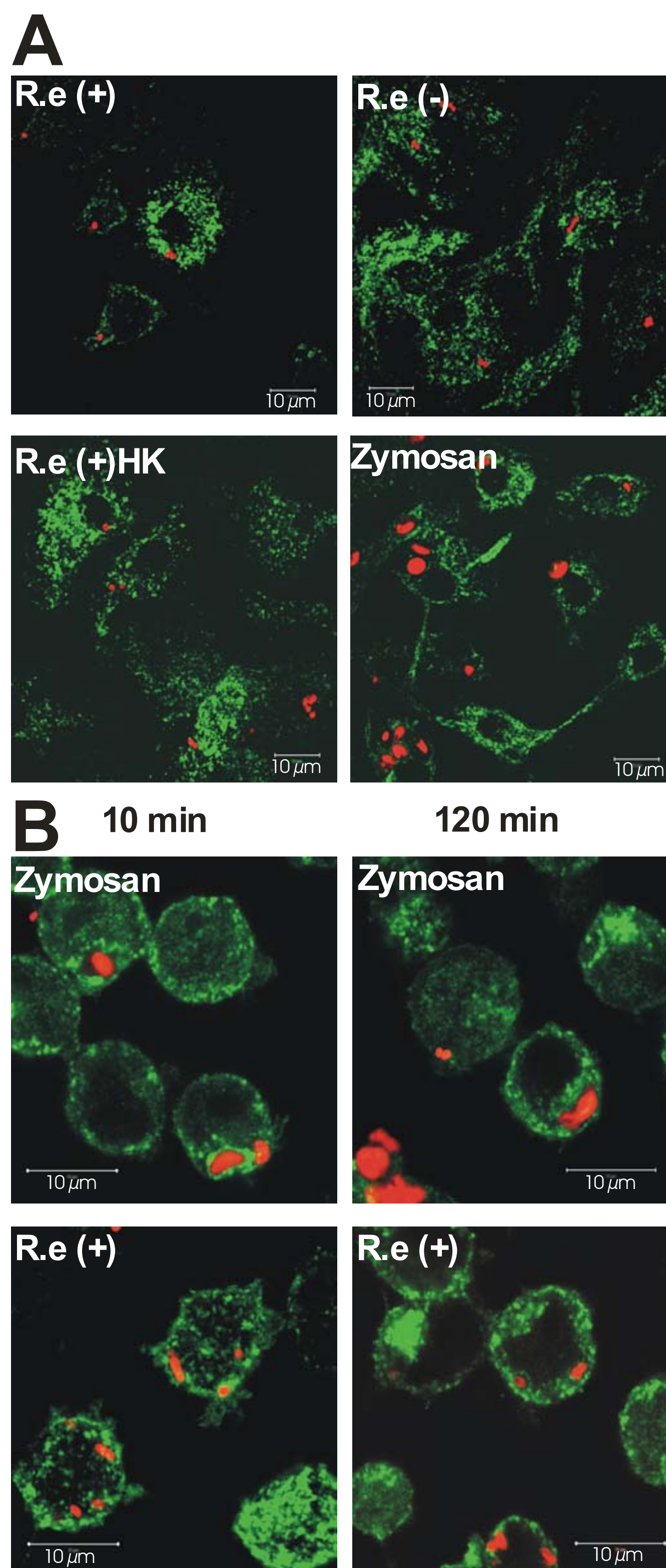

Figure 12: RCVs lack transferrin receptor.
BMMs or J774E cells were infected with prelabeled bacteria for up to 120 minutes. Samples were then fixed and immunofluorescence assays were performed to visualize the endocytic marker TfR. (A) CLSM micrographs of BMMs stained with antibodies against TfR, phagocytic probes are: live *R.equi* 33701+ [*R.e*(+)], live *R.equi* 33701- [*R.e*(-)], heat-killed *R.equi* 33701+ [*R.equi*(+)HK], and zymosan, infection time:120 min. (B) CLSM micrographs of J774E cells stained with antibodies against TfR, phagocytic probes are: zymosan and live *R.equi* 33701+ [*R.e*(+)]. Three independent experiments were done with similar results. Bars indicate 10 μm.

3.4.3- Acquisition of LAMP-1 and LAMP-2 by RCVs

Lysosome-Associated Membrane Proteins 1 and 2 (LAMP-1 and LAMP-2) have been used widely to characterize bacteria-containing phagosomes [Barker *et al*. 1997, Lührmann *et al*. 2001, Kuehnel *et al*. 2001]. They are mostly found in late endosomes and lysosomes in non-infected cells. According to Desjardins *et al*., after phagosome formation, the phagosome membrane is low in LAMP-1 and -2 [1994]. As the phagosome mature, it concentrates these marker proteins until they are equilibrated with late endocytic organelles. However, both marker proteins follow a complex recycling route via early endosomes, trans-Golgi network and the plasma membrane [Griffiths 1996, Kuehnel *et al*. 2001]. Thus, although LAMP´s are considered late endosomal/lysosomal markers, they are also present on the surface of the macrophage as well as in vesicles being transported from the Golgi apparatus to endosomes [Fukuda 1991].

To ascertain whether RCVs are enriched for LAMP-1 and LAMP-2, the occurrence of these markers were studied, using J774E and BMMs as host cells. At 10 minutes postinfection in J774E cells, relative few RCVs co-localized with LAMP-1 (between 20 and 30%, Figure 13). No difference was observed between RCVs containing viable *R.equi*(+), viable *R.equi*(-) or heat- or formaldehyde-treated bacteria [*R.equi*(+)HK and *R.equi*(+)FK]. Over the same infection period, 60% of zymosan-containing phagosomes acquired LAMP-1. However, at 180 minutes postinfection, the majority of either phagosome type contained LAMP-1 (75-94%). The same pattern was observed when BMMs were used as host cells: at 10 minutes postinfection, the majority of RCVs did not co-localize with LAMP-1 (lesser than 10%), but did at 180 minutes postinfection and at 24 hours postinfection. (approximately 90%, Figure 11). Thus, the data obtained indicate that, within murine macrophages, RCVs acquire and retain the endosomal marker LAMP-1, independent from the viability of the bacteria or the possession of the virulence-associated plasmid.

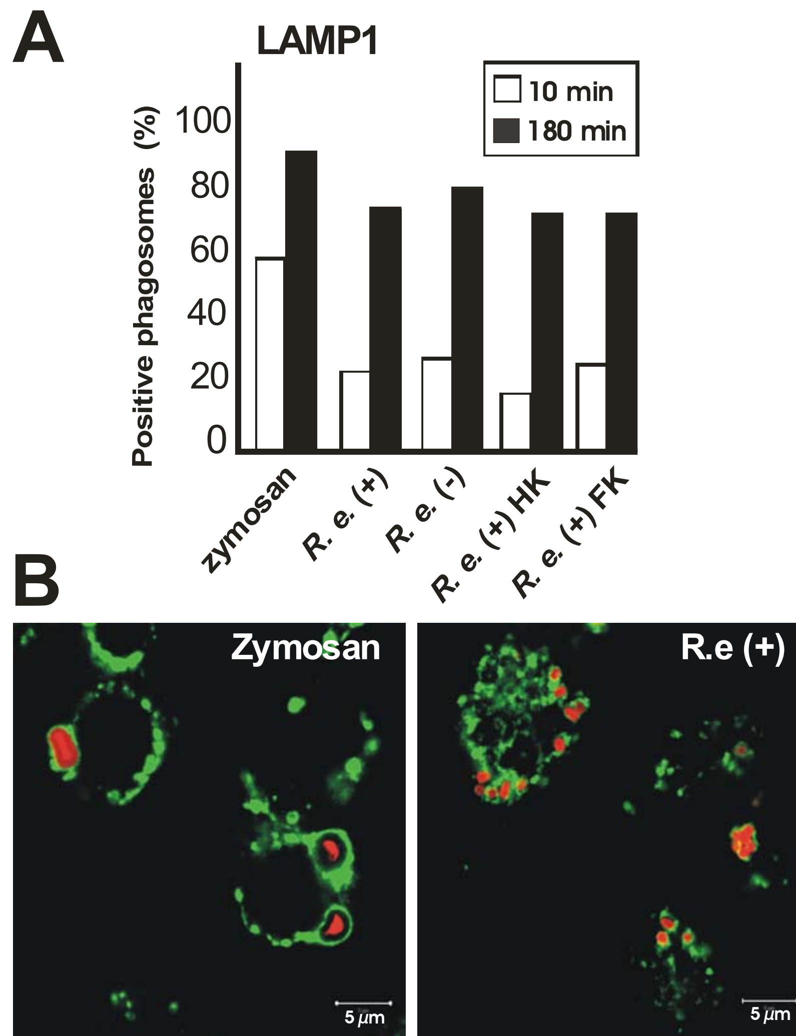

Figure 13: RCVs acquire the marker LAMP-1.
J774E were infected with prelabeled bacteria for 10 and 180 minutes. Samples were then fixed and immunofluorescence assays were performed to visualize the endocytic marker LAMP-1. Co-localization frequencies were quantified using CLSM and expressed in % of co-localization of particles with the marker. (A) Co-localization percentages of the following phagocytic probes in J774E cells: zymosan, live *R.equi* 33701+ [*R.e.*(+)], live *R.equi* 33701- [*R.e.*(-)], heat-killed *R.equi* 33701+ [*R.e.*(+)HK], and formaldehyde-killed *R.equi* 33701+ [*R.e.*(+)FK]. Data are the means of 2 experiments with a minimum of 50 phagosomes counted per experiment and sample. (B) CLSM micrographs of J774E stained with antibodies against LAMP-1, phagocytic probes are zymosan and live *R.equi* 33701+ [*R.e.*(+)], infection time: 180 min. Bars indicate 5µm.

The pattern of acquisition of LAMP-2 by RCVs in J774E and BMMs was similar to that obtained with LAMP-1. At 10 minutes postinfection in J774E, low levels of co-localization of LAMP-2 with RCVs were observed (Figure 14). Zymosan-containing phagosomes co-localized with LAMP-2 at higher levels (47%) compared with RCVs (19-28%). At 180 minutes postinfection, the majority of RCVs had acquired LAMP-2 (59-86%). Interestingly, phagosomes containing heat-killed *R.equi* (86%) or zymosan (98%) co-localized at higher levels at 180 minutes postinfection compared with phagosomes containing formaldehyde-treated bacteria (59%) or viable *R.equi* [60% for *R.equi*(+) and 55% for *R.equi*(-)]. Moreover, over the same infection period, vacuoles containing viable or formaldehyde-treated *R.equi* co-localized at higher levels (75-83%) with LAMP-1 than with LAMP-2 (55-60%), suggesting a different behaviour of the markers in J774E. At 24 hours postinfection in BMMs (Figure 11), more than 90% of RCVs co-localized with both markers (LAMP-1 and -2), showing a similar distribution. Thus, the results indicate that, in murine macrophages, the majority of RCVs acquire and retained the markers LAMP-1 and LAMP-2.

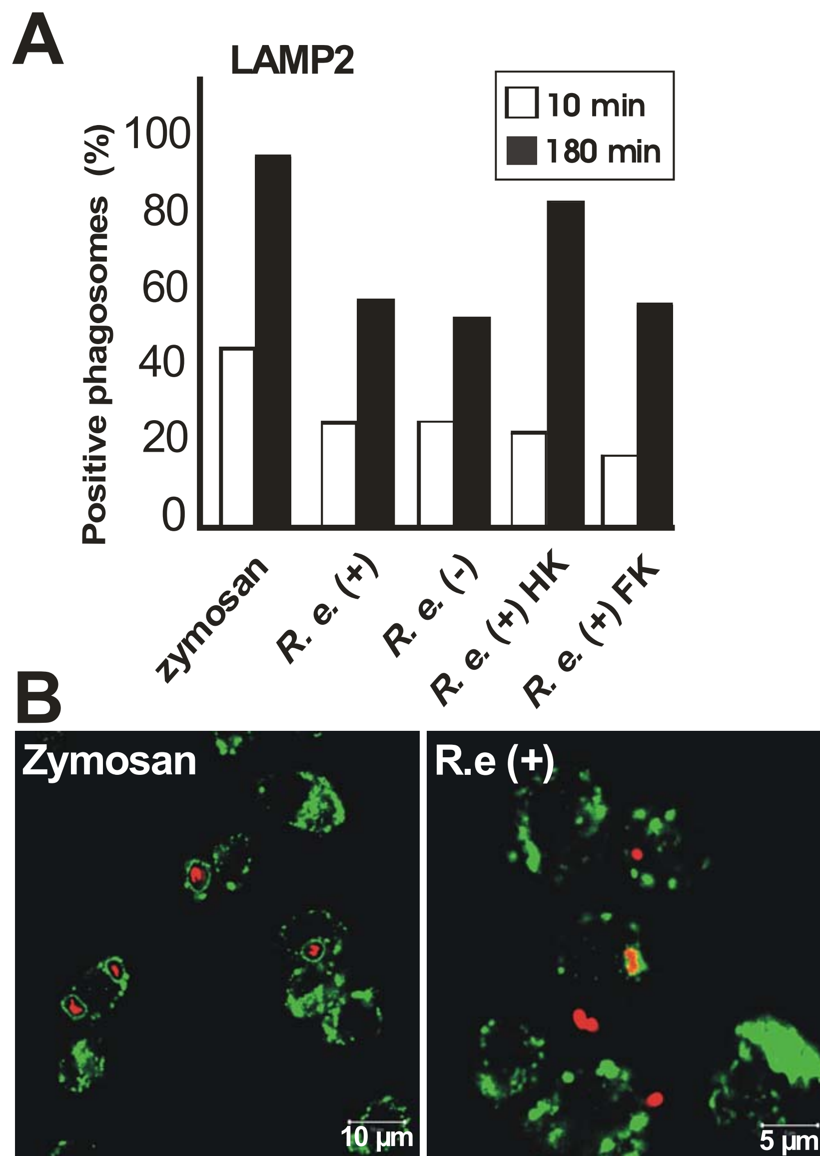

Figure 14: RCVs acquire the marker LAMP-2.
J774E were infected with prelabeled bacteria for 10 and 180 minutes. Samples were then fixed and immunofluorescence assays were performed to visualize the endocytic marker LAMP-2. Co-localization frequencies were quantified using CLSM and expressed in % of co-localization of particles with the marker. (A) Co-localization percentages of the following phagocytic probes in J774E cells: zymosan, live *R.equi* 33701+ [*R.e.*(+)], live *R.equi* 33701- [*R.e.*(-)], heat-killed *R.equi* 33701+ [*R.e.*(+)HK], and formaldehyde-killed *R.equi* 33701+ [*R.e.*(+)FK]. Data are the means of 2 experiments with a minimum of 50 phagosomes counted per experiment and sample. (B) CLSM micrographs of J774E stained with antibodies against LAMP-2, phagocytic probes are zymosan and live *R.equi* 33701+ [*R.e.*(+)], infection time: 180 minutes. Bars indicate 5µm.

