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## To cite this version:

Samantha Passey, Andrew Bradley, Harry Mellor. E. coli isolated from bovine mastitis invade mammary cells by a modified endocytic pathway. Veterinary Microbiology, 2008, 130 (1-2), pp.151. 10.1016/j.vetmic.2008.01.003 . hal-00532385

**HAL Id: hal-00532385**

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Submitted on 4 Nov 2010

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# Accepted Manuscript

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PII: S0378-1135(08)00005-9
DOI: doi:10.1016/j.vetmic.2008.01.003
Reference: VETMIC 3936

To appear in: *VETMIC*

Received date: 5-7-2007
Revised date: 3-1-2008
Accepted date: 11-1-2008

Please cite this article as: Passey, S., Bradley, A., Mellor, H., E. coli isolated from bovine mastitis invade mammary cells by a modified endocytic pathway, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.01.003

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# E. coli isolated from bovine mastitis invade mammary cells by a modified endocytic pathway

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**Keywords:** mastitis, *Escherichia coli,* confocal microscopy, endocytosis, invasion

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**Abstract**

*E. coli* is a major pathogen in the aetiology of bovine mastitis. Although classically considered to be an environmental pathogen causing mainly transient infection, the incidence of persistent *E. coli* mastitis infections may be increasing, suggesting an adaptation of this pathogen to the bovine udder environment. Mastitis *E. coli* strains have been demonstrated to enter bovine mammary cells *in vitro* but little is known about the invasion mechanism or the intracellular fate of the bacteria. In order to further understand the pathogenesis of persistent *E. coli* bovine mastitis we investigated the intracellular trafficking of mastitis *E. coli* isolates in primary bovine mammary cells using confocal microscopy and fluorescent markers of endocytic compartments.

Consistent with other studies, mastitis *E. coli* were found to invade primary bovine mammary cells *in vitro*. This process did not involvement rearrangement of the actin cytoskeleton. Intracellular bacteria were observed within membrane-bound compartments that labelled with the early endosomal marker phosphatidylinositol 3-phosphate (PtdIns(3)P) and also within late endosome-like compartments labelled with the small GTPase Rab7, indicating an endocytic mechanism of bacterial internalization. Bacteria were not observed within acidified lysosomal compartments or autophagic vacuoles, suggesting that the internalized bacteria are not targeted for lysosomal degradation via either the classical endocytic pathway or the autophagic response. Our findings are consistent with an endosomal survival niche for the internalised bacteria, allowing them to evade host immune responses and establish an infection reservoir that could later re-emerge as a recurrent clinical mastitis episode.

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## Introduction

Bovine mastitis is the most economically important disease affecting the UK dairy industry, with an annual incidence in excess of 50 quarter cases per 100 cows (Bradley et al., 2007) and an estimated annual cost of £168 million (Bradley, 2002). 80% of UK bovine mastitis cases are caused by just five pathogens; *Escherichia coli, Streptococcus uberis, Staphylococcus aureus, Streptococcus dysgalactiae and Streptococcus agalactiae* (Bradley, 2002). Mastitis pathogens are classified as either 'contagious' or 'environmental' (Blowey and Edmondson, 1995). Contagious pathogens survive within the host, causing persistent infection and spreading between udder quarters and cows. Environmental pathogens are opportunistic, causing transient infections that are rapidly eliminated by the host immune response. Of the five major causative agents of mastitis, *S. aureus, Str. dysgalactiae and Str. agalactiae* are considered to be contagious whereas *E. coli* and *Str. uberis* are environmental pathogens (Bradley, 2002). Since 1967 contagious cases have decreased with a concomitant increase in environmental cases. In particular *E. coli* cases have increased from 4.5% to 34.6% in 1998 (Bradley, 2002).

DNA fingerprinting data strongly suggests some *E. coli* strains have adapted to survive within the udder and cause recurrent infection (Bradley and Green, 2001). However there does not appear to be one single factor responsible for persistence within the udder (Bean et al., 2004; Kaipainen et al., 2002). Persistent bacterial infections can occur as a result of host-cell invasion by the pathogen, and invasion and persistence of the contagious mastitis pathogen *S. aureus* has been demonstrated *in vitro* (Lammers et al., 1999). Although previously considered to be a non-invasive pathogen in bovine mastitis, *E. coli* can invade both the bovine mammary epithelial cell line MAC-T (Dogan et al., 2006; Döpfer et al., 2000) and primary bovine mammary cells *in vitro* (Döpfer et al., 2001). *E. coli* is localised within

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membrane-bound vacuoles in infected MAC-T cells (Döpfer et al., 2000), and remains viable and replicates for at least 48h (Dogan et al., 2006).

Relatively little is known about how mastitis *E. coli* invade and persist within the mammary gland, although host-cell protein kinases and lipid kinases are required for bacterial invasion (Dogan et al., 2006; Döpfer et al., 2001). Here we present research aimed at further understanding the persistence of recurrent mastitis-causing strains of *E. coli* within primary bovine mammary cells. We show *E. coli* occupy a modified endocytic vacuole, and can persist in this compartment for up to 24h. This is comparable to other persistent bacteria, such as *Salmonella typhimurium*, however, detailed analysis of the *E. coli* compartment shows that it has unique and distinctive properties.

## Materials and Methods

### Reagents and antibodies

Mouse monoclonal anti $\alpha$-smooth muscle actin (Clone 1A4), anti-pan cytokeratin antibody mixture (clones C-11, PCK-26, Cy-60, KS1A3, M20 and A53-B/A2) and anti-vimentin antibody (clone VIM 13.2) were obtained from Sigma-Aldrich, UK. Cy5-conjugated donkey anti-mouse, FITC-conjugated donkey anti-goat and Cy3-conjugated donkey anti-rabbit antibodies were from Jackson Immunoresearch (Cambridgeshire, UK). Alexa488- and Alexa594-conjugated phalloidin, CellTracker Green CMFDA, CellTracker Blue CMAC and Lysotracker Red DND-99 were obtained from Molecular Probes (Paisley, UK). Goat anti-salmonella CSA-1 antibody was obtained from Kirkegaard and Perry Labs (Wembley, UK). Dulbecco's Modified Eagles Medium (DMEM) was obtained from Sigma-Aldrich and foetal calf serum (FCS) was obtained from Invitrogen (Paisley, UK).

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**Bacterial strains and plasmids**

*Escherichia coli* strain LT2649-M was isolated as part of a larger study on persistent bovine mastitis (Bradley and Green, 2001), from a 9th parity cow which experienced recurrent cases of mild clinical *E. coli* mastitis in the right hind quarter on days 275 and 297 of lactation. The *E. coli* lab strain HB101 was as described (Lacks and Greenberg, 1977). Stationary phase cultures of clinical isolates of *E. coli* were grown in DMEM at 37°C, 5% $CO_2$ without shaking. HB101 was grown in LB broth overnight with shaking at 37°C, then centrifuged and resuspended in DMEM for growth to mid log-phase for infection of mammalian cells. The wild-type virulent *Salmonella enterica* serovar *typhimurium* strain SL1344 was kindly provided by Mark Jepson (University of Bristol, UK), and overnight cultures were grown in LB broth at 37°C without shaking. The Green Fluorescent Protein (GFP) bacterial plasmid GFP pFPV25.1 was kindly provided by Mark Jepson and has been previously described (Valdivia and Falkow, 1996). The GFP LT2649-M strain of *E. coli* was generated by electroporation of the parent LT2649-M strain. Briefly, 2ml of log-phase culture of LT2649-M was collected by centrifugation, washed twice in ice-cold water and then resuspended in 40μl ice-cold water. GFP pFPV25.1 DNA (2μg) was added to the bacterial suspension and dispensed into a 0.2cm electroporation cuvette and pulsed at 2.5 kV using a Bio-Rad Genepulser. Immediately following electroporation 1ml SOC media was added to the bacteria, the bacterial suspension was transferred to a 1.5ml microfuge tube and incubated at 37°C for 30 minutes with shaking. Positive transformants were selected on LB agar plates supplemented with 100μg/ml ampicillin.

