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1

2 **E. coli isolated from bovine mastitis invade mammary cells by**
3 **a modified endocytic pathway**

4

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

28 **Abstract**

29

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

40

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

54

55

56 Introduction

57 Bovine mastitis is the most economically important disease affecting the UK dairy
58 industry, with an annual incidence in excess of 50 quarter cases per 100 cows
59 (Bradley et al., 2007) and an estimated annual cost of £168 million (Bradley, 2002).
60 80% of UK bovine mastitis cases are caused by just five pathogens; *Escherichia coli*,
61 *Streptococcus uberis*, *Staphylococcus aureus*, *Streptococcus dysgalactiae* and
62 *Streptococcus agalactiae* (Bradley, 2002). Mastitis pathogens are classified as either
63 'contagious' or 'environmental' (Blowey and Edmondson, 1995). Contagious
64 pathogens survive within the host, causing persistent infection and spreading
65 between udder quarters and cows. Environmental pathogens are opportunistic,
66 causing transient infections that are rapidly eliminated by the host immune response.
67 Of the five major causative agents of mastitis, *S. aureus*, *Str. dysgalactiae* and *Str.*
68 *agalactiae* are considered to be contagious whereas *E. coli* and *Str. uberis* are
69 environmental pathogens (Bradley, 2002). Since 1967 contagious cases have
70 decreased with a concomitant increase in environmental cases. In particular *E. coli*
71 cases have increased from 4.5% to 34.6% in 1998 (Bradley, 2002).

72
73 DNA fingerprinting data strongly suggests some *E. coli* strains have adapted to
74 survive within the udder and cause recurrent infection (Bradley and Green, 2001).
75 However there does not appear to be one single factor responsible for persistence
76 within the udder (Bean et al., 2004; Kaipainen et al., 2002). Persistent bacterial
77 infections can occur as a result of host-cell invasion by the pathogen, and invasion
78 and persistence of the contagious mastitis pathogen *S. aureus* has been
79 demonstrated *in vitro* (Lammers et al., 1999). Although previously considered to be a
80 non-invasive pathogen in bovine mastitis, *E. coli* can invade both the bovine
81 mammary epithelial cell line MAC-T (Dogan et al., 2006; Döpfer et al., 2000) and
82 primary bovine mammary cells *in vitro* (Döpfer et al., 2001). *E. coli* is localised within

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

85

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

95

96

97 **Materials and Methods**

98

99 **Reagents and antibodies**

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

111

112 **Bacterial strains and plasmids**

113 *Escherichia coli* strain LT2649-M was isolated as part of a larger study on persistent
114 bovine mastitis (Bradley and Green, 2001), from a 9th parity cow which experienced
115 recurrent cases of mild clinical *E. coli* mastitis in the right hind quarter on days 275
116 and 297 of lactation. The *E. coli* lab strain HB101 was as described (Lacks and
117 Greenberg, 1977). Stationary phase cultures of clinical isolates of *E. coli* were grown
118 in DMEM at 37°C, 5% CO₂ without shaking. HB101 was grown in LB broth overnight
119 with shaking at 37°C, then centrifuged and resuspended in DMEM for growth to mid
120 log-phase for infection of mammalian cells. The wild-type virulent *Salmonella enterica*
121 serovar *typhimurium* strain SL1344 was kindly provided by Mark Jepson (University
122 of Bristol, UK), and overnight cultures were grown in LB broth at 37°C without
123 shaking. The Green Fluorescent Protein (GFP) bacterial plasmid GFP pFPV25.1 was
124 kindly provided by Mark Jepson and has been previously described (Valdivia and
125 Falkow, 1996). The GFP LT2649-M strain of *E. coli* was generated by electroporation
126 of the parent LT2649-M strain. Briefly, 2ml of log-phase culture of LT2649-M was
127 collected by centrifugation, washed twice in ice-cold water and then resuspended in
128 40µl ice-cold water. GFP pFPV25.1 DNA (2µg) was added to the bacterial
129 suspension and dispensed into a 0.2cm electroporation cuvette and pulsed at 2.5 kV
130 using a Bio-Rad GenePulser. Immediately following electroporation 1ml SOC media
131 was added to the bacteria, the bacterial suspension was transferred to a 1.5ml
132 microfuge tube and incubated at 37°C for 30 minutes with shaking. Positive
133 transformants were selected on LB agar plates supplemented with 100µg/ml
134 ampicillin.