3.4.4- Acquisition of LBPA by RCVs

The poorly degradable phospholipid lysobisphosphatidic acid (LBPA) is found in internal membranes of late endosomes and it is involved in protein and lipid trafficking through late endocytic compartments [Kobayashi *et al*. 1998, 1999, Matsuo *et al*. 2003]. Since LBPA is found exclusively in internal membranes of late endosomes [Kobayashi *et al*. 1998] it can be used as a marker to examine interactions of bacteria-containing phagosomes with these endocytic organelles. In this section, kinetics of acquisition of LBPA by RCVs was examined, using J774E as host cells.

The data obtained indicate that LBPA was initially excluded from RCVs, but the number of LBPA-positive RCVs increased over time (Figure 15). At 10 minutes postinfection, 7-8% of vacuoles containing all the *R.equi* tested [*R.equi*(+),*R.equi*(-), and *R.equi*(+) HK] were positive for the marker. However, at 60 minutes postinfection, a difference was observed between RCVs containing viable (21-34%) and heat-killed *R.equi* (46%). At 120 minutes, RCVs containing *R.equi*(+) or *R.equi*(-) showed a peak in LBPA staining (41-44%), while RCVs containing *R.equi*(+) HK showed a decrease in frequency of co-localization with LBPA (31%). These findings indicate that kinetics of association of LBPA is different in RCVs containing viable bacteria when compared with RCVs containing heat-killed bacteria: while phagosomes containing non-viable *R.equi* acquired rapidly this marker but then show a loss of it, RCVs containing viable *R.equi* acquire LBPA slowly but steadily. Unfortunately, BMMs did not stain well with anti-LBPA while J774E did, preventing 24 h analysis of LBPA co-localization. Acquisition of LBPA by RCVs containing *R.equi*(+) followed the same pattern than RCVs containing *R.equi*(-): they reside in vacuoles that acquired steadily LBPA.

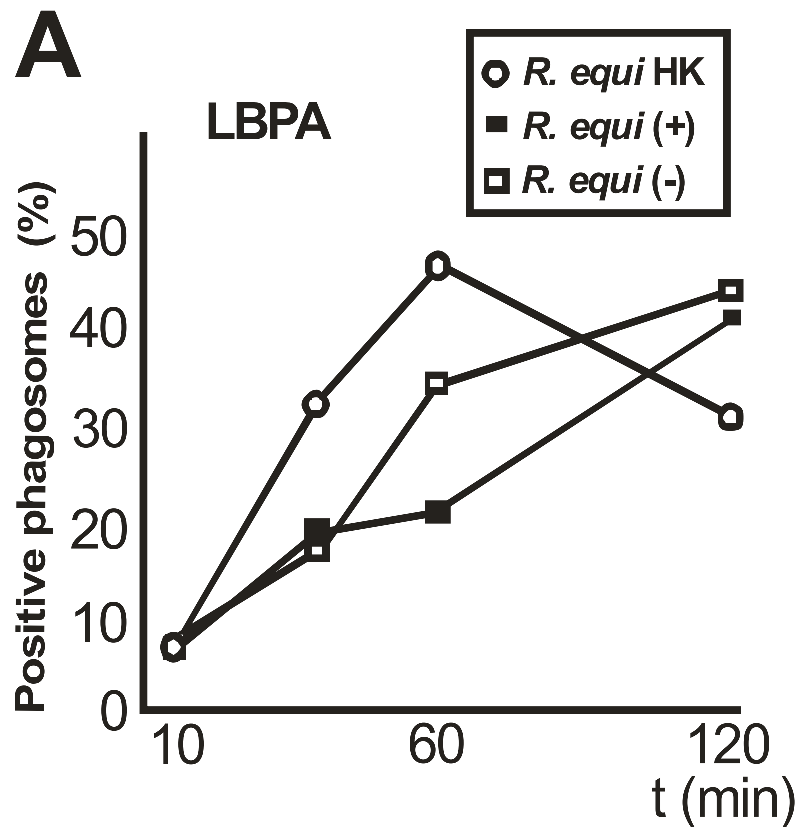

Figure 15A: Kinetics of acquisition of LBPA by RCVs.
J774E were infected with prelabeled bacteria for up to 120 minutes. Samples were then fixed and immunofluorescence assays were performed to visualize the endocytic marker LBPA. Co-localization frequencies were quantified using CLSM and expressed in % of co-localization of particles with the marker. (A) Co-localization percentages of the following phagocytic probes: live *R.equi* 33701+ [*R.equi*(+)], live *R.equi* 33701- [*R.equi*(-)], and heat-killed *R.equi* 33701+ [*R.equi*(+)HK]. Data are the means of 3 experiments with a minimum of 50 phagosomes counted per experiment and sample. Standard deviations from the mean were omitted for clarity, but never exceeded 7%.

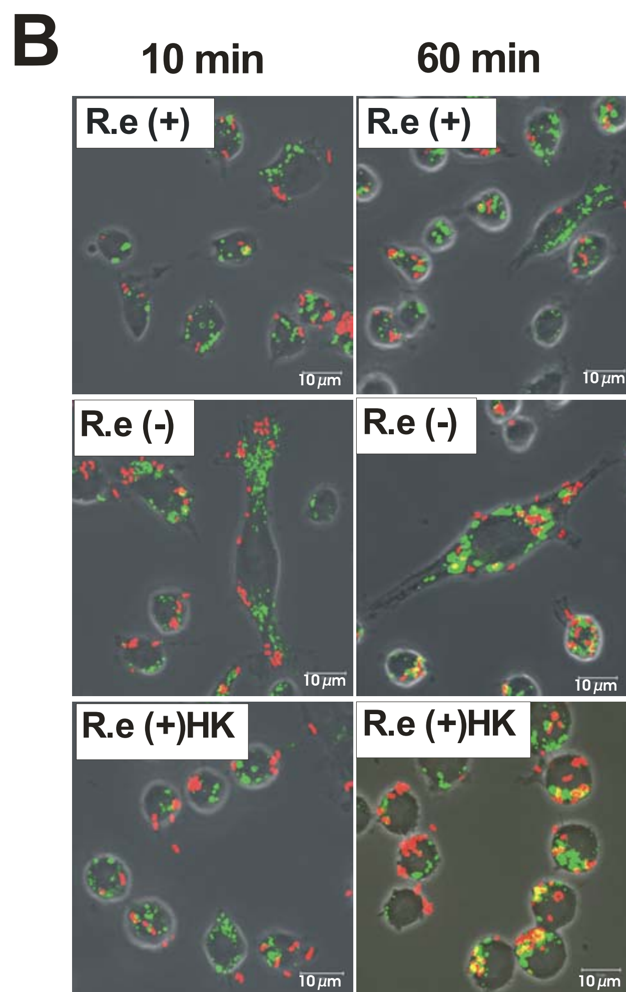

Figure 15B: Kinetics of acquisition of LBPA by RCVs.
CLSM micrographs of samples stained with antibodies against LBPA, phagocytic probes are the same as in (A). Bars indicate 10 µm.

3.4.5- Acquisition of vATPase by RCVs

The data obtained using the acidotropic probe LysoTracker (section 3.3) suggested that *R.equi* resides in phagosomes which are poor acidified. Since phagosome acidification is attributed principally to the activity of vATPases [Hackam *et al*. 1997], poor acidification in RCVs indicates either exclusion or inactivation of vATPases in the membrane of the vacuoles. To address these possibilities, the occurrence of vATPase in RCVs was assessed, using BMMs and J774E as host cells.

The results obtained suggest that, in BMMs, the majority of RCVs did not acquire vATPase (Figure 16). At 10 minutes postinfection, only 3% of all the samples tested co-localized with the marker, as expected for early phagosomes. At 180 minutes postinfection, still only 8% of RCVs enclosing live *R.equi*(+) co-localized with the vATPase, suggesting that the majority of mature RCVs lack the vATPase. When BMMs where infected with opsonized *E.coli*/GFP and the occurrence of vATPase was assessed, approximately 60% of opsonized *E.coli*/GFP-containing phagosomes co-localized with vATPase at 180 minutes postinfection (Figure 16B), as expected for normally maturing phagosomes [Baorto *et al*. 1997]. Thus, the results indicate that, in murine macrophages, the majority of *R.equi* resides in poor acidified vacuoles because they do not acquire vATPase. In addition, at 24 hours postinfection, the majority of RCVs lack vATPase, confirming that *R.equi* resides in vacuoles poor acidified that do not merge with lysosomes (Figure 11).

Interestingly, at 180 minutes postinfection in BMMs (Figure 16), 28% of RCVs containing heat-killed *R.equi*(+) co-localized with vATPase, but only 8% of *R.equi*(+), in agreement with the findings that RCVs enclosing heat-killed *R.equi* acquired the late endocytic markers calcein and dextran-texas red at higher percentages than live bacteria (section 3.2).

RCVs containing *R.equi*(-) co-localized with vATPase at higher levels than RCVs containing *R.equi*(+) (Figure 16). At 180 minutes postinfection, 15% of RCVs enclosing *R.equi*(-) co-localized with vATPase, but only 8% of RCVs containing *R.equi*(+) in agreement with the finding that, in J774E, RCVs containing *R.equi*(-) acquired lysosomal calcein, twice as often than *R.equi*(+). Together, the results suggest that factor(s) encoded by virulence-associated plasmid in *R.equi* may contribute to the establishment of RCV.

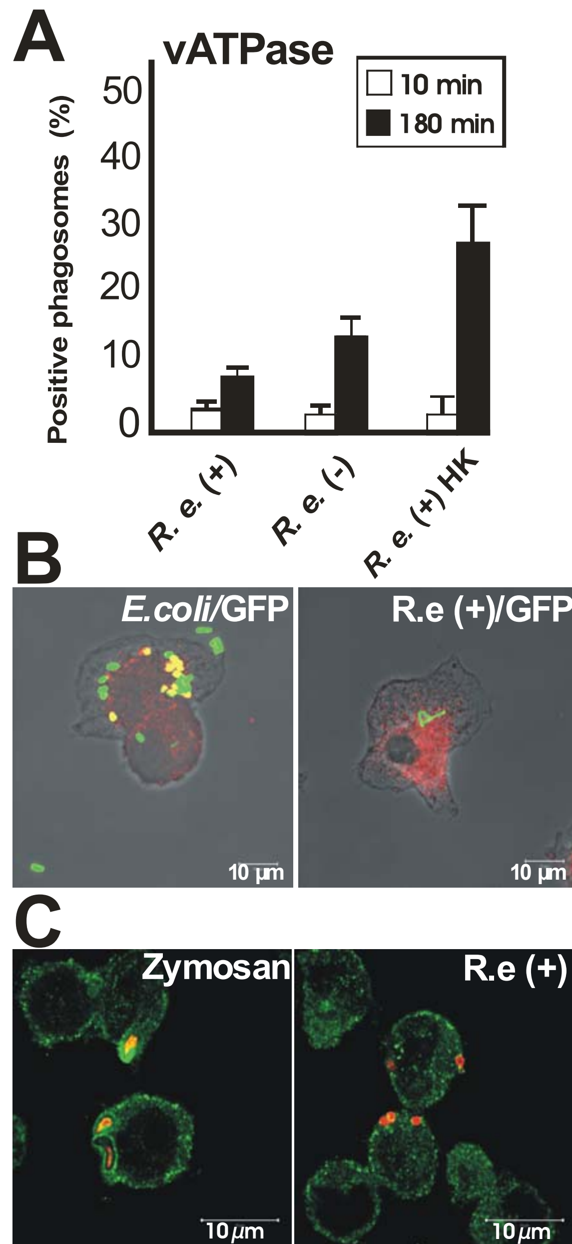

Figure 16A, B, C: Kinetics of acquisition of vATPase by RCVs.
BMMs or J774E cells were infected with prelabeled bacteria for up to 180 minutes. Immunofluorescence assays were performed to visualize vATPase. Co-localization frequencies were quantified using CLSM and expressed in % of co-localization of particles with the marker. (A) Co-localization percentages of the following phagocytic probes in BMMs: live *R.equi* 33701+ [*R.e.*(+)], live *R.equi* 33701- [*R.e.*(-)], and heat-killed *R.equi* 33701+ [*R.e.*(+)HK]. Data are the means of 3 experiments with a minimum of 50 phagosomes counted per sample.(B) CLSM micrographs of BMMs stained with antibodies against vATPase, phagocytic probes are: live *R.equi* 33701+/GFP [*R.e.*(+)/GFP] and *E. coli*/GFP, infection time: 180 minutes. (C) CLSM micrographs of J774E stained with antibodies against vATPase, phagocytic probes are: zymosan and live *R.equi* 33701+ [*R.e.*(+)], infection time: 180 minutes. Bars indicate 10µm.

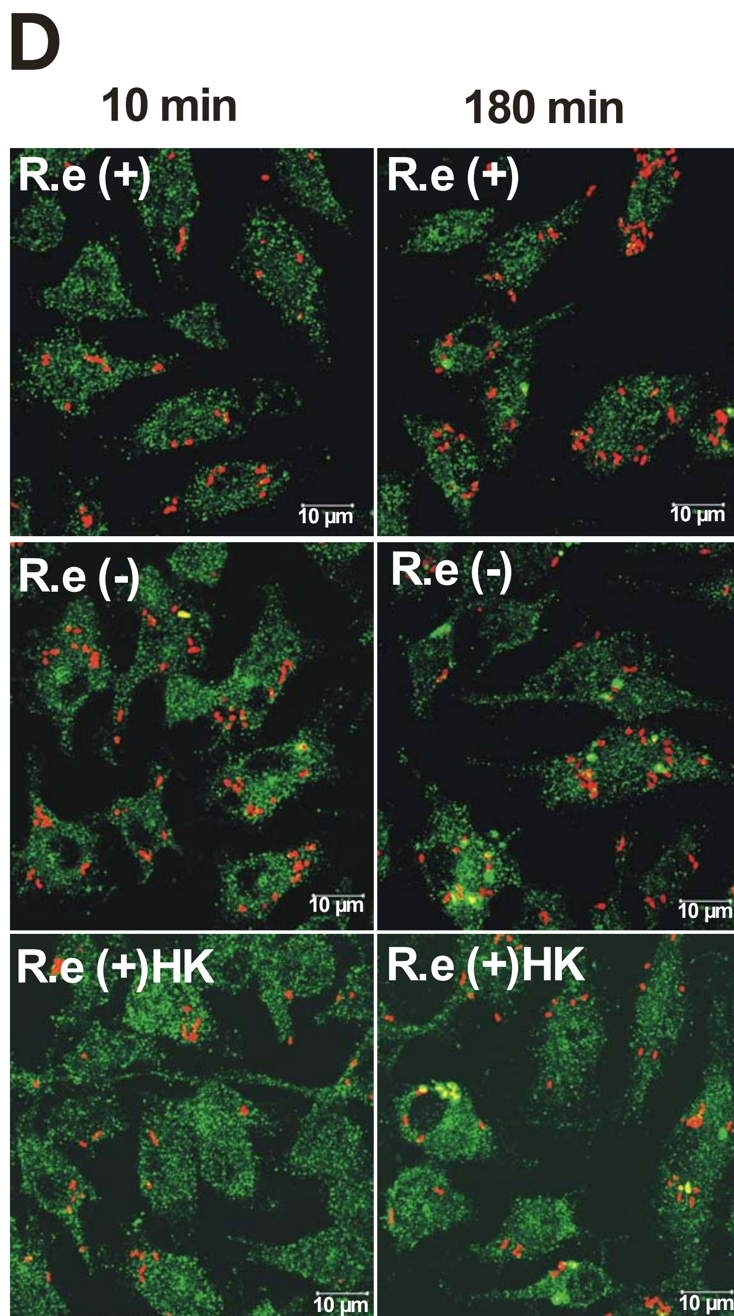

Figure 16D: Kinetics of acquisition of vATPase by RCVs.
CLSM micrographs of BMMs stained with antibodies against vATPase, phagocytic probes are the same as in (A). Bars indicate 10 µm.