**Mammalian expression plasmids**

GFP-Rab4, GFP-Rab5, GFP-Rab7 and GFP-Rab11 constructs were a generous gift from Robert Lodge (Université du Quebec, Canada) and have been previously

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described (Hunyady et al., 2002). The GFP-PLCδ-PH construct encoding the PH domain of PLCδ was kindly provided by Matilda Katan (Institute of Cancer Research, London, UK) (Takeuchi et al., 2000). GFP-2xFYVE (Gillooly et al., 2000) and GFP-ARNO-PH (Venkateswarlu et al., 1998) were generous gifts from Peter Cullen (University of Bristol, UK). Yellow Fluorescent Protein (YFP)-tagged LC3 was a kind gift from Jon Lane (University of Bristol, UK).

**Primary bovine mammary cell isolation and culture**

Tissue culture plates were coated with Type I calf skin collagen (Sigma-Aldrich, UK) at 0.2mg/ml overnight at 4°C or for 1h at room temperature. The collagen solution was removed by aspiration immediately prior to use. Dairy cows were selected prior to slaughter on the basis of the absence of mammary infection as determined by the California Mastitis Test (Schalm and Noorlander, 1957) and culture of milk samples. The udder was collected immediately following slaughter, surgically prepared, and then 3 $cm^3$ pieces of secretory tissue were dissected out and placed in ice-cold DMEM supplemented with 2mM glutamine, 100U/ml penicillin and 100□g/ml streptomycin. Tissue pieces were processed according to the method of Rose *et. al* (Rose et al., 2002). Briefly, tissue was minced to a fine paste with a sterile blade and incubated in DMEM supplemented with 600U/ml collagenase type III (Worthington Biochemicals, Reading, UK), 10% foetal calf serum (FCS), 2mM glutamine, 100U/ml penicillin and 100μg/ml streptomycin for 30-60 minutes at 37°C with occasional mixing. Digested tissue was pushed through a steel mesh of pore size 105□m and cells were collected from the filtrate by centrifugation at 400 x g and 4°C. Cells were washed three times in DMEM, and then resuspended in DMEM supplemented with 10% FCS, 2mM glutamine, 100U/ml penicillin and 100□g/ml streptomycin (Cell Culture media) and plated in 10cm collagen-coated tissue culture dishes. Medium was changed daily for the first three days to remove non-adherent cells and floating

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debris and cells were cultured for approximately 6 days before passaging by trypsinisation. Cells derived by this method were used between passages 3 and 10 for experimental work.

**Transfection of mammary cells**

Glass coverslips were coated with 0.2mg/ml type I calf-skin collagen at 4°C overnight or at room temperature for 4-6h, which was then aspirated prior to cell seeding. Mammary cells were plated at 1 x $10^5$ cells/ml on collagen-coated coverslips and incubated overnight at 37°C, 5% $CO_2$. Cells were transfected using GeneFector (Venn Nova Inc. Florida, USA) according to the manufacturer's instructions, adding 1μg of the appropriate DNA plasmid and 2μl GeneFector reagent per well of a 12-well plate. Cells were incubated at 37°C, 5% $CO_2$ in the presence of transfection reagents for 4-6h then the medium was replaced with Cell Culture media. Cells were used for experiments at 18-24h post-transfection.

**Bacterial infection of mammary cells**

Primary bovine mammary cells were plated on collagen-coated coverslips as described and cultured for 16-24h prior to infection experiments. Infection cultures were prepared by inoculating 1ml of stationary phase bacterial culture into 10ml DMEM and incubating at 37°C, 5% $CO_2$ for approximately 3h until they reached mid log-phase with an $OD_{600}$ of 0.6. Ampicillin was added at 100 μg/ml to GFP LT2649-M cultures to maintain the GFP plasmid. Mammary cells were washed three times into serum-free antibiotic-free DMEM 1h prior to infection. For *E. coli* infections cell culture media was removed from each well and replaced with bacterial suspension at an MOI of approximately 150, for *Salmonella* infections bacteria were added to wells at a 1:200 dilution. Cells were incubated in the presence of bacteria for 15 minutes or 3h, and washed rigorously three times with PBS prior to fixation to remove

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extracellular bacteria. For long-term incubations, the cell media was then replaced at this stage with media containing 50μg/ml gentamicin for a further 21h.

**Fixation and processing for immunofluorescence**

Cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilised with 0.1% Triton X-100 in PBS for 5 minutes and then quenched with 0.1% $NaBH_4$ for 10 minutes. Primary antibodies were diluted in PBS containing 1% BSA, secondary antibodies were diluted in PBS. Antibodies were incubated on coverslips for 1h each. DAPI (1μg/ml) was incubated on coverslips for 5 minutes after removal of the secondary antibody. Coverslips were washed three times in PBS between each processing step. CellTracker green CMFDA or CellTracker Blue CMAC were used to label the cell cytoplasm at 1μM according to the manufacturer's instructions, Lysotracker Red DND-99 was used at 50nM according to the manufacturer's instructions. Cells were imaged by confocal laser scanning microscopy using a Leica TCS-SP2-AOBS attached to a Leica DM IRE2 inverted epifluorescence microscope.

## Results

**Invasion of primary bovine mammary cells by mastitis *E. coli***

In order to investigate the invasive behaviour of mastitis *E. coli* in a physiologically relevant cell culture system, we prepared primary bovine mammary cells from bovine udder (Rose et al., 2002). The identity of the cells present in the culture was determined by immunofluorescence staining (Figure 1). Mammary epithelial cells showed a typical 'cobblestone' morphology and stained positive for pan-cytokeratin. Myoepithelial cells surrounded the islands of epithelial cells and stained positive for α-smooth muscle actin. These cells also contained thick contractile actomyosin

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bundles. No positive staining was observed for vimentin, indicating an absence of fibroblasts in the culture.

We examined the ability of the persistent mastitis *E. coli* strain LT2649-M to invade these cells and compared this to the invasive pathogen *Salmonella Enterica* serovar *typhimurium*. At 3h post-infection both strains were seen to be associated with bovine mammary cells after washing (Figure 2). Confocal microscopy allowed us to distinguish between bacteria adhered to the surface of the host cell and invaded bacteria. Both *E. coli* and Salmonella were observed in the cell cytoplasm (Figure 2), confirming previous reports that *E. coli* can act as an invasive pathogen on bovine mammary cells. In comparison, a control laboratory *E. coli* strain HB101 showed no invasion into these cells (data not shown).

**Invasion of bovine mammary cells by mastitis *E. coli* is not accompanied by actin rearrangement**

Many invasive bacteria cause dramatic rearrangements of the actin cytoskeleton at the cell surface to aid internalisation. To investigate possible cytoskeleton rearrangements induced by host cell adhesion of mastitis *E. coli*, bovine mammary cells were infected for 15 minutes then non-adhered bacteria were removed by extensive washing in PBS prior to fixation. *Salmonella* is well documented for its ability to induce dramatic actin rearrangements upon adhesion to the host cell surface (Francis et al., 1993) and we used these bacteria as a control.