135 **Mammalian expression plasmids**

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

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

144

145 **Primary bovine mammary cell isolation and culture**

146 Tissue culture plates were coated with Type I calf skin collagen (Sigma-Aldrich, UK)
147 at 0.2mg/ml overnight at 4°C or for 1h at room temperature. The collagen solution
148 was removed by aspiration immediately prior to use. Dairy cows were selected prior
149 to slaughter on the basis of the absence of mammary infection as determined by the
150 California Mastitis Test (Schalm and Noorlander, 1957) and culture of milk samples.
151 The udder was collected immediately following slaughter, surgically prepared, and
152 then 3 cm³ pieces of secretory tissue were dissected out and placed in ice-cold
153 DMEM supplemented with 2mM glutamine, 100U/ml penicillin and 100 μ g/ml
154 streptomycin. Tissue pieces were processed according to the method of Rose *et. al*
155 (Rose et al., 2002). Briefly, tissue was minced to a fine paste with a sterile blade and
156 incubated in DMEM supplemented with 600U/ml collagenase type III (Worthington
157 Biochemicals, Reading, UK), 10% foetal calf serum (FCS), 2mM glutamine, 100U/ml
158 penicillin and 100 μ g/ml streptomycin for 30-60 minutes at 37°C with occasional
159 mixing. Digested tissue was pushed through a steel mesh of pore size 105 μ m and
160 cells were collected from the filtrate by centrifugation at 400 x g and 4°C. Cells were
161 washed three times in DMEM, and then resuspended in DMEM supplemented with
162 10% FCS, 2mM glutamine, 100U/ml penicillin and 100 μ g/ml streptomycin (Cell
163 Culture media) and plated in 10cm collagen-coated tissue culture dishes. Medium
164 was changed daily for the first three days to remove non-adherent cells and floating

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

168

169 **Transfection of mammary cells**

170 Glass coverslips were coated with 0.2mg/ml type I calf-skin collagen at 4°C overnight
171 or at room temperature for 4-6h, which was then aspirated prior to cell seeding.

172 Mammary cells were plated at 1×10^5 cells/ml on collagen-coated coverslips and
173 incubated overnight at 37°C, 5% CO₂. Cells were transfected using GeneFactor
174 (Venn Nova Inc. Florida, USA) according to the manufacturer's instructions, adding
175 1µg of the appropriate DNA plasmid and 2µl GeneFactor reagent per well of a 12-
176 well plate. Cells were incubated at 37°C, 5% CO₂ in the presence of transfection
177 reagents for 4-6h then the medium was replaced with Cell Culture media. Cells were
178 used for experiments at 18-24h post-transfection.

179

180 **Bacterial infection of mammary cells**

181 Primary bovine mammary cells were plated on collagen-coated coverslips as
182 described and cultured for 16-24h prior to infection experiments. Infection cultures
183 were prepared by inoculating 1ml of stationary phase bacterial culture into 10ml
184 DMEM and incubating at 37°C, 5% CO₂ for approximately 3h until they reached mid
185 log-phase with an OD₆₀₀ of 0.6. Ampicillin was added at 100 µg/ml to GFP LT2649-M
186 cultures to maintain the GFP plasmid. Mammary cells were washed three times into
187 serum-free antibiotic-free DMEM 1h prior to infection. For *E. coli* infections cell
188 culture media was removed from each well and replaced with bacterial suspension at
189 an MOI of approximately 150, for *Salmonella* infections bacteria were added to wells
190 at a 1:200 dilution. Cells were incubated in the presence of bacteria for 15 minutes or
191 3h, and washed rigorously three times with PBS prior to fixation to remove

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

194

195 **Fixation and processing for immunofluorescence**

196 Cells were fixed in 4% paraformaldehyde for 15 minutes, permeabilised with 0.1%
197 Triton X-100 in PBS for 5 minutes and then quenched with 0.1% NaBH₄ for 10
198 minutes. Primary antibodies were diluted in PBS containing 1% BSA, secondary
199 antibodies were diluted in PBS. Antibodies were incubated on coverslips for 1h each.
200 DAPI (1µg/ml) was incubated on coverslips for 5 minutes after removal of the
201 secondary antibody. Coverslips were washed three times in PBS between each
202 processing step. CellTracker green CMFDA or CellTracker Blue CMAC were used to
203 label the cell cytoplasm at 1µM according to the manufacturer's instructions,
204 LysoTracker Red DND-99 was used at 50nM according to the manufacturer's
205 instructions. Cells were imaged by confocal laser scanning microscopy using a Leica
206 TCS-SP2-AOBS attached to a Leica DM IRE2 inverted epifluorescence microscope.