3.5- Exogenously administered transferrin as a marker for sorting and recycling endosomes

The data obtained using immunofluorescence techniques to visualize the early endosomal marker TfR suggest that *R.equi* resides in vacuoles that lack this marker (section 3.4.2). To test this, *R.equi*-infected macrophages were incubated with fluorescent transferrin preparations, and potential acquisition of transferrin was studied using CLSM.

3.5.1- Co-localization of transferrin-alexa fluor with antibodies against transferrin receptor and LAMP-1

Several controls were performed in order to test whether transferrin-alexa fluor was properly internalized and did not traffic through late endosomes/lysosomes. First, non-infected cells were incubated in the presence of transferrin-alexa fluor and analysed by CLSM. Green fluorescent punctate structures were observed within compartments and also in the plasma membrane of the cells. That is the expected for transferrin-TfR complexes, which cycle between the plasma membrane and early endosomes [Dautry-Varsat *et al*. 1983; Sönnichsen *et al*. 2000]. When cells were incubated in the presence of a mixture of transferrin-alexa fluor and an excess of unlabeled holotransferrin (section 2.17.1), fluorescent transferrin was out-competed by unlabeled transferrin and no signal was observed within cells or in the cell surface. Moreover, co-localization of transferrin with TfR or LAMP-1 was tested in non-infected cells (section 2.17.1). In CLSM analysis, a sample was considered positive when the red signal coming from the antibodies totally covered the green fluorescence coming from transferrin-alexa fluor. As expected, transferrin-alexa fluor almost completely co-localized with TfR. No co-localization was observed between transferrin-alexa fluor and the late endosomal/lysosomal marker LAMP-1, indicating that transferrin-alexa fluor did not traffic through late endosomes and lysosomes (Figure 17B). These experiments suggest that transferrin-alexa fluor is internalized and follow a route similar to that expected for transferrin, without interacting with late endosomes/lysosomes.

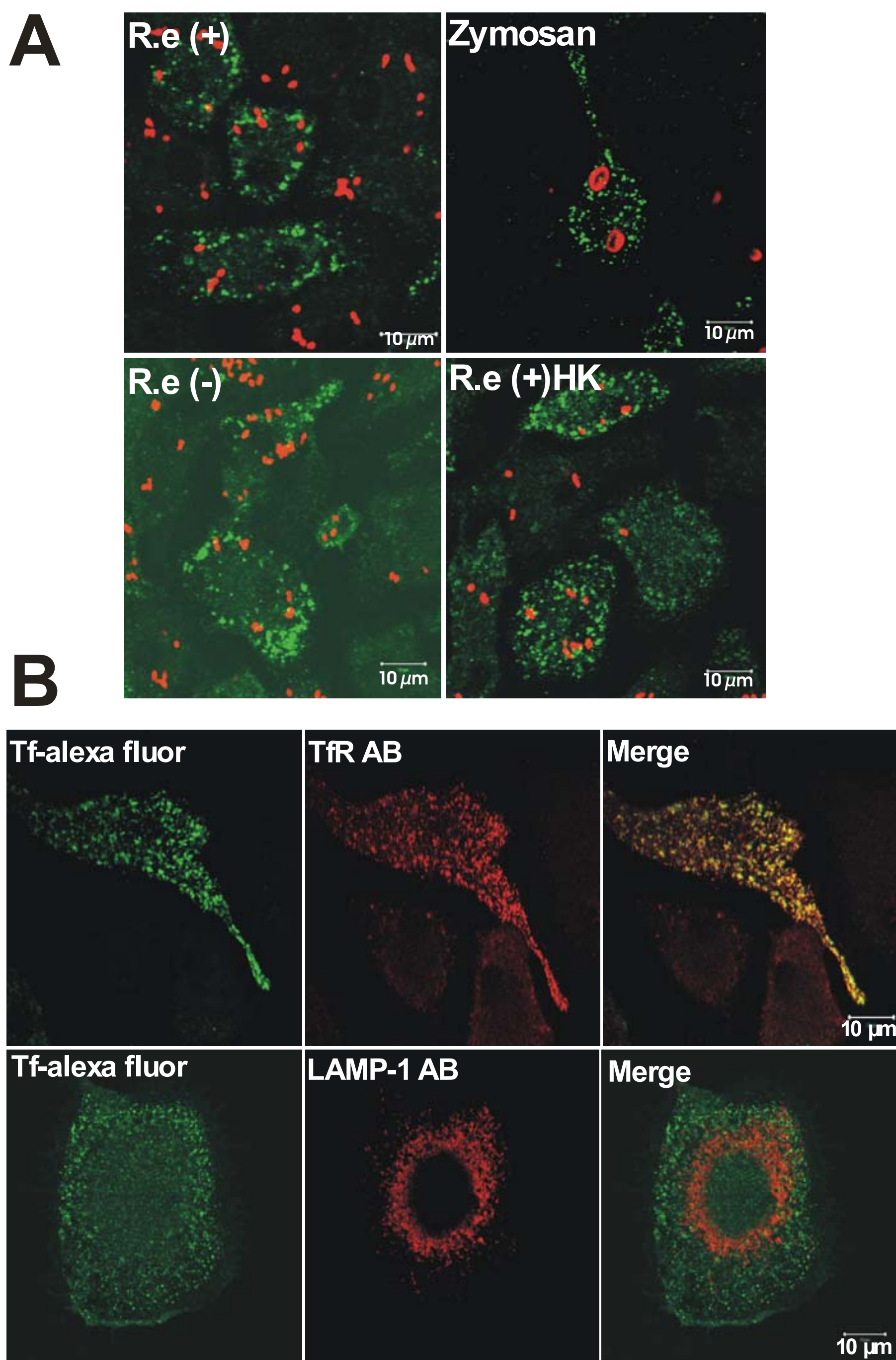

Figure 17: RCVs do not intersect with the transferrin recycling pathway.
BMMs were infected with *R. equi* for 2 h, cleared of loaded transferrin receptors, Alexa 488- labeled transferrin was added for 30 min, samples were fixed and co-localization between the marker and phagocytic probes was analysed using CLSM. (A) Transferrin-Alexa 488 and *R. equi* 33701(+) [*R.e.*(+)], *R.equi* 33701(-) [*R.e.*(-)], heat-killed *R.equi* 33701(+) [*R.e.*(+)HK], and zymosan (B) Transferrin-Alexa 488 and antibody-detected TfR (upper panel) or LAMP-1 (lower panel). Shown are single channels and the computer-assessed superimpositions (right panels). Same results were obtained in a second, independent experiment.

3.5.2- Access of RCV to exogenously administered transferrin

BMMs growing on coverslips were infected with prelabeled *R.equi* or zymosan for 30 minutes (section 2.17.2) and chased for 2 hours, followed by incubation at 4°C in the presence of holotransferrin coupled with alexa fluor to bind transferrin to TfR on the cell surface. Cells were incubated at 37°C for 10 minutes to allow the internalization of the receptor-ligand complexes, fixed, and analysed using CLSM, as described in section 2.14.2. Transferrin-alexa fluor would be delivered to RCVs only if these vacuoles were able to interact with TfR-positive endosomes, thus acquiring the TfR-transferrin-alexa fluor complexes.

At 2 hours postinfection, only approximately 2% of RCVs and phagosomes containing zymosan acquired transferrin-alexa fluor (Figure 17). No differences were observed between the samples tested [*R.equi*(+), *R.equi*(-) and *R.equi*(+) HK, and zymosan]. These results are similar with the finding that, at 2 hours postinfection within BMMs and J774E cells, the majority of RCVs lack TfR (section 3.4.2), independent from the viability of *R.equi* or the possession of the virulence plasmid. This indicates that, in murine macrophages, *R.equi* resides in phagosomes that do not interact with TfR/Tf-positive endosomes.

3.6- Compartment analysis of RCVs using EGFP fusion proteins

In the following sections, non-immune detection using Enhanced Green Fluorescence Protein (EGFP) was used to study the pattern of acquisition of different endocytic markers by RCVs. This approach was performed in order to avoid problems of antibody cross reaction with the surface of *R.equi* bacteria described in section 3.4. In addition, it allows the visualization of some proteins which are normally found in too low concentrations to be detected reliably by immunofluorescence.

Utilization of J774E and BMMs as host cells was hampered by the low efficiency of transfection obtained in both macrophage cell line and primary cells, limiting the number of transfected cells available for analysis. For this reason, two model systems were employed in the following sections: Raw 264.7 cells and CHO-FcRγII cells. Raw cells were chosen because, like J774E, they are murine professional phagocytes, and an efficient transfection procedure for this cell line was obtained using

the transfection reagent jetPEIman. CHO-FcRγII is a line of engineered phagocytes generated by stable transfection of cDNA encoding the human FcRγIIA receptor into normally non-phagocytic Chinese Hamster Ovary cells (CHO cells) [Downey *et al*. 1999]. The expression of Fcγ receptors on the surface of CHO cells confers this cell line the ability to internalize IgG-coated particles, and the resulting phagosomes mature in a manner similar to that of professional phagocytes [Indik *et al*. 1995; Downey *et al*. 1999]. This cell line was chosen because it is easier to transfect than myelomonocytic cell lines, giving relatively high levels of transient transfection, and it behaves like phagocyte cell lines. In order to evaluate whether the results obtained with transiently transfected Raw cells were influenced by activation of the macrophages, acquisition of fluorescent marker proteins by RCVs was also tested in CHO-FcRγII cells, which are engineered phagocytes and thus can not be activated. In addition, Raw cells and CHO-FcRγII cells have been used before to visualize the acquisition of GFP fusion proteins by phagosomes containing intracellular pathogens [Kagan *et al*. 2002; Vieira *et al*. 2003].

To determine the acquisition of EGFP fusion proteins by RCVs*,* monolayers of Raw 264.7 cells growing logarithmically on coverslips were transiently transfected using the transfection reagent jetPEIman (section 2.18.4). Pre-labeled bacteria or zymosan were given to the transfected cells and culture dishes were centrifuged at 4°C for 30 minutes. This centrifugation step allows bacteria to make close contact with mammalian cells and also helps to synchronize phagocytosis. Cold medium was then withdrawn and replaced for fresh warm medium at 37°C, being that set as 0 min. After incubating the samples at 37°C for up to 210 minutes, samples were fixed, mounted on slides and analysed using CLSM, as described in section 2.14.2. When CHO-FcRγII cells were used as host cells, cell monolayers growing logarithmically on coverslides were transiently transfected using Fugene 6, as described in section 2.18.6. Prelabeled bacteria or zymosan (previously opsonized with specific antibodies) were given to the cells, and culture dishes were centrifuged for 30 minutes at 4°C. Ingestion was then initiated by giving warm medium (37°C) to the cells. Samples were then incubated at 37°C for up to 210 minutes. Cells were then fixed, mounted on slides and analysed using CLSM, as described in section 2.14.2. In all cases, the presence of a green fluorescent ring surrounding the red labeled bacteria was considered a positive signal.

3.6.1- Acquisition of Rab/EGFP by RCV

Rab-GTPases are members of the Ras superfamily that regulate docking and fusion between different subcellular organelles [Pfeffer 1994, Clemens *et al*. 2000a, Sönnichsen *et al*. 2000]. One of the best studied Rab proteins on endosomal membranes is Rab5. It is required for the delivery of material from the plasma membrane to early endosomes as well as homotypic early endosome fusion [Sönnichsen *et al*. 2000, De Renzis *et al*. 2002]. Apart from Rab5, other Rabs have been also implicated in endosomal trafficking, e.g. Rab4, Rab11, and Rab7 [Bucci et al. 2000, Sönnichsen *et al*. 2000, De Renzis *et al*. 2002]. By analogy with the progression of the endocytic pathway, it is thought that Rab´s proteins regulate the sequence of fusion events that are part of the maturation process of phagosomes: Rab5 has been detected in early phagosomes, where it resides transiently [Desjardins *et al*. 1994a, Jahraus *et al*. 1998], and Rab7 is present at later stages of phagosome maturation, in late phagosomes [Desjardins *et al*. 1994b, Scianimanico *et al*. 1999].

Since Rab-GTPases play a pivotal role in regulation of membrane trafficking within eukaryotic cells, it was considered the possibility that an alteration in the acquisition of Rab´s proteins by RCVs could play a role in the altered development of these vacuoles. To test this hypothese, the distribution of the Rab-GTPases Rab5, Rab4, Rab11, and Rab7 in RCVs was examined, using infected Raw 264.7cells transfected with Rab/EGFP.

3.6.1.1- Co-localization of Rab/EGFP with antibodies against EEA-1 and LAMP-1

To test whether the distribution of Rabs/EGFP was the expected for these proteins, Raw 264.7 cells were transfected with Rab5/EGFP or Rab7/EGFP and immunofluorescence analysis was performed to detect EEA-1 and LAMP-1 (section 2.18.7).

Practically all compartments labeled with antibodies against EEA-1 also co-localized with Rab5/EGFP. Although the majority of Rab5/EGFP-labeled compartments co-localized with EEA-1, some Rab5-positives compartments that did not co-localize with EEA-1 were observed. In contrast, the great majority of compartments labeled with LAMP-1 antibodies did not co-localize with Rab5/EGFP, as expected (Figure 18A). Since Rab5 regulates binding of a large number of effector proteins including EEA-1, some Rab5-positive compartments are expected to be void of EEA-1. The majority of

Rab7/EGFP labeled compartments did not co-localize with EEA-1. In contrast, the majority of Rab7/GFP (but not all) co-localized with LAMP-1 (Figure 18B). LAMP-1 positive compartments that did not co-localize with Rab7/EGFP were also observed, as expected. LAMP-1, as a late endosomal/lysosomal marker, is expected to co-localize only partially with Rab7. Thus, immunofluorescence analysis confirmed that the distribution of Rabs/EGFP was similar than that expected for wild type Rabs.