As expected, dramatic actin ruffles were observed upon adhesion of *Salmonella* to the mammary cells (Figure 3). In contrast, invasion of *E. coli* LT2649-M was not associated with actin ruffles, or with any rearrangement of actin in the vicinity of the bacteria (Figure 3). This remained the case even at later time points when more bacteria had adhered to the cell surface (data not shown).

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**Mastitis *E. coli* traffic through early and late endosomal compartments.**

In order to determine the identity of the *E. coli* invasion compartment we used specific markers of the endocytic pathway and of the signalling lipids that control endosomal trafficking. The lipid PtdIns(3,4,5)$P_3$ transiently accumulates at the phagocytic cup and at the site of entry of *Salmonella* (Cullen et al., 2001). We used a well-characterised GFP-biosensor for PtdIns(3,4,5)$P_3$ (Venkateswarlu et al., 1998) to determine whether mastitis *E. coli* also stimulated PtdIns(3,4,5)$P_3$ accumulation. Mastitis *E. coli* infection failed to cause any apparent formation or redistribution of PtdIns(3,4,5)$P_3$ (Figure 4). The signalling lipid PtdIns(4,5)$P_2$ is found on the plasma membrane and is involved in phagocytosis and clathrin-mediated endocytosis (Jost et al., 1998); however, mastitis *E. coli* also caused no increase in PtdIns(4,5)$P_2$ production and PtdIns(4,5)$P_2$ was not recruited to membranes surrounding internalised bacteria (Figure 4). In contrast, vacuolar structures containing internalized LT2649-M stained strongly with a GFP-biosensor for the signalling lipid PtdIns(3)P (Figure 4). PtdIns(3)P marks early endosomes (Roth, 2004) and also vacuoles containing internalised *Salmonella* (Cullen et al., 2001). From this we conclude that invasive mastitis *E. coli* reside within the endocytic pathway, but enter through a process that does not involve production of PtdIns(4,5)$P_2$ or PtdIns(3,4,5)$P_3$, and is therefore distinct from the classical phagocytic mechanism.

To fully characterize the nature of the *E. coli* compartment in bovine mammary cells, we looked at the co-localisation between internalised bacteria and well-defined Rab GTPase markers of endocytic subcompartments (Zerial and McBride, 2001). Internalised LT2649-M bacteria did not co-localise with the early endosome marker Rab5, the early recycling endosome marker Rab4 or the late recycling endosomal marker Rab11 (Figure 5). Vacuoles containing internalised mastitis *E. coli* labelled

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strongly with the late endosomal marker Rab7, however, and these structures were enlarged compared to normal Rab7+ endosomes (Figure 5). We performed three-colour imaging to determine if Ptd(3)P coated vacuoles were distinct from Rab7-coated vacuoles and found instead that internalised *E. coli* were found within structures that co-labelled with both markers at 3h (Figure 6), suggesting that the *E. coli* compartment is a hybrid endocytic vacuole.

**The mastitis *E. coli* compartment is distinct from the autophagic compartment and from lysosomes**

Rab7 can also associate with autophagosomes and facilitate their maturation and fusion with the lysosome (Gutierrez et al., 2004). We therefore examined whether the Rab7+ invasive *E. coli* compartment was part of the autophagy pathway. Infected cells were examined by confocal microscopy and autophagosomes were labelled with the autophagy marker LC3. LC3 is cytosolic in resting cells but translocates to the membranes of autophagosomes upon the induction of the autophagic response (Kabeya et al., 2000). Uninfected control cells showed a few LC3+ autophagic vesicles near the nucleus; however, internalised mastitis *E. coli* showed no co-localisation with this compartment (Figure 7B). Further, there was no induction of autophagic vesicles upon *E. coli* infection, whereas treatment of control cells with rapamycin, an inducer of autophagy, caused a marked increase in the number of LC3+ structures (Figure 7A).

Rab7+ late endosomes would normally fuse with lysosome to deliver their contents for degradation. We examined whether internalised mastitis *E. coli* were present in lysosomes by co-staining with the lysosomal marker Lysotracker Red DND-99 to label acidified compartments. We could not detect co-localisation between bacteria and Lysotracker Red-positive compartments by confocal microscopy, indicating that LT2649-M do not exist within an acidified compartment (Figure 8A). We were

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interested to see if internalised mastitis *E. coli* remained in Rab7+ late endosomes during long-term persistence in the cell. Cells were infected with LT2649-M as previously and then incubated in antibiotic for 21h to kill non-internalised bacteria and to prevent re-infection. Internalised bacteria could readily be detected 24h after infection; however, these bacteria were no longer contained within Rab7+ vacuoles. We were unable to find any marker of the endocytic compartment that co-localised with internalised mastitis *E. coli* at this late time point (data not shown), although Rab7+ vesicles were seen in close proximity (Figure 8B).

**Discussion**

Previous studies have highlighted the growing problem of persistent bovine mastitis in the UK dairy industry and have shown that the bacterial pathogen *E. coli* is a significant cause of such persistent infections (Bradley, 2002). Despite being traditionally considered an opportunistic environmental mastitis pathogen there is mounting evidence that the pathogen has become adapted to the udder environment (Bradley and Green, 2001), capable of invading and surviving within host mammary cells (Dogan et al., 2006; Döpfer et al., 2000; Döpfer et al., 2001). In light of these findings, this study was aimed at investigating the intracellular trafficking pathway taken by these pathogens within the host cell in order to understand the invasion process in more detail. Invasion of the mastitis *E. coli* isolate LT2649-M into primary bovine mammary cells was demonstrated by confocal microscopy. Such invasion was observed with other mastitis strains tested (data not shown) and is in agreement with published work (Dogan et al., 2006; Döpfer et al., 2000), indicating that the invasive behaviour observed is common to a number of persistent strains and

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suggesting that *E. coli* may use host cell invasion as a means to establish a persistent infection.

Bacterial invasion into host cells is accompanied by rearrangement of the host cell actin cytoskeleton in numerous bacterial species including *Salmonella, Shigella* and *Yersinia* and often involves bacterial modulation of host cell signalling pathways (Steele-Mortimer et al., 2000). The actin depolymerising agent cytochalasin D has been reported to inhibit the invasion of mastitis *E. coli* into bovine mammary cells (Dogan et al., 2006), suggesting that the actin cytoskeleton is important for invasion. However cytochalasin D has been shown to cause cell rounding and affect cell viability in similar studies (Döpfer et al., 2001), making interpretation of the involvement of the actin cytoskeleton in mastitis *E. coli* invasion difficult. We looked directly to see if polymerisation or rearrangement of F-actin accompanied entry of mastitis *E. coli* and found that it did not. This does not preclude a requirement for an intact actin network to facilitate bacterial entry. Other bacteria including *Yersinia* species have been demonstrated to enter cells via a zippering mechanism that requires the actin cytoskeleton but does not induce dramatic actin rearrangements (Young et al., 1992). Further investigation into the mode of entry of the mastitis *E. coli* is needed to determine exactly the factors that facilitate bacterial entry.

Mastitis *E. coli* LT2649-M bacteria were internalized into a membrane-bound endocytic vacuole. GFP-tagged phosphoinositide probes and Rab GTPases were used to characterize this compartment and have been previously described as markers of endocytic compartments (Cullen et al., 2001; Hunyady et al., 2002). Intracellular bacteria were identified within a compartment with early endosome-like characteristics as shown by positive labelling with a fluorescent probe for PtdIns-3P. No colocalisation was observed with probes for PtdIns(4,5)$P_2$ or PtdIns(3,4,5)$P_3$, indicating that the bacteria are not internalized through a classical phagocytic

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mechanism. These data are comparable to the invasion pathway of *Salmonella* that resides in a membrane-bound Salmonella Containing Vacuole (SCV) and actively modulates the PtdIns components on this compartment through depletion of PtdIns(4,5)$P_2$ and retention of PtdIns(3)P (Hernandez et al., 2004).