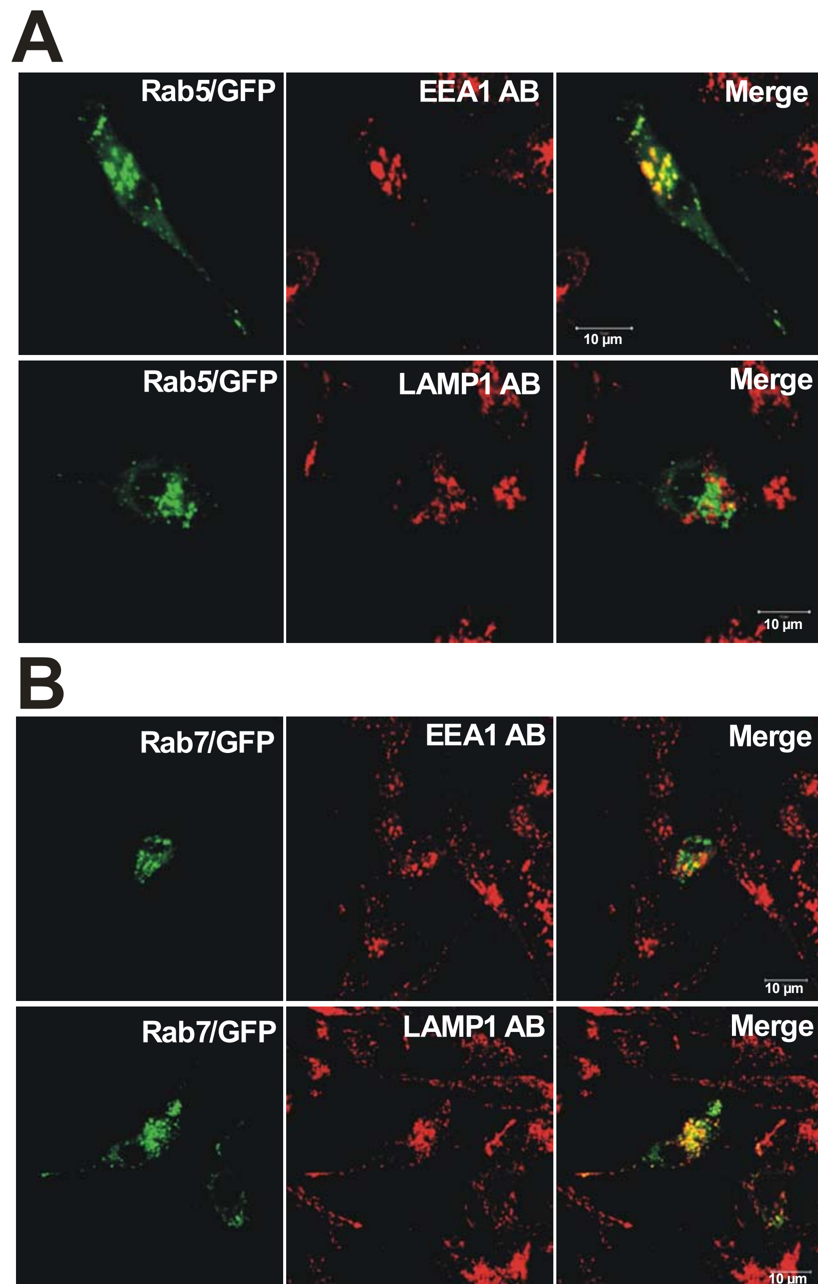

Figure 18: Co-localization of Rabs/EGFP with LAMP-1 and EEA-1.
RAW264.7 macrophage cells were transiently transfected with either EGFP-Rab5 or EGFP-Rab7. Samples were fixed and immunofluorescence assays were performed to visualize the endocytic markers LAMP-1 or EEA-1. Samples were analysed using CLSM. Shown are single channels and the computer assessed superimpositions (right panels). (A) Co-localization of EGFP-Rab5 with EEA-1 (upper panel) and LAMP-1 (lower panel). (B) Co-localization of EGFP-Rab7 with EEA-1 (upper panel) and LAMP-1 (lower panel). Bars indicate 10 µm.

3.6.1.2- Acquisition of Rabs/EGFP by RCVs

Analysis of acquisition of Rab5/EGFP and Rab7/EGFP by RCVs indicates that RCVs acquire transiently Rab5, and that loss of Rab5 is followed by acquisition of Rab7, which is retained by RCVs (Figure 19). While 33% of RCVs were positive for Rab5/EGFP at 5 minutes postinfection, only 6% were positive at 15 minutes, and the marker was completely absent at 120 minutes. The same result was obtained with control phagosomes containing zymosan. Rab7/EGFP was acquired as Rab5/EGFP was lost, with 86% of RCVs being positive at 60 minutes postinfection. Moreover, at 240 minutes postinfection, RCVs were still positive for Rab7/EGFP, suggesting that this marker is retained by these vacuoles. Zymosan-containing phagosomes acquired Rab7/EGFP faster than RCVs did, probably due to faster uptake of zymosan particles. Rab4/EGFP and Rab11/EGFP were only sporadically observed to label RCVs or control phagosomes (containing zymosan). The results obtained with transiently transfected RAW 264.7 macrophages were also in agreement with data obtained using Raw 264.7 cells stably transfected with Rabs/EGFP (section 2.18.5). Thus, the data obtained in this section suggest that RCVs acquire characteristics of early endosomes (visualized by acquisition of Rab5/EGFP), but quickly loose them, as they acquire Rab7/EGFP, which is retained at least for the first 240 minutes of infection. These results are supported by data obtained using immunofluorescence (section 3.4): RCVs acquire transiently the early endocytic marker EEA-1 followed by acquisition and retention of LBPA, LAMP-1 and LAMP-2. Together the data indicate that *R.equi* inhibit phagosome maturation at a stage downstream of the early phagosomal stage.

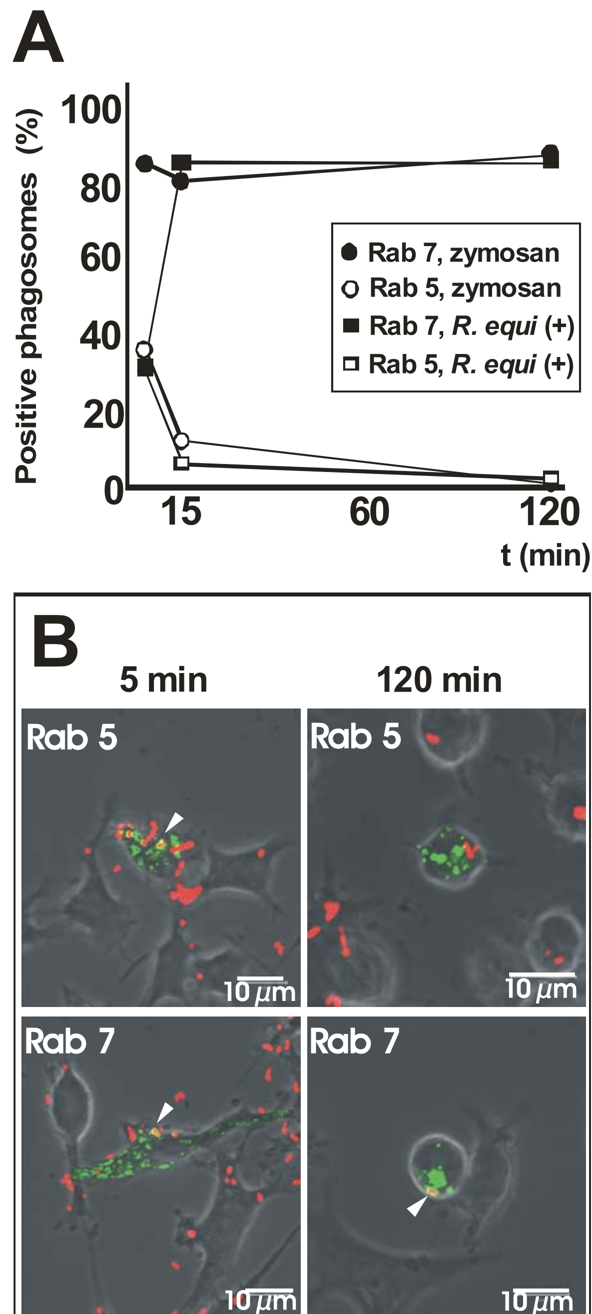

Figure 19: Rab5 is quickly lost by phagosomes which acquire Rab7 within minutes.
RAW264.7 macrophage cells were transiently transfected with either EGFP-Rab5 or EGFP-Rab7 and infected with either prelabeled zymosan or *R. equi* 33701(+) [*R.equi* (+)] for 5, 15, or 120 min. Samples were prepared for and analysed by CLSM. Closed arrowheads point to positive phagosomes. Data are the means of two independent experiments with a minimum of 50 phagosomes counted per experiment and sample. Bars indicate 10 µm.

3.6.2- Acquisition of LAMP-1/ EGFP by RCVs

The results obtained using immunofluorescence (section 3.4) suggest that, in murine macrophages, RCVs acquire gradually the late endosomal/lysosomal marker LAMP-1. To test whether RCVs acquire and retain this marker, kinetics of acquisition of LAMP-1/EGFP by RCVs were performed using transiently transfected Raw 264.7 cells and CHO-FcRγII cells. Control experiments were also performed in order to determine whether the distribution of LAMP-1/GFP resembles that observed in normal non-transfected cells.

3.6.2.1- Co-localization of LAMP-1/EGFP with antibodies against EEA-1 and LAMP-1

To determine whether the kinetics of acquisition of LAMP-1/EGFP by RCVs reflect the situation in non-transfected cells, distribution of LAMP-1/EGFP in transiently transfected Raw 264.7 cells was studied using antibodies against LAMP-1 and EEA-1 (section 2.18.7).

Cells expressing LAMP-1/EGFP contained green-labeled punctuate structures distributed within the cytosol of the cells which co-localized with antibodies directed against LAMP-1. In contrast, no co-localization was observed between the GFP fusion protein and EEA-1 antibodies. Thus, the results suggest that the distribution of the EGFP fusion protein LAMP-1 in transfected cells resembles the distribution of the wild type protein within macrophages.

3.6.2.2- Kinetics of acquisition of LAMP-1/EGFP by RCVs

Kinetics of appearance of LAMP-1/EGFP in RCVs is shown in Figure 20. At 5 minutes postinfection, only 2% of RCVs co-localized with LAMP-1/EGFP. However, as the infection period increased, RCVs acquired gradually LAMP-1/EGFP, being 72% at 60 minutes postinfection. Finally, at 120 minutes postinfection, the majority of RCVs (81%-84%) co-localized with LAMP-1/EGFP. No significant difference was observed when the acquisition of LAMP-1/EGFP by RCVs containing *R.equi*(+) and *R.equi*(-) was compared.

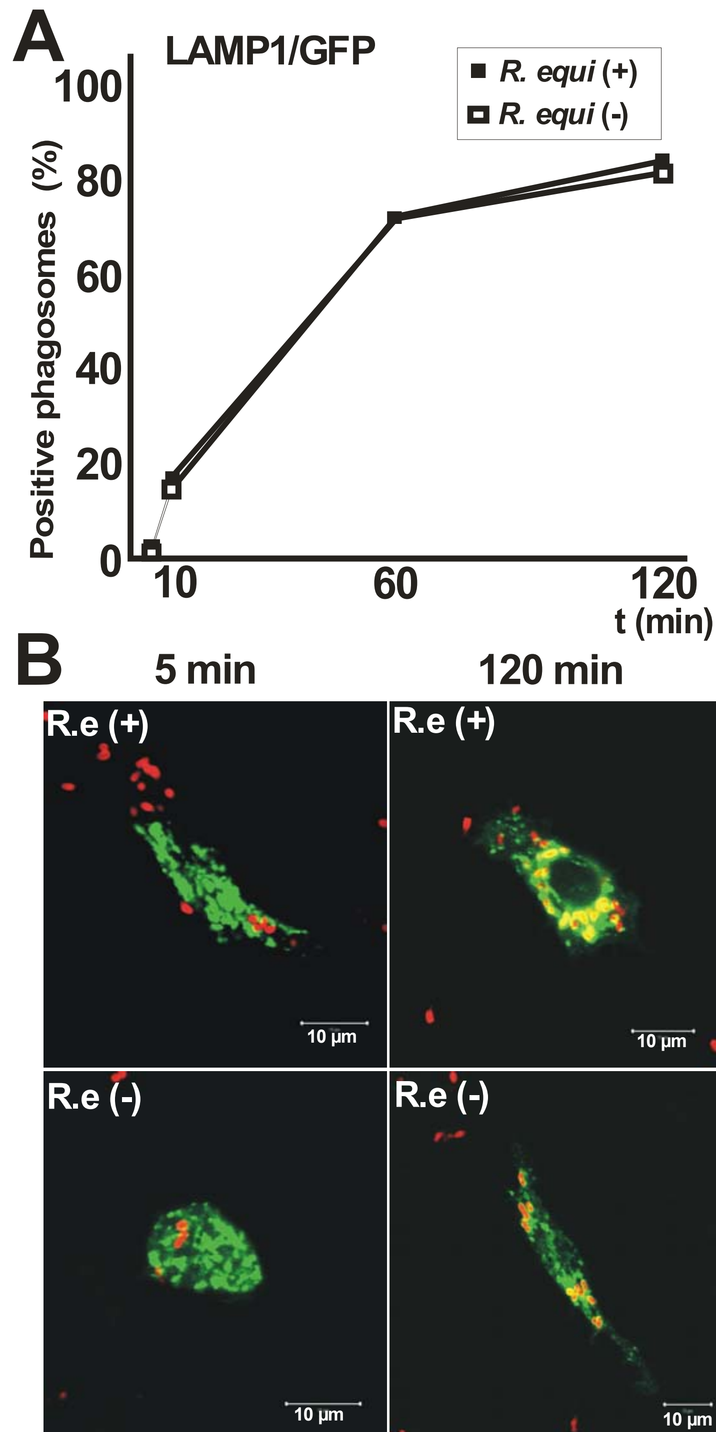

Figure 20: RCVs acquire LAMP-1/EGFP.
RAW264.7 macrophage cells were transiently transfected with LAMP-1/EGFP and infected with either *R. equi* 33701(+) [*R.equi* (+)] or *R. equi* 33701(-) [*R.equi* (-)] for 5, 10, 60, or 120 min. Samples were prepared for and analysed by CLSM. Co-localization frequencies were quantified using CLSM and expressed in % of co-localization of particles with the marker. (A) Co-localization percentages of the phagocytic probes described above. Data were obtained from 1 experiment with a minimum of 50 phagosomes counted per sample. (B) CLSM micrographs of Raw 264.7 macrophages transfected with LAMP-1/EGFP, phagocytic probes are the same described in (A). Bars indicate 10µm.

The same pattern was observed when CHO-FcRγII cells transiently transfected with LAMP-1/EGFP were used as host cells. At 10 minutes postinfection, approximately 2% of RCVs or zymosan-containing phagosomes co-localized with LAMP-1/EGFP, as expected for early phagosomes. However, at 120 minutes postinfection, approximately 90% of RCVs or zymosan-containing phagosomes co-localized with LAMP-1/EGFP (Figure 21).