*E. coli* containing vacuoles also stained strongly for the late endosomal marker Rab7. No colocalisation was observed with Rab 4 or Rab 11, indicating that the bacteria do not interact with the recycling pathways of endocytosis. Parallels can be drawn with the well-characterized *Salmonella* invasion process, as Rab7 associates with the SCV and is proposed to direct SCV maturation (Meresse et al., 1999). No bacterial compartments labelled with the early endosome marker Rab5 were observed. This is interesting in light of the previous observation of bacteria within early endosome-like PtdIns3-P positive compartments, as Rab5 and PtdIns3-P are involved in the recruitment of early endosomal antigen 1 (EEA1) and regulation of the fusion of early endosomes with other endosomal compartments (Simonsen et al., 1998; Zerial and McBride, 2001). *Salmonella* actively modulates phosphoinositide metabolism in order to control SCV maturation, resulting in retention of PtdIns3-P on the SCV membrane (Hernandez et al., 2004). In keeping with this, we found that mastitis *E. coli* could also be found in vacuoles containing both markers, strongly suggesting that this pathogen is also is capable of similar active modification of the character of its intracellular compartment.

An alternative intracellular trafficking route that is related to the endocytic pathway is the cellular autophagy pathway. Autophagy is a mechanism of self-digestion resulting in the lysosomal degradation of cellular components and exists both as a housekeeping mechanism and also as a response to stress (Gutierrez et al., 2004). Cellular components or organelles are enclosed within a membrane vacuole termed the autophagosome (Dorn et al., 2002). Rab 7 associates with the autophagosome

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membrane and facilitates its maturation and fusion with the lysosome (Gutierrez et al., 2004). Some pathogens including *Coxiella burnetii, Legionella pneumophila* and *Brucella abortus* can survive and even replicate within autophagosomes (Beron et al., 2002; Dorn et al., 2002). Large Rab7-positive autophagic vacuoles containing *Coxiella burnetii* accumulate in infected host cells, and it is thought that the induction of the autophagic response provides the bacteria with a niche environment rich in nutrients from the breakdown of cellular components in which the bacteria can survive and replicate (Beron et al., 2002). The autophagic response was investigated as an alternative trafficking route for internalized mastitis *E. coli*, using the autophagosome marker LC3. No co-localisation of the autophagosome marker YFP-LC3 and *E. coli* LT2649-M was observed, indicating that the bacteria do not make significant interactions with the autophagy pathway and traffic through an endocytic pathway within the host cell.

The end-point of classical endocytosis is degradation of endocytic cargo via fusion of their trafficking compartment with the lysosome. Many bacteria have evolved ways of avoiding lysosomal killing through prevention of lysosomal fusion or acidification, surviving within the harsh lysosome environment or by escaping from their membrane-bound compartment into the cytoplasm (Finlay and Falkow, 1997). No co-localisation of mastitis *E. coli* with acidified compartments was observed, indicating that the bacteria do not reside within an acidified lysosomal compartment. This suggests that the bacteria may actively direct their intracellular trafficking to avoid killing by lysosomal mechanisms and persistence in the cell. In keeping with this, at long time points, internalised mastitis *E. coli* could be found in compartments that did not label with Rab7, suggesting that they eventually modify or escape the Rab7/PtdIns(3)P compartment. The precise nature of this final resting place is unknown.

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In conclusion this study has revealed that mastitis *E. coli* are capable of invading primary bovine mammary cells through a mechanism that does not result in visible actin cytoskeleton rearrangement. We propose a model for *E. coli* intracellular trafficking, shown schematically in Figure 8. Upon internalization bacteria reside within a membrane-bound compartment that traffics along an endocytic pathway acquiring the early and late endosomal markers PtdIns(3)P and Rab7 respectively. The trafficking route appears to be modified, as suggested by the absence of the early endosome marker Rab5 on the bacterial compartment and the observation that internalized bacteria do not co-localise with a marker of acidified lysosome-like compartments. Bacterial modulation of intracellular trafficking pathways has been observed with other intracellular pathogens including *Salmonella* (Hashim et al., 2000; Hernandez et al., 2004) and represents an important survival mechanism to establish an intracellular infection reservoir. The recent report that intracellular mastitis *E. coli* remain viable over a 48h period *in vitro* and can be detected in the mammary tissue of *in vivo* infected bovine mammary glands (Dogan et al., 2006) indicates that the establishment of a viable intracellular reservoir is a relevant pathogenic mechanism used by these bacteria to persist within the udder environment. This study has contributed to the overall understanding of persistent bovine *E. coli* mastitis and takes us one step closer to unravelling the pathogenic mechanisms used by these bacteria to establish such infections. Further investigation to elucidate the nature of the factors that allow these bacteria to adhere and invade is likely to yield important information about the bacterial contribution to the invasion process and possibly allow the identification of novel therapeutic targets to aid in the control and treatment of this problematic disease.

**Acknowledgements**

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The authors would like to thank Mark Jepson for advice and assistance. The study was supported by a CASE studentship between the BBSRC and Pfizer, and by an MRC Infrastructure Award to the School of Medical Sciences Imaging Centre.

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**Figures**

**Figure 1.** Primary Bovine Mammary cell cultures consist of a co-culture of myoepithelial cells and epithelial cells. Confocal micrographs of primary bovine mammary cells isolated as described in Materials and Methods. Specific antibodies (red) were used to identify the cell types present – cytokeratin (epithelial cells), α-smooth muscle actin (myoepithelial cells) and vimentin (fibroblasts). Alexa 488-conjugated phalloidin was used to visualise the actin cytoskeleton (green). Scale bar = 10μm.

**Figure 2.** The persistent mastitis *E. coli* strain LT2649-M is capable of invading primary bovine mammary cells *in vitro*. Primary bovine mammary cells were infected with *Salmonella typhimurium* strain SL1344 or *E. coli* LT2649-M for 3h as described in Materials and Methods. The cell cytoplasm was labelled by incubation of cells with CellTracker Green CMFDA (green), *Salmonella* bacteria were labelled with anti-*Salmonella* CSA-1 antibody (red). *E. coli* and host-cell nuclei were labelled with DAPI (shown in red for clarity). Bottom panels show confocal cross-sections through the cells to show the intracellular bacteria (arrows). The dashed line in each top panel indicates the plane of the section. Scale bar = 10μm.

**Figure 3.** Mastitis *E. coli* invasion is not accompanied by visible actin cytoskeleton rearrangements. Primary bovine mammary cells were infected with *Salmonella typhimurium* SL1344 or mastitis *E. coli* LT2649-M for 15 minutes followed by cell fixation. The actin cytoskeleton was visualised by staining with Alexa 488-conjugated phalloidin (green). *Salmonella* bacteria were labelled with anti-*Salmonella* CSA-1 antibody (red). *E. coli* and host-cell nuclei were labelled with DAPI (red). *Salmonella* caused the formation of bright F-actin-rich ruffles beneath the adherent bacteria

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(arrow). No rearrangement or polymerisation of actin was seen in association with adherent *E. coli* (arrow). Scale bar = 10μm.