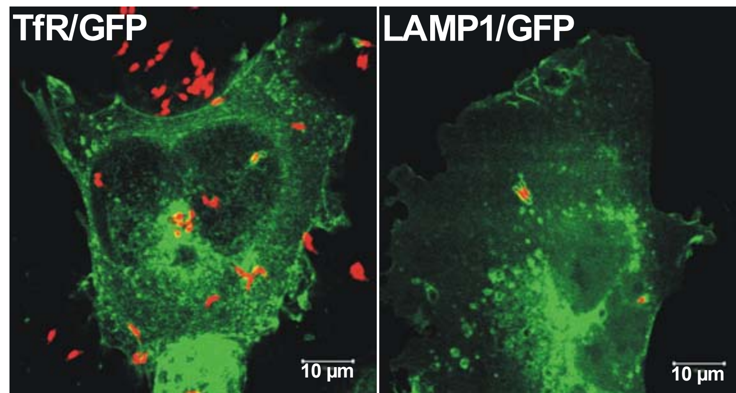

Figure 21: RCVs acquire LAMP-1/EGFP but not TfR/EGFP in CHO FcRγII cells.
CHO-FcRγII cells were transiently transfected with either LAMP-1/EGFP or TfR/EGFP and infected with opsonized *R. equi* 33701(+) for 120 minutes. Samples were prepared for and analysed by CLSM. Figure shows CLSM micrographs of CHO-FcRγII cells transfected with TfR/EGFP (left) LAMP-1/EGFP (right) and infected with *R. equi* 33701(+). Bars indicate 10µm.

3.6.3- Acquisition of transferrin receptor/EGFP with RCVs

The results described in section 3.4.2 using immunofluorescence analysis suggested that, in murine macrophages, *R.equi* resides in vacuoles that lack transferrin receptor. To test whether RCVs lack this marker, Raw 264.7 and CHO-FcRγII cells transiently expressing TfR/EGFP were used to determine the presence of this marker in the membrane of RCVs.

3.6.3.1- Co-localization of transferrin receptor/EGFP with antibodies against TfR and LAMP-1

In order to confirm that the distribution of the GFP fusion protein TfR resembles that observed for TfR in non-transfected cells, Raw 264.7 cells were transiently transfected using TfR/GFP and immunofluorescence assays were then made using antibodies to visualize TfR and LAMP-1, as described in section 2.18.7.

Cells expressing TfR/EGFP contained green-labeled punctuate structures that were distributed within the cytoplasm and the plasma membrane of the cells (Figure 22). That is the expected for TfR, which cycles between the plasma membrane and early endosomes [Dautry-Varsat *et al*. 1983; Sönnichsen *et al*. 2000]. All green fluorescence coming from the EGFP fusion protein co-localized with the red signal from the TfR antibodies. No co-localization was observed between TfR/EGFP and LAMP-1, as expected for TfR, which does not traffic to late endosomes/lysosomes. Thus, the distribution of TfR/EGFP appears to be similar of that observed with for TfR in non-transfected cells: this marker is present in the plasma membrane and also in compartments different of late endosomes/lysosomes.

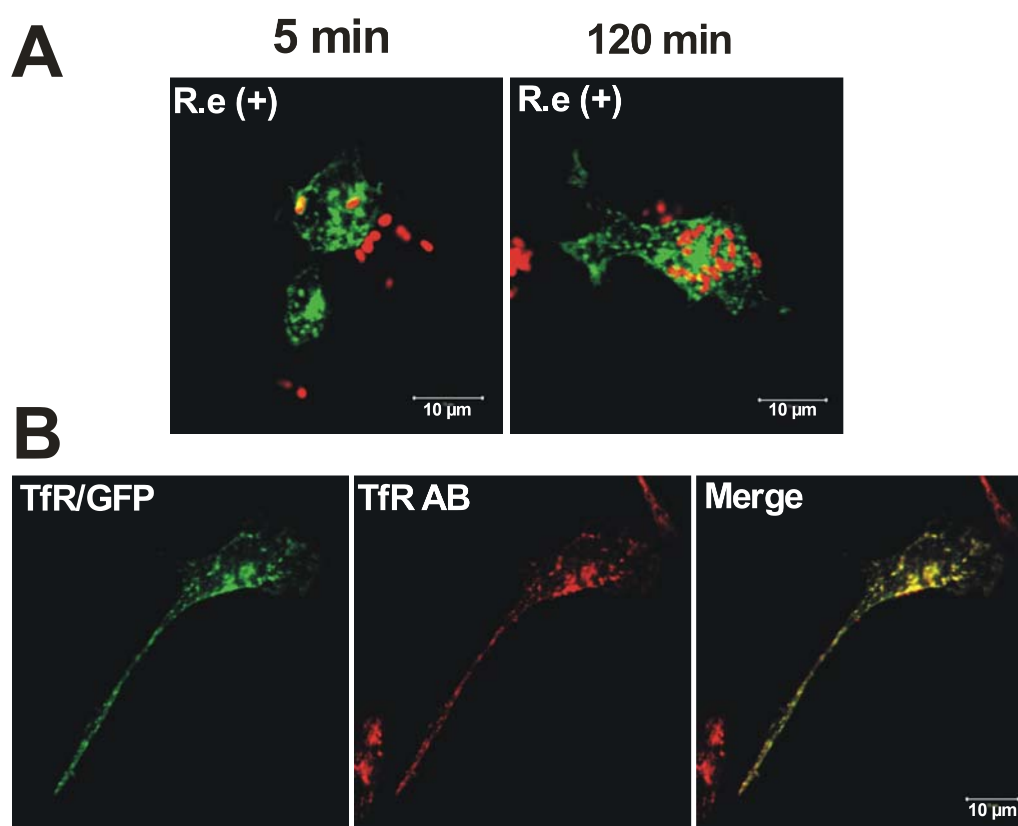

Figure 22 RCVs does not acquire TfR/EGFP.
RAW 264.7 macrophage cells were transiently transfected with TfR/EGFP and infected with *R. equi* 33701(+) [*R.e* (+)] for 5, 60, or 120 min. Samples were prepared for and analysed by CLSM. (A) CLSM micrographs of Raw 264.7 macrophages transfected with TfR/EGFP, phagocytic probes are the same described above. Data were obtained from 3 experiments with a minimum of 50 phagosomes counted per sample. (B) Co-localization of TfR/EGFP with antibodies against TfR. Shown are single channels and the computer-assessed superimpositions (right panels). Bars indicate 10 µm.

3.6.3.2- Acquisition of transferrin receptor/EGFP with RCVs

R.equi resides in vacuoles that lack transferrin receptor (Figure 22): at all time investigated, only 1-4% of RCVs co-localized with TfR/EGFP. The same result was observed using CHO-FcRγII cells as host cells (Figure 21). These results confirmed those obtained with immunofluorescence: after 120 minutes postinfection, *R.equi* resides in phagosomes that lack the early endosomal marker transferrin receptor.

3.6.4- Kinetics of acquisition of PtdIns3P by RCVs

The results obtained in previous sections indicated that, initially, the composition of RCV in murine macrophages resembles that of the host´s early endocytic compartments: in the first minutes of infection, RCVs have detectable levels of EEA-1 and Rab5. At later stages, RCVs acquire some of the proteins known to be present in late endosomes, but fail to merge with lysosomes. Thus, although rhodococal phagosomes acquire LAMP-1, LAMP-2, Rab7, and LBPA, they do not express vATPase and exclude fluid phase markers loaded into macrophage late endocytic compartments before establishment of an infection. Since the presence of PtdIns3P at early stages of phagosomal formation is critical for phagosome maturation [Vieira *et al*. 2003; Kelley & Schorey 2004], the relationship between RCVs and PtdIns3P formation and association with the vacuole was analysed. In this section, chimeric constructs of GFP and the PX domain of the 40-kDa subunit of NADPH oxidase (a PtdIns3P binding domain) were used to monitor the distribution and dynamics of PtdIns3P during the formation and progression of RCVs. This PX1/GFP construct was shown to bind avidly to PtdIns3P, having a distribution partially cytosolic and partially localized to EEA-1 positive structures [Ellson *et al*. 2001] In addition, several studies have used GFP coupled with PtdIns3P binding domains to monitor the distribution of PtdIns3P in phagosomes containing particles and also containing intracellular pathogens [Ellson *et al*. 2001, Scott *et al*. 2002, Kelley & Schorey 2004]. In this section, Raw 264.7 cells stably expressing the PX1/GFP protein were infected with prelabeled *R.equi* or zymosan, cells were centrifuged at 4°C for 30 minutes, and ingestion was initiated by warming up the samples, following by incubation at 37°C for up to 210 minutes. Samples were fixed, and analysed using CLSM, as described in section 2.18.9.

As expected for a PtdIns3P binding protein, the distribution of the PX1/GFP in non-infected Raw 264.7 cells was partially cytosolic and partially concentrated in punctuate structures. At 5 minutes postinfection, strong co-localization (51%-69%) was observed between phagosomes containing *R.equi* or zymosan and this GFP fusion protein (Figure 23). However, this association was only transient: at 60 minutes postinfection, between 5% and 22%of the phagosomes enclosing *R.equi* or zymosan did not co-localize with PX1/GFP, and at 120 minutes postinfection, between 0% and 6% of the phagosomes containing *R.equi* or zymosan associated with this GFP fusion protein. No significant difference was observed between all the samples tested [*R.equi*(+), *R.equi*(-), *R.equi*(+) HK or

zymosan-containing phagosomes]. These results are in agreement with those obtained using antibodies to visualize EEA-1 (section 3.4.1): RCVs acquired the early marker EEA-1 but only transiently, and this pattern was independent from the viability of *R.equi* or the possession of the virulence-associated plasmid. Thus, the results obtained using Raw 264.7 cells stably transfected with a PtdIns3P binding probe suggest that RCVs acquire PtdIns3P but only transiently and that this pattern of acquisition resembles that observed with model phagosomes.

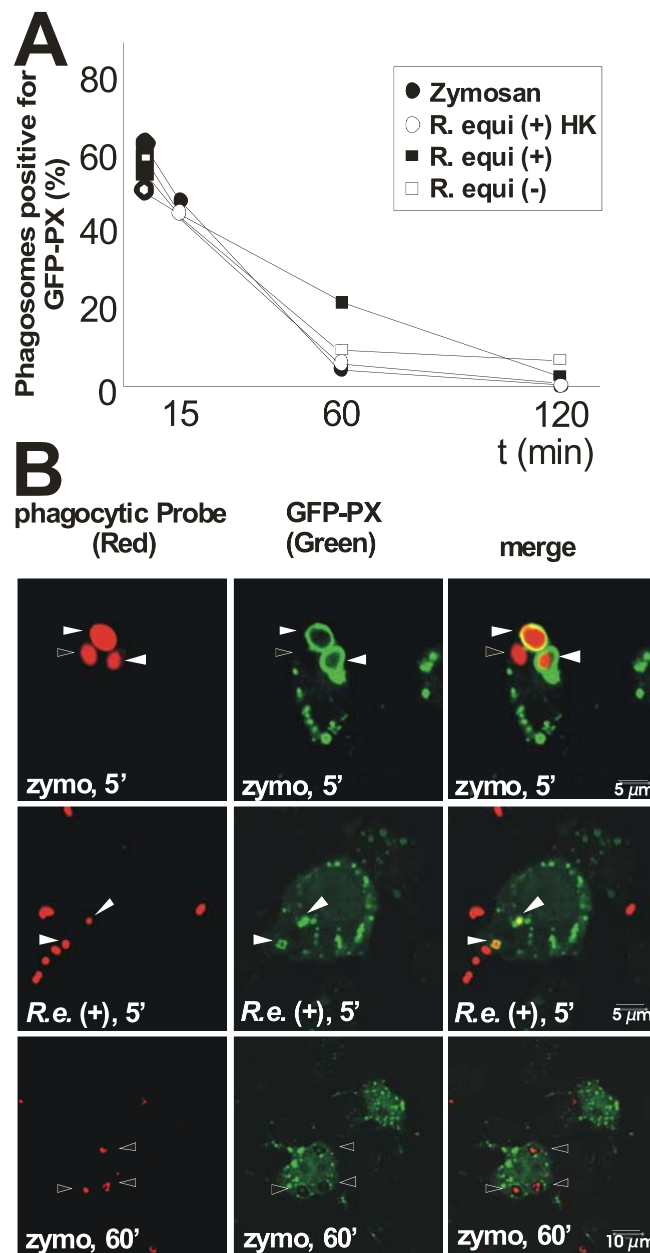

Figure 23: Phosphatidylinositol 3-phosphate [PtIns(3)P] is acquired by RCVs and lost within minutes.
RAW 264.7 macrophage cells stably expressing a fusion of EGFP to the PI(3)P binding phox[40] domain were infected for 5, 15, 60 or 120 min. Samples were fixed and prepared for fluorescence microscopy. Co-localization between EGFP fusion protein and phagocytic probes was quantified using CLSM. (A) Co-localization percentages of the phagocytic probes: live *R.equi* 33701+ [*R.equi.*(+)], live *R.equi* 33701- [*R.equi* (-)], heat-killed *R.equi* 33701+ [*R.equi* (+)HK], and zymosan. Data shown are the means of 3 independent experiments with a minimum of 50 phagosomes counted per experiment and sample. Standard deviations from the mean were omitted for clarity, and never exceeded 11 %.(B) Samples as analysed in (A) with R.equi 33701 [*R. e.*(+)] or zymosan shown in red (left panel), the PtIns(3)P specific probe in green (middle panel), and the computer-assessed superimpositions added (right panel). Closed arrowheads point at representative phagosomes positive for PtIns(3)P, open arrowheads mark negative phagosomes. Please note different sizing bars (5 or 10 µm) in upper and lower panels.

4- Discussion

Interactions between pathogens and phagocytic cells of the mononuclear and polymorphonuclear lineages are decisive for the development of an infection. Elimination of potential pathogens is effected primarily by phagocytosis, a process by which the pathogens are engulfed into a plasma membrane-derived organelle called phagosome. The environment within the phagosome gradually changes to generate degradative conditions, a process that is controlled by a succession of molecules that cooperate with each other and enable the maturing phagosome to fuse sequentially with early endosomes, late endosomes, and later with lysosomes, thus acquiring new properties [Desjardins 1994a]. This process is called phagolysosome biogenesis [Desjardins 1995]. Paradoxically, some pathogens take advantage of the environment within phagocytes to establish and maintain an infection: these pathogens are able to gain access to the phagocyte interior and, subverting the maturation process, become intracellular parasites. The fate of these intracellular parasites depends on their ability to establish an intracellular niche where they can hide from the immune system. Previous work has shown that *Rhodococcus equi* is an intracellular bacterium, which is able to inhibit phagolysosome formation in primary foal alveolar macrophages [Zink *et al*. 1987, Hietala & Ardans 1987]. In this work, the precise compartmentation of *R.equi* in murine macrophages was analysed using laser scanning confocal fluorescence and transmission electron microscopy methods.