**Figure 4.** *E. coli* LT2649-M are internalized into a PtdIns(3)P containing endosomal compartment. Primary bovine mammary cells were transfected with GFP-tagged biosensors for the signalling lipids PtdIns(4,5)P2 (PLCδ-PH domain), PtdIns(3,4,5)P3 (ARNO PH domain) or PtdIns(3)P (2xFYVE) for 18-24h then infected with LT2649-M for 3h followed by cell fixation. Cells were labelled for bacterial DNA and cell nuclei with DAPI (red) and the relevant PtdIns probe (green). The right-hand panels show magnified sections of the images as indicated by the dashed boxes. Internalised *E. coli* could be seen in swollen vacuoles that labelled only with PtdIns(3)P (arrowhead). Scale bar = 10μm.

**Figure 5.** *E. coli* LT2649-M are internalized into a compartment with late endosome-like characteristics. Primary bovine mammary cells were transfected with GFP-tagged Rab4, Rab5, Rab7 and Rab11 for 18-24h then infected with LT2649-M for 3h followed by fixation and processing for immunofluorescence. Cells were labelled with DAPI (red) to stain cell nuclei and bacterial DNA and the relevant GFP-tagged Rab protein (green). The right-hand panels show magnified sections of the images as indicated by the dashed boxes. Internalised *E. coli* could be seen in swollen vacuoles that labelled only with Rab7 (arrowhead). Scale bar = 10μm.

**Figure 6.** The E. coli mastitis vacuole is a hybrid endocytic compartment. Primary bovine mammary cells were transfected with the GFP-tagged biosensor for PtdIns(3)P (2xFYVE) and CFP-tagged Rab7 and then infected with mastitis *E. coli* LT2649-M for 3h followed by fixation and processing for immunofluorescence. Cells were labelled with DAPI (red) to stain cell nuclei and bacterial DNA, the GFP-tagged



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PtdIns(3)P probe (green) and CFP-Rab7 (blue). The right-hand panels show magnified sections of the images as indicated by the dashed boxes. Internalised *E. coli* could be seen in swollen vacuoles that labelled with both PtdIns(3)P and Rab7 (arrowhead). Scale bar = 10μm.

**Figure 7.** Internalized *E. coli* LT2649-M do not reside within an LC3-positive autophagic compartment. Primary bovine mammary cells were transfected with the autophagosome marker YFP-LC3 for 18-24h. (A) Cells were treated with 25nM rapamycin for 2h to demonstrate induction of the autophagosome compartment. (B) Cells were alternatively infected with *E. coli* LT2649-M for 3h followed by fixation and processing for immunofluorescence. The right-hand panel shows a magnified section of the image as indicated by the dashed box. Cells were labelled with DAPI (red) to visualise cell nuclei and bacterial DNA and YFP-LC3 (green) to label autophagic compartments. Internalised E. coli did not reside in autophagocytic vacuoles and infection did not visibly up-regulate the autophagocytic compartment. Scale bar =10μm.

**Figure 8.** Internalized *E. coli* LT2649-M do not reside within lysosomes. (A) Primary bovine mammary cells were infected with GFP-expressing *E. coli* LT2649-M (green) for 3h. The lysosomal compartment was labelled with LysoTracker Red DND-99 (Red). The cell cytoplasm was labelled with Cell Tracker Blue CMAC (blue). Internalised *E. coli* (arrowheads) were not labelled with lysosomal markers. (B) Primary bovine mammary cells were transfected with GFP-tagged Rab7and then infected with LT2649-M for 3h followed by 21h of further incubation with antibiotic. Cells were labelled with DAPI (red) to stain cell nuclei and bacterial DNA and GFP-tagged Rab7 (green). At long incubations, invaded *E. coli* could be found that no longer labelled with Rab7 (arrowhead) or lysosomal marker (data not shown). The



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right-hand panels show magnified sections of the images as indicated by the dashed boxes. Scale bar = 10μm.

**Figure 9.** A model for *E. coli* invasion into mammary cells during mastitis infection. A proposed model of *E. coli* invasion in bovine mastitis drawn in parallel to *Salmonella* invasion, modified from (Knodler and Steele-Mortimer, 2003). SCV, Salmonella containing vacuole; ECCV, *E. coli* containing vacuole.

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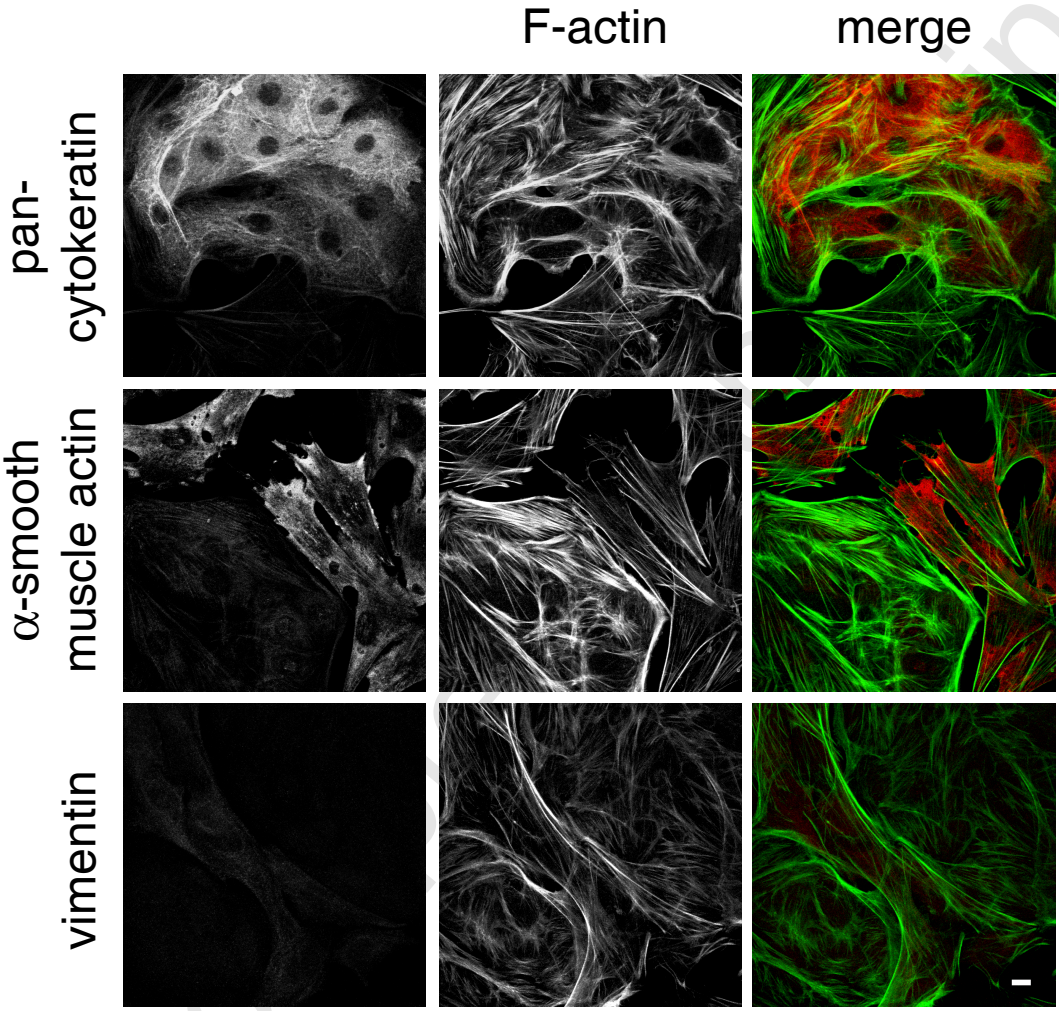


Figure 1

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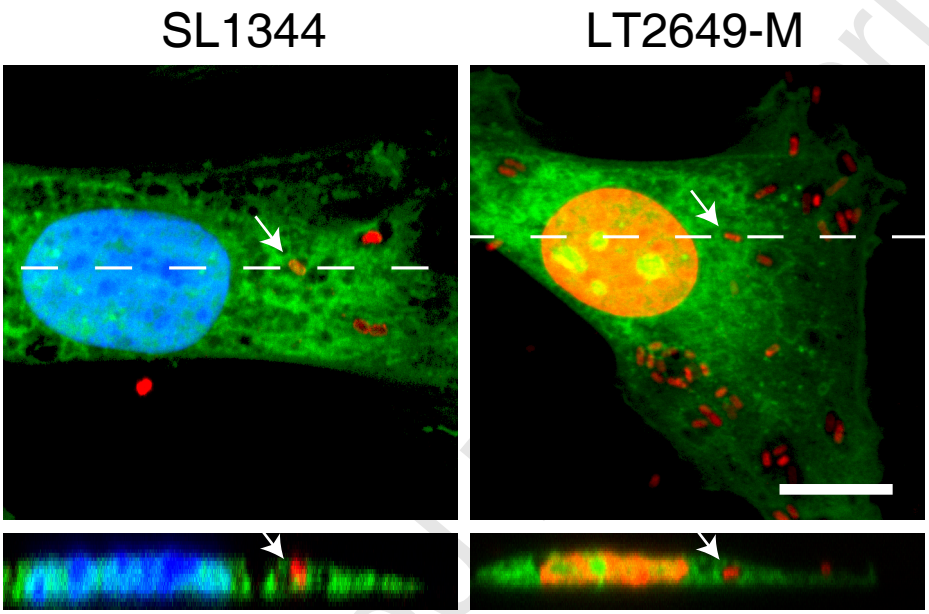


Figure 2

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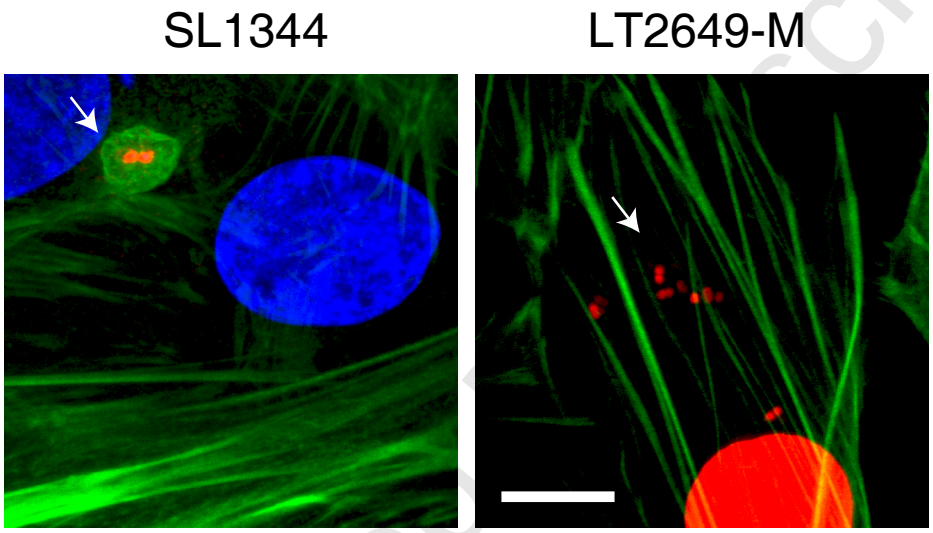


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Figure 3

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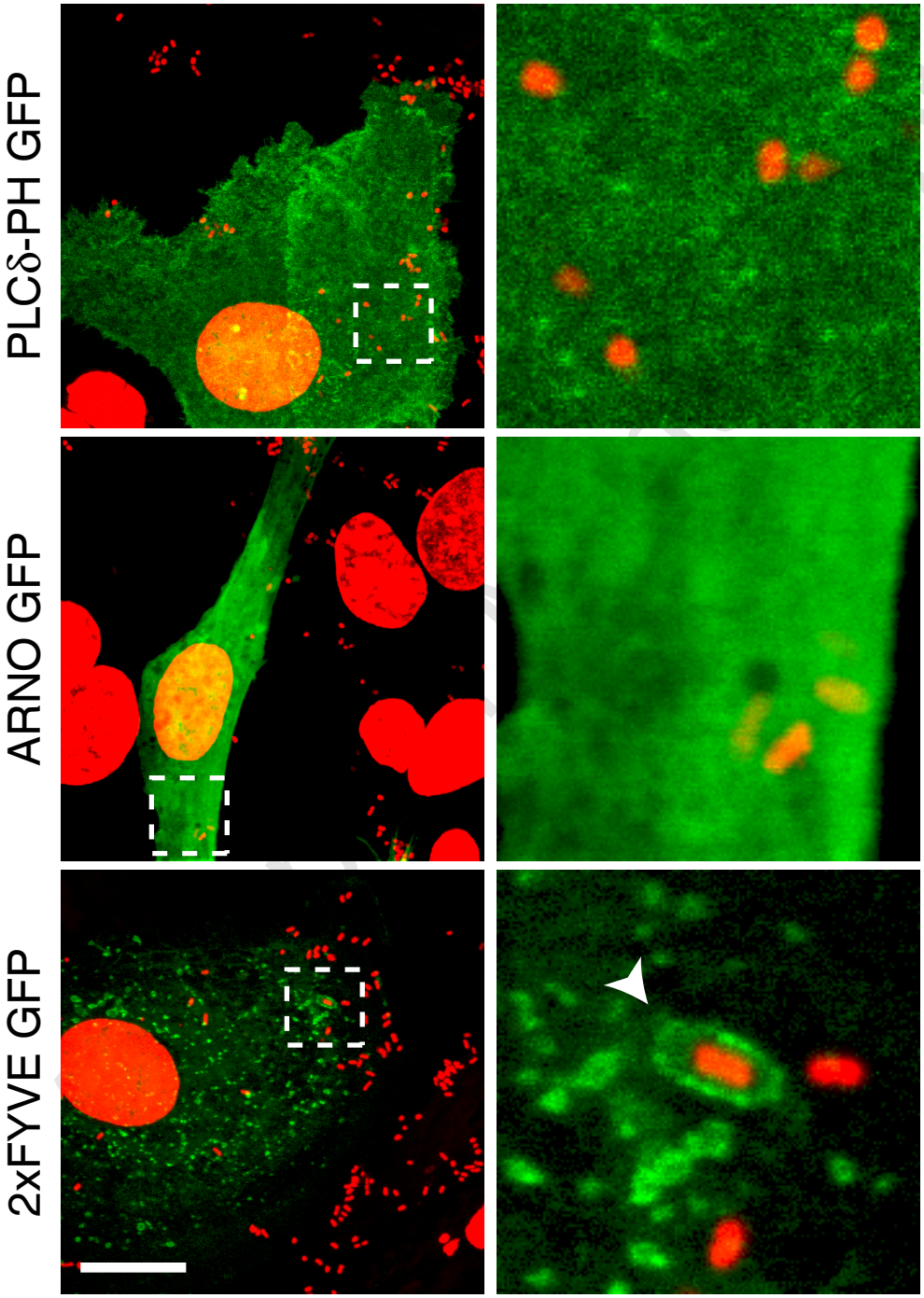