4.1- RCV compartmentation: use of fluid phase endocytic markers

Previous studies indicated that, in primary foal alveolar macrophages, *R.equi* resides within membrane-enclosed vacuoles that do not fuse with lysosomes [Zink *et al*. 1987, Hietala & Ardans 1987]. This indicates that *R.equi* is capable to divert phagosome maturation. In order to analyse whether *R.equi* is able to inhibit phagolysosome formation, lysosomes of J774E-macrophages were labeled with the fluid-phase markers dextran-texas red or calcein, followed by infection with prelabeled bacteria. The results showed that the majority of RCVs failed to merge with lysosomes while most positive control phagosomes (containing zymosan) acquired these markers under the same conditions. In agreement with these data, purified RCVs did not contain appreciable amounts of lysosome acid β-galactosidase (Marco Polidori, personal communication). One possible explanation

for these results could be that *R.equi*, in the same way as *Listeria monocytogenes* [Goebel & Kuhn 2000], is able to disrupt the phagosome membrane and find its intracellular niche in the cytosol of the macrophage. However, transmission electron analysis of infected BMMs and J774E cells revealed that ingested *R.equi* was present within membrane-closed phagosomes, similar to the situation within primary foal alveolar macrophages [Zink *et al*. 1987, Hietala & Ardans 1987]. Therefore, *R.equi* is able to divert phagosome maturation.

In general, the percentages of co-localization of all the phagocytic probes with the lysosomal markers were higher in the assay using dextran-texas red (10000 Da) than in the assay using calcein (622 Da), possibly due to a slower clearance of dextran-texas red from late endosomes into lysosomes, since smaller solute probes move more efficiently and easier through the endocytic pathway than larger solutes [Berthiaume *et al*. 1995]. Both fluorophores were cleared into late compartments because they co-localized with LAMP-1 and -2, and were negative for EEA-1. Percentages of co-localization shown in the dextran labeling assay may be due to phagosome interaction with both late endosomes and lysosomes, whereas co-localization shown in the calcein-labeling assay mainly due to phagosome-lysosome interacting only.

Most intracellular parasites remain within membrane bound vesicles for the entirety of their intracellular replication. Some of these parasites are able to arrest the maturation of their phagosomes in a pre-lysosomal stadium, but maintain communication with the endocytic pathway, situation that enables them to gain access to nutrients. This maturation arrest can occur at an early phagosomal stage, as the case of phagosomes containing *Mycobacterium tuberculosis* [Sturgill-Koszycki *et al*. 1996], or at a late phagosomal stage, as the case of *Salmonella*-containing phagosomes [Brumell & Grinstein 2004]. Others manipulate the traffic of organelle membranes to take up residence in a non-phagosomal organelle which is completely isolated from the endocytic pathway. This is the case with *Legionella pneumophila* [Joshi *et al.* 2001]. The ability of *R.equi* to inhibit fusion of its phagosome with lysosomes suggests that this bacterium can alter normal phagosome maturation. RCVs could, however, have access to material from the endocytic pathway, or be completely isolated from it. To study these two possibilities, J774E-macrophages were first infected with prelabeled bacteria and then the fluid-phase marker dextran-texas red was used to label the endocytic pathway. The results showed that RCVs maintained interaction with the endocytic pathway: 62% of RCVs acquired the

endocytic marker, a percentage similar of that obtained with control phagosomes containing zymosan. Therefore, *R.equi* is able to block phagosome maturation, but resides in vacuoles that remain in contact with or are part of the endocytic pathway. Interestingly, vacuoles containing viable *R.equi* co-localized more frequently with the endocytic marker than vacuoles containing formaldehyde-killed or heat-killed *R.equi*. Phagosomes usually become less fusogenic, the older they are. Acording to this, phagosomes containing dead *R.equi*, which acquired more lysosomal markers than RCVs containing viable *R.equi*, interacted lesser with the endocytic organelles and probably took longer to acquire the endocytic marker than RCVs containing viable *R.equi*. Another possible explanation for this may be that viable *R.equi* promote fusion of their vacuoles with endocytic organelles, and by this way bacteria may acquire nutrients. In contrast, vacuoles containing non-viable bacteria may be less fusogenic than vacuoles containing viable *R.equi*, thus acquiring less endocytic marker.

4.2- RCV compartmentation: use of EGFP-constructs and immunofluorescence

Together, the data obtained using fluid-phase markers suggest that *R.equi* belongs to the intracellular pathogens that are able to arrest normal phagosome maturation and maintain communication with the endocytic pathway. However, RCVs may display early endocytic characteristics, such as *Mycobacterium tuberculosis*-containing phagosomes [Sturgill-Koszycki *et al*. 1996], or characteristics of late phagosome-like stage, like *Salmonella*-containing phagosomes [Brumell & Grinstein 2004]. To analyse the exact compartment composition of RCVs in murine macrophages, acquisition of different early and late endocytic markers by RCVs was analysed in infected J774E and BMMs, using immunofluorescence and non-immune detection with Enhanced Green Fluorescence Protein (EGFP) fusion proteins. As BMMs and J774E cells are poorly transfectable [Kelley & Schorey 2003], the better transfectable RAW 264.7 murine macrophage-like cell line was used in EGFP experiments.

Immunofluorescence data showed an acquisition of early endosomal Rab5, EEA-1 and PtdIns3P, followed by a loss of these markers within minutes, clearly suggesting initially undisturbed phagosome maturation. Kinetic dissection of phagosome maturation showed that Rab5 was acquired by RCVs shortly after uptake and quickly lost again: while 33% of RCVs acquired the marker at 5 minutes postinfection, the marker was completely lost by 120 minutes. The same was observed with control phagosomes containing zymosan, in agreement with the transient association of Rab5 observed with

latex bead phagosomes [Vieira *et al*. 2003]. In addition, analysis of association of phosphatidylinositol 3-phosphate (PtIns3P) by RCVs suggested a pattern of acquisition and loss of the marker which resembled that observed by model phagosomes: at 5 minutes postinfection, approximately 60% of the RCVs contained PtIns3P, but this percentage decreased over time, and after 120 minutes all phagosomes were void of the marker. In the same manner, the early endocytic marker EEA-1 was acquired transiently by RCVs: 27% of RCVs were positive with the marker at 5 minutes postinfection, but only 5% at 60 minutes. Interestingly, the percentage of *R.equi*(+)-containing vacuoles positive for EEA-1 at 120 minutes (17%) was higher than the percentage at 60 minutes (5%), suggesting accumulation of this marker over time. EEA-1, a Rab5 effector, serves as a critical organelle-tethering molecule by bridging membranes for fusion [Simonsen *et al*. 1998, Christoforidis *et al*. 1999a], thus contributing to the fusion process. EEA-1 recruitment and association with endosomal membranes is achieved by interactions of this molecule with Rab5, and is strengthened by the binding of its FYVE domain to PtdIns3P [Simonsen *et al*. 1998]. However, analysis of association of Rab5 and PtdIns3P in RCVs clearly indicated that both molecules are acquired only transiently, suggesting undisturbed phagosome maturation. In addition, immunoblotting analysis of isolated RCVs showed an accumulation of EEA-1 at 10 minutes postinfection, but at 120 minutes, the marker was absent from the isolated RCVs, and the same was observed with the early marker coronin (Marco Polidori, personal communication). Posterior acquisition of late endocytic markers by RCVs also suggested that inhibition of phagosome maturation may be downstream the early-phagosomal stage. Finally, analysis of EEA-1 at 24 hours of infection clearly indicated that RCVs are devoid of EEA-1, confirming that this marker is acquired but not retained by RCVs. Thus, the increase of RCVs positive for EEA-1 at 120 minutes observed using immunofluorescence is unlikely to be related with inhibition of phagosome maturation. The above data suggested that RCVs acquire early sorting endosome marker characteristics transiently. However, RCVs could interact with the early recycling endosome system, like the phagosomes containing *Mycobacterium tuberculosis* do. They accumulate transferrin receptors (TfR), are positive for Rab11, and have access to externally added transferrin [Clemens & Horwitz 1995, Clemens & Horwitz 1996, Fratti *et al.* 2000]. To test whether RCVs interact with recycling endosomes, the occurrence of different recycling endosomal markers was assessed. The results clearly indicated that RCVs did not communicate with recycling endosomes: they lacked the recycling endosomal markers Rab4, Rab11, and TfR at all times tested (for up to 2 hours), and failed to accumulated externally added transferrin. Altogether, analysis of acquisition of early markers by

RCVs clearly indicated undisturbed early phagosome maturation and suggested that the inhibition of phagosome maturation may involve a step downstream the early-phagosomal stage. Similar to *R.equi*, the intracellular pathogens *Brucella abortus* [Pizarro-Cerdá *et al*. 1998, Chaves-Olarte *et al*. 2002] and *Salmonella enterica* serovar Typhimurium [Steele-Mortimer *et al*. 1999] are able to arrest phagosome maturation downstream the early phagosomal stage, thus acquiring early endocytic markers only transiently.

Study of late endosomal markers indicated that RCVs failed to acquire a typical late endocytic composition. Immunofluorescence analysis showed that while control phagosomes acquired the vATPase complex, RCVs did not, even after 24 hours of infection. This finding was corroborated with analysis of phagosome acidification using the acidotropic substance LysoTracker Red DND-99: at 2 hours postinfection only 13% of all RCV were positive for LysoTracker, a percentage that very well agrees with the small percentage of RCV that acquired the proton pump (8%), and also with the percentage that received lysososomal calcein (9%). Acidification of the phagosome is a critical component of the microbicidal response: low pH is directly toxic to many microorganisms and enhances the efficiency of other bactericidal mechanisms, such as the efficiency of lytic enzymes. Phagosomal acidification has been attributed largely to the activity of the vATPase complex [Hackam *et al*. 1997], which is incorporated into the phagosome through fusion with pre-existing acidic late endosomes or through fusion of early endocytic compartments with Golgi-derived vesicles [Fratti *et al*. 2003]. Thus, the lack of this enzyme complex in RCVs may be critical for the fate of the phagosome. Besides *R.equi*, two other members of the pathogenic actinomycetales, *Mycobacterium tuberculosis* [Sturgill-Koszycki *et al*. 1994] and *Nocardia asteroides* [Black *et al*. 1986] are able to inhibit acidification of their phagosomes. It has been suggested that a specific mycobacterial glycolipid, mannose-capped lipoarabinomannan (ManLAM), alters phagosome maturation by blocking the delivery of cargo from the TGN, thus preventing the acquisition of vATPase and lysosomal enzymes, such as the immature form of cathepsin D [Fratti *et al*. 2003]. Because the three members of actinomycetales that have shown to inhibit phagosome acidification (*R.equi, M.tuberculosis, and N.asteroides*) possess unusual, mycolic acid-containing glicolipids, a mechanism based on lipid-mediated inhibition of phagosomal acidification seems possible.

RCVs acquired and retained LAMP-1 and LAMP-2: while few RCVs stained positive for LAMP-1 and LAMP-2 at 10 minutes postinfection, the majority did at 180 minutes, at a frequency similar to that obtained with control phagosomes. Moreover, most RCVs stained positive for LAMP-1 and -2 after 24 hours of infection, suggesting that the markers are retained by RCVs. Although LAMP-1 and -2 are widely used as late endosomal/lysosomal markers, both markers are also present in *trans*-Golgi network, in early endosomal compartments [Rohrer *et al*. 1996], and on the surface of macrophages [Fukuda 1991, Barker *et al*. 1997]. Thus, intracellular pathogens that are able to arrest the normal maturation process of their phagosomes, may acquire LAMP-1 and -2 independent of fusion with late endosomes. Accordingly, pathogenic *Mycobacterium* species reside in phagosomes that are clearly early endocytic organelles, but are positive for LAMP-1 [Barker *et al*. 1997; Schaible *et al*. 1998, Kuehnel *et al*. 2001]. Interestingly, while LAMP-1 is, at 180 minutes postinfection, acquired equally by all phagosomes tested, LAMP-2 was acquired by fewer RCVs (60%) than positive control phagosomes (98%). At 24 hours postinfection in BMMs, the majority of RCVs co-localized with both markers, showing a similar co-localization percentage (approximately 90%). These findings can be interpreted in two ways. First, there may be a difference in the trafficking routes of LAMP-1 and -2, as already observed in cells infected with *Mycobacterium paratuberculosis* [Kuehnel *et al*. 2001]. According to this, despite the difference observed in J774E-macrophages, the pattern of acquisition of the markers in BMMs could be the same. Second, kinetics of acquisition of LAMP-2 may be slower than that of LAMP-1. Consistent with this, at 180 minutes postinfection, a difference in the percentages of co-localization of LAMP-1 and -2 in RCVs was observed, but when the infection time increases (e.g. 24 hours) this difference no longer exists.

Analysis using EGFP fusion proteins showed that the late endosomal GTPase Rab7 was acquired by RCVs as the early endosomal Rab5 was lost (86% positive at 15 minutes). Moreover, the majority of RCVs was still positive for Rab7 at 4 hours of infection, suggesting that this marker is retained by RCVs. Zymosan-containing phagosomes acquired Rab7 faster than RCVs did, probably due to faster uptake of zymosan particles. Despite the role of Rab7 in late endosome-lysosome fusion [Bucci *et al.* 2000], the acquisition of this GTPase alone is not sufficient to mediate this process: *Legionella*- and *Mycobacterium*-containing phagosomes in HeLa cells overexpressing Rab7 are still able to inhibit phagosome maturation, suggesting that, beside Rab7, other molecules may be necessary to interact with late endosomes/lysosomes [Clemens *et al*. 2000b]. Moreover, the authors proposed an

intermediate compartment between the classical early and late compartments, rich in Rab7 but devoid of typical late and lysosomal markers [Clemens *et al*. 2000b]. In another study using phagosomes containing sheep red blood-cells, Vieira *et al*. [2003] also suggest that the presence of Rab7 in the phagosomal membrane is not sufficient to mediate fusion of the phagosomes with late endosomes and lysosomes. These authors proposed a phagosomal stage in which Rab7 is present in the phagosomal membrane but fusion with late endosomes is still to occur, suggesting that a possible phagosomal Rab7 source may be a non-late endosomal, Rab7-positive organelle. Hence, acquisition of Rab7 by RCVs does not necessarily indicate a typical late endosome stadium. Instead, RCVs could have characteristics of an intermediate compartment between early and late phagosomal-maturation stadium, positive for Rab7 but negative for other late endocytic markers.

The kinetics of association of LBPA, a lipid marker for multivesicular bodies (MVBs), differed between RCVs containing viable and heat-killed *R.equi*. While RCVs containing heat-killed *R.equi* showed a peak of acquisition of the marker at 60 minutes postinfection followed by a drop in the percentage of positive phagosomes, RCVs containing viable bacteria acquired low level for staining, but showed clearly an increase in concentration over time. Lysobisphosphatidic acid (LBPA) is abundant in internal membranes of multivesicular bodies (MBVs), and is involved in protein and lipid trafficking through late endosomes [Kobayashi *et al.* 1998]. Hence, the increase in LBPA by RCVs containing viable *R.equi* may correspond to the deposition of intravacuolar vesicles, as RCVs interact with MVB, in agreement with the presence of internal vesicles in RCVs observed using electron microscopy.

4.3- RCV vs other priviledged compartments

Altogether, the analysis of acquisition of endocytic markers by RCVs presented in this work indicates that RCV is a compartment which has left the early phagosomal-maturation stage but fails to acquire a typical late endocytic composition: all early endosomal markers tested (Rab5, EEA-1, and PtdIns3P) are acquired only transiently by RCV, followed by acquisition of some late endocytic and/or lysosomal markers (LAMP-1, LAMP-2, Rab7, and LBPA) but exclusion of others (vATPase, lysosomal fluid-phase markers). Moreover, compartmentation does not change appreciably once RCVs have passed the early endosome-like stage: RCVs lack EEA-1, TfR, and vATPase, but the frequency of vacuoles positive for LAMP-1 and LAMP-2 increases with time. These above findings are in nice agreement with data obtained using purified phagosomes: at 10 minutes postinfection, RCVs were positive for EEA-1 and coronin (also known as TACO protein) and negative for LAMP-1, Rab7, and vATPase. At 2 hours, isolated RCVs were devoid of all early endosomal markers tested as well as of vATPase and cathepsin D, but positive for LAMP-1 and Rab7 (Marco Polidori, personal communication). Table 1 summarizes all the data obtained in this study. The arrested phagosome stage displayed by RCVs shares some features with vacuoles containing either of two other intracellular pathogens: *Salmonella enterica* serovar Typhimurium (*S*. Typhimurium), or *Mycobacterium tuberculosis*. Early stages in the maturation of *Salmonella*-containing vacuoles resemble those observed with control phagosomes and with RCVs: they acquire Rab5, EEA-1, and accumulate PtIns3P [Scott *et al*. 2002, Brumell & Grinstein 2004]. These early markers are rapidly lost from the vacuoles containing *Salmonella*, which possess then characteristics of a intermediate phase of phagosomal maturation: they acquire Rab7 (but only transiently), LAMP-1 and LAMP-2 [Meresse *et al.* 1999a], but fail to accumulate LBPA [Brumell *et al*. 2001], mannose-6-phosphate receptors [Garcia-del Portillo & Finlay 1995], and processed cathepsin D [Mills & Finlay 1998]. However, in contrast to *Rhodococcus*, *Salmonella* reside in vacuoles that acquire vATPase and acidify quickly [Brumell & Grinstein 2004]. Like RCVs, vacuoles containing *Mycobacterium tuberculosis* exclude vATPase, fail to acidify, and acquire LAMP-1, but, in contrast to RCVs, are positive for Rab5, TfR, and acquire exogenously administered transferrin [Russell 2001].

Table 1- Results of the studies to determine RCV compartmentation

Compartment[1]	Protein/lipid investigated	t(min)	Mammalian cell[2]	Immunofluorescence		Immunoblotting[4]	
				R.equi	***R.equi* HK[3] or zymosan**	***R.equi***	***R.equi* HK[3] or *L.innocua***
SE	Coronin-1	10	J774E	ND[5]	ND[5]	+	+
	EEA-1	5	BMMs	+	+	ND[5]	ND[5]
		10	BMMs	+	+	+	+
		15	J774E	+	ND[5]	ND[5]	ND[5]
		120	BMMs, J774E	-(J774E) +(BMMs)	-	-	-
		24 h	BMMs	-	-	ND[5]	ND[5]
	Rab4 (EGFP)	5-120	Raw 264.7	-	-	ND[5]	ND[5]
	Rab5 (EGFP)	5	Raw 264.7	+	+	ND[5]	ND[5]
		15	Raw 264.7	-	-	ND[5]	ND[5]
		120	Raw 264.7	-	-	ND[5]	ND[5]
	PtdIns3P (EGFP)	5	Raw 264.7	++	++	ND[5]	ND[5]
		15	Raw 264.7	++	++	ND[5]	ND[5]
		60	Raw 264.7	+	+	ND[5]	ND[5]
		120	Raw 264.7	-	-	ND[5]	ND[5]

[1]SE: early ,'sorting' endosome, RE: early ,'recycling' endosome, LE: late endosome, lys: (phago)lysosome; [2]BMMs: bone marrow-derived macrophages, Raw 264.7: Raw 264.7 macrophage cell line (in transfection experiments), J774E: J774E macrophage cell line; [3]*R.equi*(+) heat-killed; [4]Data provided by Marco Polidori; [5]ND: no data available; [6]Both EGFP constructs and specific antibodies were used in fluorescence microscopy; [7]Live *E.coli* were used as control organism; [8]Loading of macrophage lysosomes with this compound before infection; [9]Enzymatic activity in purified phagosomes.

Table 1- (cont.)

Compartment[1]	Protein/lipid investigated	t(min)	Mammalian cell[2]	Immunofluorescence		Immunoblotting[4]	
				R.equi	*R.equi* HK[3] or zymosan	*R.equi*	*R.equi* HK[3] or *L.innocua*
RE	Rab11 (EGFP)	5-120	Raw 264.7	-	-	ND[5]	ND[5]
	TfR (also EGFP)[6]	10	BMMs, J774E, CHO-FcRγII	-	-	ND[5]	ND[5]
		120	BMMs, J774E, CHO-FcRγII	-	-	ND[5]	ND[5]
		24 h	BMMs	-	-	ND[5]	ND[5]
	Transferrin added	120	BMMs	-	-	ND[5]	ND[5]
LE	LBPA	10	J774E	-	-	ND[5]	ND[5]
		30	J774E	+	++	ND[5]	ND[5]
		60	J774E	+	+++	ND[5]	ND[5]
		120	J774E	++	++	ND[5]	ND[5]
	vATPase	10	BMMs	-	-	-	-
		120	BMMs, J774E	*R.e*(+): - *R.e*(-): +	++	*R.e*(+): - *R.e*(-): +	+
		24 h	BMMs	-	ND[5]	ND[5]	ND[5]

[1]SE: early ,'sorting' endosome, RE: early ,'recycling' endosome, LE: late endosome, lys: (phago)lysosome; [2]BMMs: bone marrow-derived macrophages, Raw 264.7: Raw 264.7 macrophage cell line (in transfection experiments), J774E: J774E macrophage cell line; [3]*R.equi*(+) heat-killed; [4]Data provided by Marco Polidori; [5]ND: no data available; [6]Both EGFP constructs and specific antibodies were used in fluorescence microscopy; [7]Live *E.coli* were used as control organism; [8]Loading of macrophage lysosomes with this compound before infection; [9]Enzymatic activity in purified phagosomes.

Table 1- (cont.)

Compartment[1]	**Protein/lipid investigated**	**t(min)**	**Mammalian cell[2]**	**Immunofluorescence**		**Immunoblotting[4]**	
				R.equi	***R.equi* HK[3] or zymosan**	***R.equi***	***R.equi* HK[3] or *L.innocua***
LE	LAMP-1 or LAMP-2 (also EGFP)[6]	10	J774E, BMMs, CHO-FcRγII, Raw 264.7	+	+	-	-
		120	J774E, CHO-FcRγII, Raw 264.7	++	++	++	++
		180	BMMs, J774E, CHO-FcRγII, Raw 264.7	++	++	ND[5]	ND[5]
		24 h	BMMs	++	++	ND[5]	ND[5]
	Rab7 (EGFP)	5	Raw 264.7	+	+++	ND[5]	ND[5]
		10	J774E	ND[5]	ND[5]	-	+
		15	Raw 264.7	+++	+++	ND[5]	ND[5]
		120	J774E, Raw 264.7	+++	+++	++	++
		240	Raw 264.7	+++	ND[5]	ND[5]	ND[5]

[1]SE: early ,'sorting' endosome, RE: early ,'recycling' endosome, LE: late endosome, lys: (phago)lysosome; [2]BMMs: bone marrow-derived macrophages, Raw 264.7: Raw 264.7 macrophage cell line (in transfection experiments), J774E: J774E macrophage cell line; [3]*R.equi*(+) heat-killed; [4]Data provided by Marco Polidori; [5]ND: no data available; [6]Both EGFP constructs and specific antibodies were used in fluorescence microscopy; [7]Live *E.coli* were used as control organism; [8]Loading of macrophage lysosomes with this compound before infection; [9]Enzymatic activity in purified phagosomes.

Table 1- (cont.)

Compartment[1]	Protein/lipid investigated	t(min)	Mammalian cell[2]	Immunofluorescence		Immunoblotting[4]	
				R.equi	*R.equi* HK[3] or zymosan	*R.equi*	*R.equi* HK[3] or *L.innocua*
Lys	LysoTracker	120	J774E	-	++[7]	ND[5]	ND[5]
	dextran-texas red[8]	120	J774E	-	++	ND[5]	ND[5]
	Calcein[8]	120	J774E	-	++	ND[5]	ND[5]
	Acid β-galactosidase[9]	120	J774E, BMMs	ND[5]	ND[5]	-	++

[1]SE: early ,'sorting' endosome, RE: early ,'recycling' endosome, LE: late endosome, lys: (phago)lysosome; [2]BMMs: bone marrow-derived macrophages, Raw 264.7: Raw 264.7 macrophage cell line (in transfection experiments), J774E: J774E macrophage cell line; [3]*R.equi*(+) heat-killed; [4]Data provided by Marco Polidori; [5]ND: no data available; [6]Both EGFP constructs and specific antibodies were used in fluorescence microscopy; [7]Live *E.coli* were used as control organism; [8]Loading of macrophage lysosomes with this compound before infection; [9]Enzymatic activity in purified phagosomes.

4.4- RCV maturation and plasmid status

Inhibition of phagolysosome formation has been proposed to confer *R.equi* the ability to multiplicate inside host cells [Zink *et al*. 1987, Hondalus & Mosser 1994]. In macrophages, intracellular growth of *R.equi* is associated with the presence of a 15-17 kDa protein termed VapA [Hondalus & Mosser 1994, Jain *et al*. 2003], which is encoded by a plasmid of approximately 85 kb [Takai *et al*. 1993, Tan *et al*. 1995]. Moreover, the presence of the "VapA encoding plasmid" is associated with full virulence in foals and mice [Takai *et al*. 1991c, Giguere *et al*. 1999a]. Therefore, it is possible that the virulence-associated antigen VapA play an important role in the establishment of RCVs. However, this is still to be determined because, when the two studies that showed that *R.equi* is able to inhibit fusion of its vacuole with lysosomes were conducted [Zink *et al*. 1987, Hietala & Ardans 1987], the virulence-associated plasmid and its products were as yet unidentified. Another plasmid type, the "VapB encoding plasmid", possesses intermediary virulence between plasmid-less and VapA positive strains in experimental foal [Takai *et al*. 2000a] or mouse [Takai *et al.* 1995] infection. Analysis of the role of

factor(s) encoded by the virulence-associated plasmids in the establishment of RCVs is important to understand the virulence mechanisms employed by *R.equi*. For this purpose, intracellular compartmentation of virulent *R.equi* strains bearing virulence-associated plasmids and their cured avirulent partners in murine macrophages were compared. Interestingly, analysis of *R.equi* strain ATCC 33701 (containing a VapA-expressing plasmid) and its cured-plasmid partner showed only a modest dependency on the plasmid status of *R.equi* for the establishment and/or maintenance of RCVs, as kinetics of acquisition of EEA-1, PtdIns3P LAMP-1 and LAMP-2 were similar for either vacuole. However, RCVs containing *R.equi* 33701(-) co-localized more frequently with vATPase and lysosomal fluid phase markers than RCVs containing *R.equi* 33701 (+). The exclusion of vATPase from *R.equi*(+) has also been observed in biochemical experiments (Marco Polidori, personal communication). These results suggested that more *R.equi*(-)-containing vacuoles matured into phagolysosomes than those containing *R.equi*(+), contributing to the increased percentage of vATPase and lysosomal markers observed when RCVs containing *R.equi*(-) were analysed.

These data suggested that at least one factor encoded by the virulence-associated plasmid of *R.equi* may be directly or indirectly involved in the establishment of RCVs, and chromosomal factors may also contribute. Analysis of phagosomes containing different strains bearing either VapA- or the VapB-expressing plasmids showed no significant differences in acquisition of lysosomal calcein between plasmid-bearing and plasmid-cured bacteria-containing phagosomes. However, there was a difference in acidification with RCVs containing strain 103(+) (18% were positive for LysoTracker) and RCVs containing strain 103(-) (34% positive). This discrepancy in the results obtained using lysosomal calcein and LysoTracker may reflect differences in the sensitivity of the 2 methods. LysoTracker remains trapped in acidified organelles, labeling compartments with acidic pH, such as lysosomes. In contrast, the majority of calcein should label only lysosomes. Differences would then be explained by a late endosomal and lysosomal vs only lysosomal localization of RCVs. In summary, the plasmid status does not affect phagosome trafficking over a two hour period of infection with the notable exception of vATPase, which tends to be more abundant on RCVs containing plasmid-less strains.

4.5- Vitality of bacteria and compartmentation

For many intracellular pathogens, viability is required for inhibition of phagosome maturation. Oh & Straubinger [1996] showed that, while phagosomes containing viable *Mycobacterium avium* were able to inhibit phagosome maturation and did not acquired lysosomal markers, the majority of phagosomes containing non-viable *M.avium* acquired lysosomal markers and thus matured into phagolysosomes. Similar studies have demonstrated the role of viability in the establishment of intracellular niches of different parasites, such as *Mycobacterium marinum* [Barker *et al*. 1997], *Salmonella typhimurium* [Hashim *et al*. 2000], *Afipia feli*s [Lührmann *et al*. 2001], *Listeria monocytogenes* [Alvarez-Dominguez *et al*. 1997], *Brucella suis* [Naroeni *et al*. 2001], or *Brucella abortus* [Arenas *et al*. 2000]. To analyse whether bacterial viability is required for the establishment of RCVs, *R.equi* bacteria were killed before infection, and the intracellular compartmentation of the bacteria was then assessed. The data obtained showed that neither heat- nor formaldehyde-killing of bacteria before infection changed intracellular compartmentation dramatically: while acquisition and loss of early markers followed the same pattern in RCVs containing viable and non-viable *R.equi*, co-localization with vATPase and lysosomal fluid-phase markers were approximately three times as much in vacuoles containing non-viable *R.equi* than in those containing viable bacteria. However, the majority of RCVs containing non-viable *R.equi* were still able to prevent acquisition of these markers. In agreement with this result, a previous study also reported that non-viable (formaldehyde-killed) *R.equi* was able to prevent phagolysosome formation to the same extent as viable *R.equi* did [Zink *et al*. 1987]. RCVs containing dead *R.equi* quickly acquired LBPA, a marker for MVBs, followed by a slight loss. Vacuoles containing viable *R.equi* acquired LBPA slowly but steadily. This suggests that, although viability is not strictly required to inhibit phagolysosome formation, it may contribute for the establishment and maintenance of RCVs. All data obtained in this work suggests that, heat-insensitive factors, such as lipids in the bacterial cell periphery, may play a major role in the establishment of the RCV, although a heat sensitive factor may also be involved. Killing of *Nocardia asteroides*, another member of the pathogenic actinomycetales does not hamper its capacity to prevent phagosome maturation either [Black *et al*. 1986]. Because both *Rhodococcus* and *Nocardia* possess unusual glycolipids in their cell envelope, lipid-mediated mechanisms may be plausible.

4.6- Is the RCV an endocytic compartment?