Figure 4

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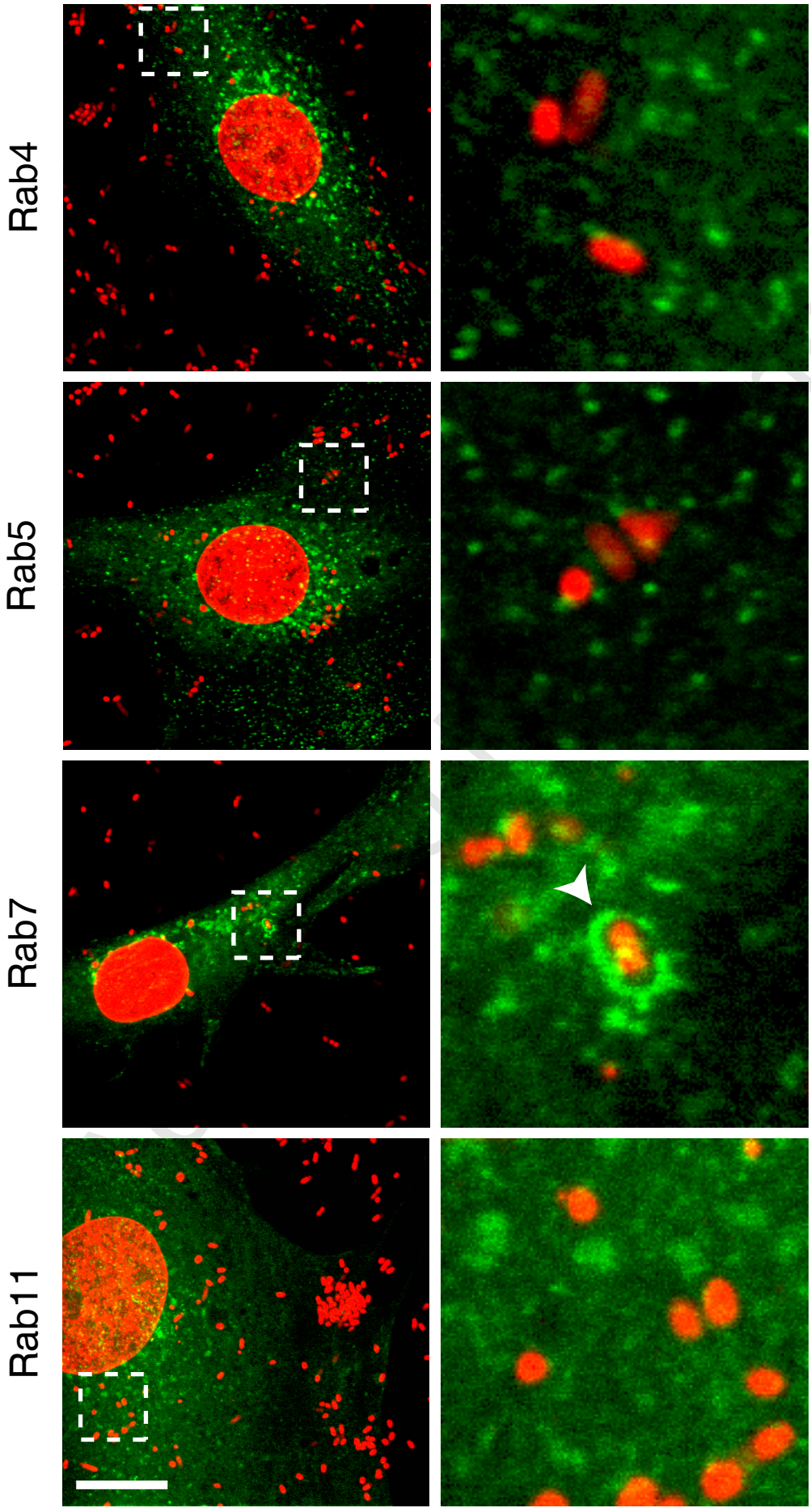


Figure 5

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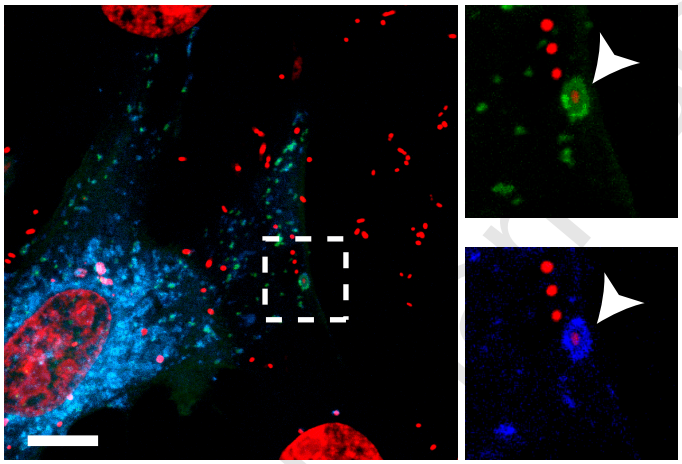

Figure 6

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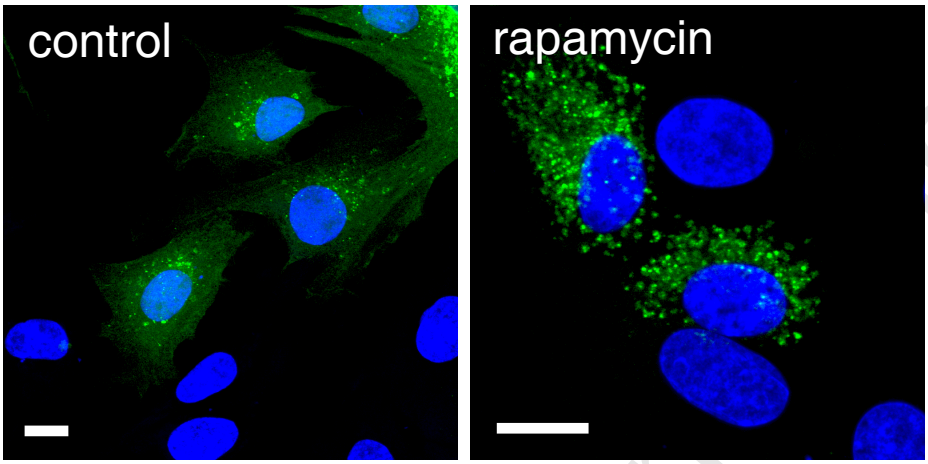
A

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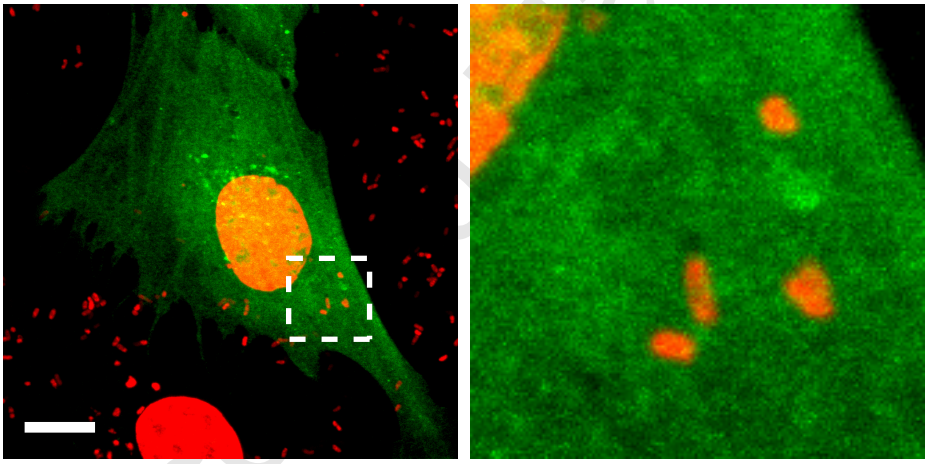