The above data indicate that *R.equi* resides in phagosomes that maintain communication with the endocytic pathway, and have left the early-endosomal stage but do not acquire all the typical characteristics of late endosomes. Alternatively, *R.equi* could promote association of its vacuole with host organelles such as mitochondria or endoplasmic reticulum, and by this way acquiring nutrients for survival and multiplication, such as the case of *Brucella abortus*. Within macrophages, *Brucella*-containing phagosomes acquire characteristics of early endosomes transiently [Chaves-Olarte *et al*. 2002]; follow by acquisition of the late endosomal/lysosomal LAMP-1 but not cathepsin D [Moreno & Gorvel 2004]. *Brucella*-containing phagosomes do not mature into phagolysosomes [Frenchick *et al*. 1985, Naroeni *et al*. 2001], but instead, fuse with cisterns of the endoplasmic reticulum, where the bacteria establish their replication niche [Moreno & Gorvel 2004]. Moreover, at later infection times, *R.equi* could eventually be able to escape into the cytoplasm by rupturing its phagosome, like *Listeria monocytogenes* [Smith *et al*. 1995], *Shigella flexneri* [High *et al*. 1992], or *Rickettsia conorii* [Gouin *et al*. 1999]. To analyse the development of the RCV over time, transmission electron microscopy of infected primary BMMs and J774E cells was performed. At 2 hours postinfection, all *R.equi* organisms were found in membrane-surrounded compartments which contain one or two bacteria and approximately 50% of the vacuoles containing viable *R.equi* had either tightly apposed membranes or loosely fitting membranes that contained numerous membranous vesicles. Furthermore, *R.equi* organisms were never found free in cytoplasm (only after 24 hours in decaying macrophages). These results agree with previous data using foal alveolar macrophages, where the majority of *R.equi* was observed within “loose” phagosomes, in which the phagosome membrane was widely separated from the bacteria [Zink *et al*. 1987]. In contrast, membranes of phagosomes containing non-viable *R.equi* or live *Escherichia coli* were almost always closely apposed. Tight membrane apposition has been suggested as a re-routing of mycobacterial phagosome maturation and establishment of the vacuole as a permanent early endosome [de Chastellier & Thilo 1997]. However, this phagosome phenotype appears to be different from the “loose phagosomes” observed enclosing *R.equi*, probably reflecting a different bacterial mechanism that causes different compartmentation. In addition, the tightly apposed membranes observed in phagosomes containing non-viable *R.equi* and non-pathogenic *E.coli* suggested that the “loose phagosomes” with vesicular material may reflect a different compartmentation of RCVs containing viable bacteria.

After 24 hours of infection in BMMs, the majority of *R.equi* was morphologically intact and inhabited very spacious, multilobed vacuoles of uneven shape and many internal membranes and vesicles. Moreover, most *R.equi* were found within phagosomes, although some bacteria were found free in the cytoplasm of damaged macrophages. In agreement with these results, previous studies using transmission electron microscopy in primary alveolar macrophages showed that, after 24 hours of infection, the majority of *R.equi* appeared to be intact and were also found in phagosomes with uneven shape that possessed cytoplasmic invaginations [Zink *et al*. 1987]. These studies also found *R.equi* free in the cytoplasm of damaged macrophages after prolonged infection [Zink *et al*. 1987, Hietala & Ardans 1987]. In contrast with the RCVs observed at 2 hours postinfection, the majority of RCVs at 24 hours were communal vacuoles, usually containing more than two bacteria. Previous studies using foal macrophages have also observed that, at 24 hours postinfection, *R.equi* were found in communal vacuoles containing more than two bacteria [Zink *et al*. 1987]. Interestingly, no difference was observed in the morphology of vacuoles containing plasmid bearing *R.equi* (33701+) and its plasmid cured partner (33701-). This result is in agreement with confocal fluorescence analyses that suggested that the basic mechanisms responsible for the establishment of RCVs are not likely to be regulated by plasmid genes. In macrophages, intracellular multiplication of *R.equi* is dependent on the plasmid-encoded protein VapA [Hondalus & Mosser 1994]. It is likely that the virulence-associated plasmid plays a major role later in infection, maybe by facilitating the acquisition of nutrients for bacterial multiplication. In that case, morphological differences in RCVs containing plasmid-bearing and plasmid-cured bacteria may be apparent at later infection times.

In summary, the results obtained using transmission electron microscopy indicated that, within host macrophages, *R.equi* resides in membrane-enclosed vacuoles which do not associate with host organelles such as mitochondria or endoplasmic reticulum, and which have either tightly apposed or loosely fitting membranes and internal membrane material. At later infection times (24 hours), RCVs are communal vacuoles usually containing more than two bacteria whith loose membranes and much internal material.

4.7- Summary

Altogether, the data obtained in this study can be accommodated in the following scheme (Figure 24): Following internalization by host macrophages, *R.equi* bacteria are enclosed within vacuoles that, in the same manner as normally maturing phagosomes, interact with early endocytic compartments transiently, thus acquiring early endocytic markers, such as Rab5 and EEA-1. Early endosomal markers are rapidly lost by RCVs and approximately 10% of the RCVs interact with late endosomes, acquiring from them the markers Rab7, LAMP-1, LAMP-2, LBPA, cathepsin D, and vATPase, and finally maturing into phagolysosomes with lysosomal hydrolases. In contrast, approximately 90% of the RCVs do not mature into phagolysosomes, and do not interact with late endosomes or vesicles coming from Golgi, thus not acquiring vATPase or cathepsin D (not even the immature form) (Marco Polidori, personal communication). However, RCVs do acquire the late endosomal markers Rab7, LBPA, LAMP-1 and LAMP-2, which indicate that they have left the early endosomal stage behind them but do not acquire a typical late composition. Thus, *R.equi* could be localized in vacuoles that resemble multivesicular body compartments (MVB). MVBs are transport intermediates between early and late endosomes [Gruenberg & Stenmark 2004] which contain transmembrane proteins destined for degradation, such as downregulated epidermal-growth-factor receptor (EGFR) [Gruenberg 2001, Katzmann *et al*. 2002]. MVBs display internal vesicles that are the product of inward budding on early sorting endosomes. These internal vesicles are very similar to the ones observed within RCVs using transmission electron microscopy. Because MVBs are intermediates between early and late compartments, they may acquire some late endosome characteristics as they begin to interact with late compartments, and that could be the situation of RCVs, which acquire Rab7, LAMP-1 and LAMP-2 but not vATPase or cathepsin D. LBPA is produced from phospholipids in internal membranes of MVBs, and therefore can be used as a marker for these compartments. RCVs containing heat-killed *R.equi* acquire LBPA followed by a slight loss of the marker, probably because the interactions of these vacuoles with LBPA-positive compartments are only transient. In contrast, RCVs containing viable bacteria acquire LBPA slower than RCVs containing heat-killed *R.equi*, but steadily. This may reflect continuous interactions with MVB and deposition of intravacuolar vesicles. At 24 hours postinfection, RCVs are multilobed large compartments which contain many internal vesicles and are still negative for vATPase, probably because RCVs do not fuse with late endocytic compartments and instead establish in a type of MVB-like compartment. However, the data obtained in this work do not

confirm the establishment of RCVs in a type of MVB-like compartment. Since LBPA is a marker for MVBs, analysis of LBPA accumulation by RCVs at long infection periods (24 hours) is important to confirm the establishment of RCVs as a MVB-like compartment. Additional studies are also needed to elucidate the mechanism employed by *R.equi* to arrest phagosome maturation. E.g., it remains to be determined what the pattern of association of Hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) by RCVs is. Hrs is emerging as a central coordinator of sorting to late endosomes [Chin *et al*. 2001, Raiborg *et al*. 2001a, Raiborg *et al*. 2001b, Raiborg *et al*. 2002] and it was shown to be essential for the progression of early phagosomes to phagolysosomes [Vieira *et al.* 2004]. Vieira *et al*. [2004] showed that the recruitment of Hrs by phagosomes containing virulent *Mycobacterium marinum* is considerably lesser than in that of control phagosomes. Elimination of Hrs using siRNA is associated with defective phagosome maturation, similar to maturation of mycobacterial phagosomes. Like virulent mycobacteria, *R.equi* may interfere with Hrs recruitment thus leading to an arrest in phagosome maturation.

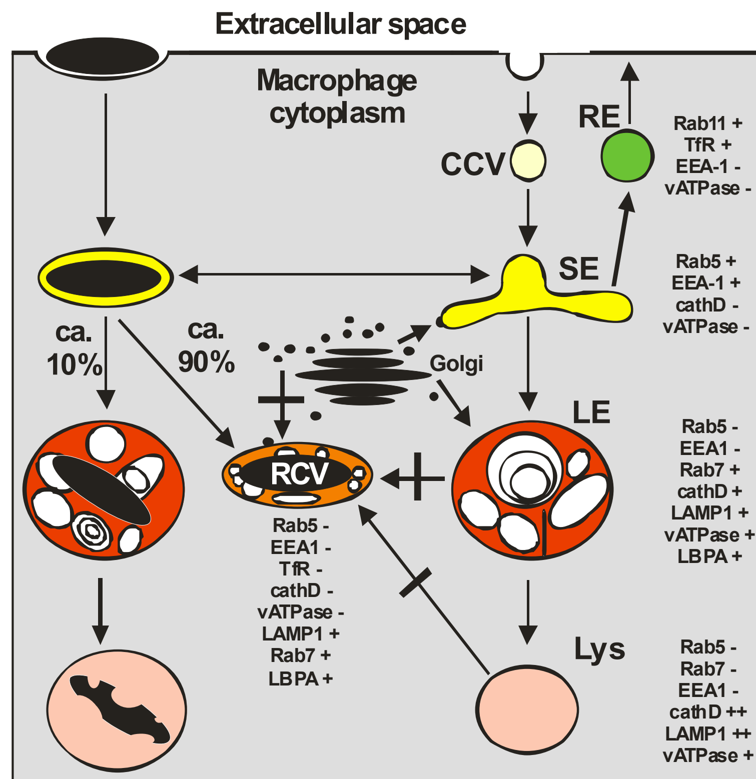

Figure 24: Working model describing the trafficking of RCVs in macrophages infected with live *R. equi*(+).
Clathrin-coated vesicles (CCV) feed extracellular liquid into the endocytic system. Phagosomes are formed at the plasma membrane and normally interact serially with early sorting endosomes (SE), late endosomes (LE), and lysosomes (Lys), but not with early Recycling Endosomes (RE) through which plasma membrane receptors can be recycled from the SE back to the surface. Some 10% of the ingested *R. equi* are transported along the degradative pathway to a (phago)lysosomal compartment and likely degraded, whereas the majority (some 90%) are directed to an unusual compartment whose composition stands between the SE and the LE. The Golgi compartiment communicates through vesicular trafficking with both, the SE / LE system and RCVs, through LEs may receive vATPase via this pathway and RCVs do not. Typical compartmental markers are indicated (cartoon courtesy of A.Haas).

4.8- What is the inhibitory component?

The finding that non-viable *R.equi* was still able to inhibit phagosome maturation (although not in the same way as viable *R.equi* did) suggests that heat-insensitive factors, such as cell periphery lipids, may play a major role in inhibition of phagosome maturation, although heat-sensitive factor(s) may also be involved. The fact that glycolipids of *Mycobacterium tuberculosis*, a member of actinomycetales and phylogenetically related to *R.equi*, are important for the inhibition of phagolysosome formation [Chua *et al*. 2004] suggests that *R.equi*, like other pathogenic actinomycetales, could employ lipid-mediated mechanisms to block phagosome maturation. Similarly, killing of *Nocardia asteroides*, another pathogenic bacterium related to *R.equi*, did not interfere with the inhibition of maturation of its phagosome [Black *et al*. 1986]. Further studies are needed in order to identify the factors, lipids or protein, that may be involved and how these factors get delivered to the proper site of action within host macrophages. These studies are important to understand the virulence mechanisms employed by *R.equi* as well as the pathogenesis of *R.equi* infection. Although the main mechanism responsible for inhibition of phagosome maturation is unlikely to be regulated by associated-virulence plasmid of *R.equi*, factor(s) encoded by this plasmid may contribute to the establishment of RCV, as vacuoles containing plasmid-cured bacteria co-localized more frequently with vATPase than those containing plasmid-bearing bacteria, and also reside in more acidic vacuoles. Additional studies using *R.equi* mutants lacking different regions of the virulence-associated plasmid may give an insight of the role of plasmid-encoded factors in the establishment of RCVs. It also remains to be determined whether the establishment of RCV is important for survival and replication of *R.equi* within macrophages and whether possession of virulence-associated plasmids by *R.equi* is important for intracellular survival or *R.equi*-induced host cell necrosis.

5- Summary

Rhodococcus equi is a Gram-positive intracellular pathogen which can cause severe bronchopneumonia in foals. In recent years, the role of this bacterium as human pathogen has been noted, as *R.equi* infections in humans have increased in frequency. This increase is associated with the rise in immunosupressed individuals, specially AIDS patients, where infection leads to symptoms and pathology similar to those seen in foals with a high mortality rate. Due to its capability to survive and multiply in murine and equine macrophages, *R.equi* has been classified as a facultative intracellular bacterium. *R.equi* is found frequently in macrophages in alveolar infiltrate from infected animals. The pathogenicity of *R.equi* depends on its ability to exist and multiply inside macrophages and has been associated with the presence of virulence plasmids. It has been observed that, inside foal alveolar macrophages, *R.equi*-containing vacuoles (RCVs) do not mature into phagolysosomes. However, most of the intracellular events during *R.equi* infection have not been investigated in detail.
The aim of this study was to elucidate the intracellular compartmentation of *R.equi* and the mechanism by which the bacteria avoid destruction in host macrophages. The importance of the virulence-associated plasmids of *R.equi* for the establishment of RCVs was also evaluated. Furthermore, the intracellular fate of viable and non-viable *R.equi* was compared in order to study whether viability of *R.equi* influenciates the establishment of RCVs.

In this study, the RCV was characterized by using a variety of endocytic markers to follow the path of the bacteria through murine macrophages. Transmission electron microscopy-based analysis showed that *R.equi* was found equally frequently in phagosomes with loosely or tightly apposed membranes, and RCV often contains numerous membranous vesicles. Laser scanning microscopy of infected macrophages showed that the majority of phagosomes containing *R.equi* acquired transiently the early endosomal markers Rab5, PtIns3P, and EEA-1, suggesting initially undisturbed phagosome maturation. Although the RCV acquired some late endosomal markers, such as Rab7, LAMP-1, and LAMP-2, they did not acquire vATPase, did not interact with pre-labeled lysosomes, and failed to acidify. These data clearly suggest that the RCV is a compartment which has left the early endosomal stage but fails to acquire a typical late endocytic composition. *R.equi* could be localized in vacuoles that resemble multivesicular body compartments (MVB), which are transport intermediates between early and late endosomes and display internal vesicles very similar to the ones observed within RCVs.

Analysis of several *R.equi* strains containing either VapA- or VapB-expressing plasmids or neither demonstrated that the possession of the virulence-associated plasmids does not affect phagosome trafficking over a two hour period of infection. The finding that non-viable *R.equi* was still able to inhibit phagosome maturation (although not to the same extent as viable *R.equi* did) suggests that heat-insensitive factors, such as cell periphery lipids, may play a major role in inhibition of phagosome maturation, although heat-sensitive factors may also be involved.

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