Figure 7

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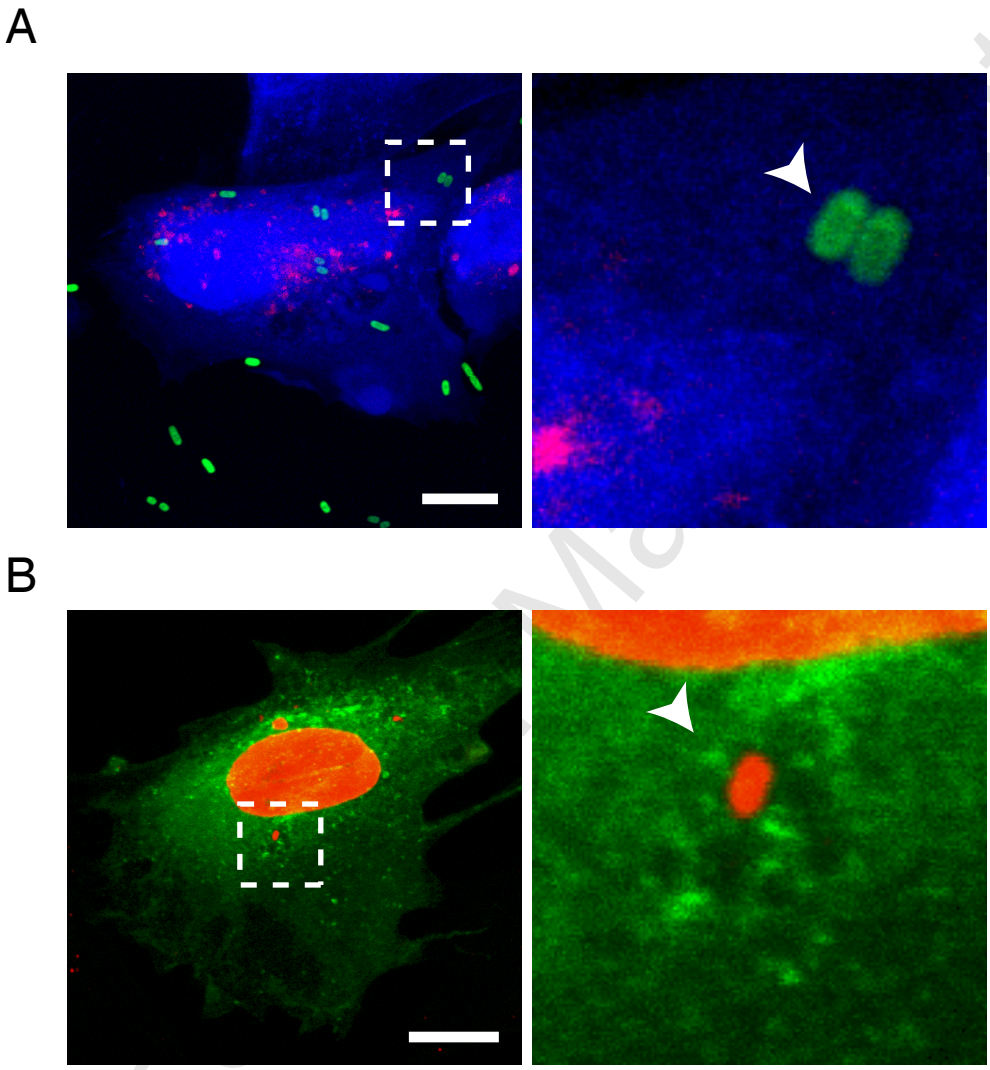


Figure 8

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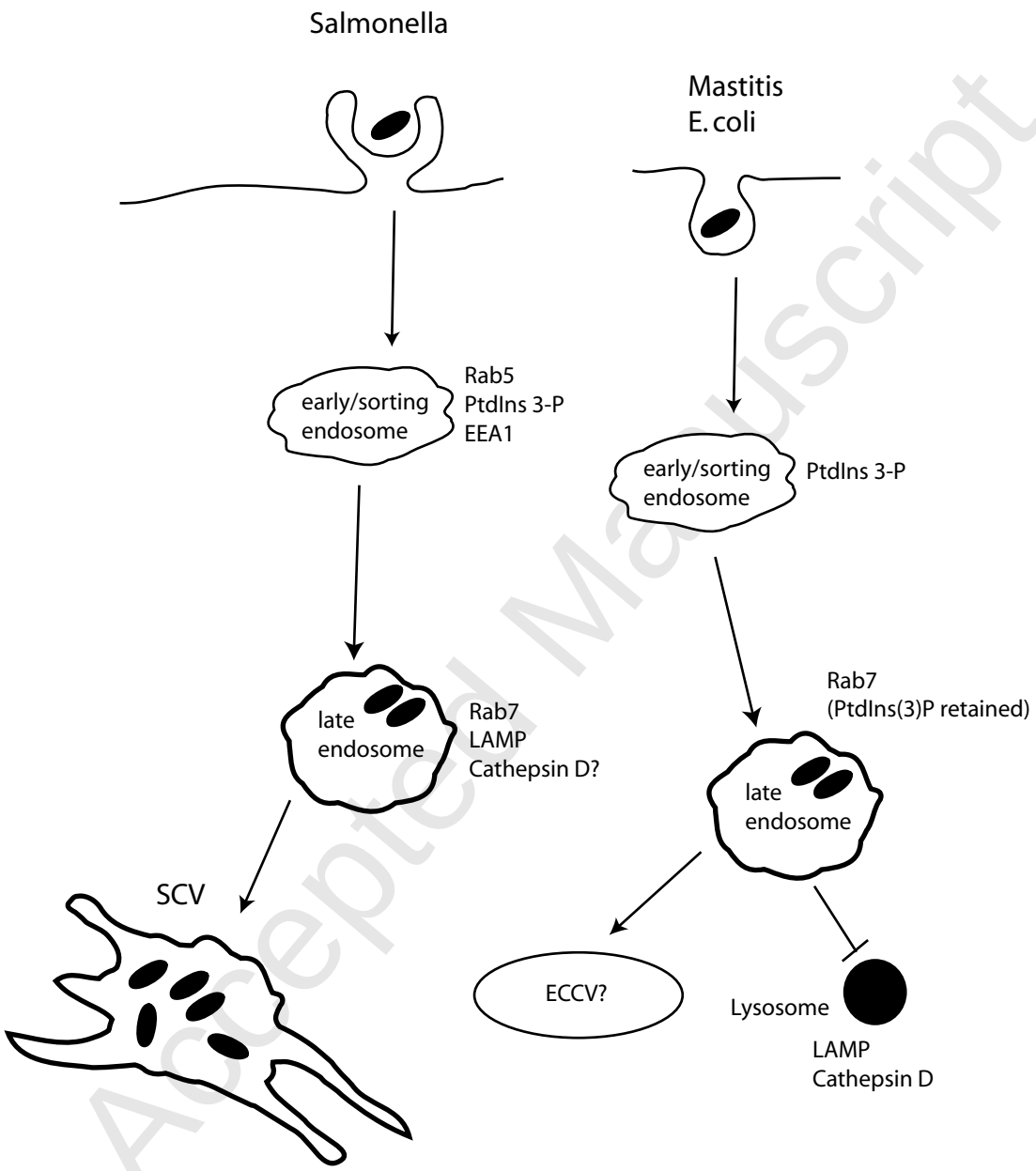


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Figure 9