207

208 **Results**

209

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

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

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

220

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

230

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

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

240

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

246

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

248 In order to determine the identity of the *E. coli* invasion compartment we used
249 specific markers of the endocytic pathway and of the signalling lipids that control
250 endosomal trafficking. The lipid PtdIns(3,4,5)P₃ transiently accumulates at the
251 phagocytic cup and at the site of entry of *Salmonella* (Cullen et al., 2001). We used
252 a well-characterised GFP-biosensor for PtdIns(3,4,5)P₃ (Venkateswarlu et al., 1998)
253 to determine whether mastitis *E. coli* also stimulated PtdIns(3,4,5)P₃ accumulation.
254 Mastitis *E. coli* infection failed to cause any apparent formation or redistribution of
255 PtdIns(3,4,5)P₃ (Figure 4). The signalling lipid PtdIns(4,5)P₂ is found on the plasma
256 membrane and is involved in phagocytosis and clathrin-mediated endocytosis (Jost
257 et al., 1998); however, mastitis *E. coli* also caused no increase in PtdIns(4,5)P₂
258 production and PtdIns(4,5)P₂ was not recruited to membranes surrounding
259 internalised bacteria (Figure 4). In contrast, vacuolar structures containing
260 internalized LT2649-M stained strongly with a GFP-biosensor for the signalling lipid
261 PtdIns(3)P (Figure 4). PtdIns(3)P marks early endosomes (Roth, 2004) and also
262 vacuoles containing internalised *Salmonella* (Cullen et al., 2001). From this we
263 conclude that invasive mastitis *E. coli* reside within the endocytic pathway, but enter
264 through a process that does not involve production of PtdIns(4,5)P₂ or
265 PtdIns(3,4,5)P₃, and is therefore distinct from the classical phagocytic mechanism.
266

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

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

279

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

282 Rab7 can also associate with autophagosomes and facilitate their maturation and
283 fusion with the lysosome (Gutierrez et al., 2004). We therefore examined whether
284 the Rab7+ invasive *E. coli* compartment was part of the autophagy pathway.

285 Infected cells were examined by confocal microscopy and autophagosomes were
286 labelled with the autophagy marker LC3. LC3 is cytosolic in resting cells but
287 translocates to the membranes of autophagosomes upon the induction of the
288 autophagic response (Kabeya et al., 2000). Uninfected control cells showed a few
289 LC3+ autophagic vesicles near the nucleus; however, internalised mastitis *E. coli*
290 showed no co-localisation with this compartment (Figure 7B). Further, there was no
291 induction of autophagic vesicles upon *E. coli* infection, whereas treatment of control
292 cells with rapamycin, an inducer of autophagy, caused a marked increase in the
293 number of LC3+ structures (Figure 7A).

294

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

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

309

310

311

312 **Discussion**

313

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

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

330

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

347

348 Mastitis *E. coli* LT2649-M bacteria were internalized into a membrane-bound
349 endocytic vacuole. GFP-tagged phosphoinositide probes and Rab GTPases were
350 used to characterize this compartment and have been previously described as
351 markers of endocytic compartments (Cullen et al., 2001; Hunyady et al., 2002).
352 Intracellular bacteria were identified within a compartment with early endosome-like
353 characteristics as shown by positive labelling with a fluorescent probe for PtdIns-3P.
354 No colocalisation was observed with probes for PtdIns(4,5)P₂ or PtdIns(3,4,5)P₃,
355 indicating that the bacteria are not internalized through a classical phagocytic

356 mechanism. These data are comparable to the invasion pathway of *Salmonella* that
357 resides in a membrane-bound Salmonella Containing Vacuole (SCV) and actively
358 modulates the PtdIns components on this compartment through depletion of
359 PtdIns(4,5)P₂ and retention of PtdIns(3)P (Hernandez et al., 2004).

360

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

377

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

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

397

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

411

412 In conclusion this study has revealed that mastitis *E. coli* are capable of invading
413 primary bovine mammary cells through a mechanism that does not result in visible
414 actin cytoskeleton rearrangement. We propose a model for *E. coli* intracellular
415 trafficking, shown schematically in Figure 8. Upon internalization bacteria reside
416 within a membrane-bound compartment that traffics along an endocytic pathway
417 acquiring the early and late endosomal markers PtdIns(3)P and Rab7 respectively.
418 The trafficking route appears to be modified, as suggested by the absence of the
419 early endosome marker Rab5 on the bacterial compartment and the observation that
420 internalized bacteria do not co-localise with a marker of acidified lysosome-like
421 compartments. Bacterial modulation of intracellular trafficking pathways has been
422 observed with other intracellular pathogens including *Salmonella* (Hashim et al.,
423 2000; Hernandez et al., 2004) and represents an important survival mechanism to
424 establish an intracellular infection reservoir. The recent report that intracellular
425 mastitis *E. coli* remain viable over a 48h period *in vitro* and can be detected in the
426 mammary tissue of *in vivo* infected bovine mammary glands (Dogan et al., 2006)
427 indicates that the establishment of a viable intracellular reservoir is a relevant
428 pathogenic mechanism used by these bacteria to persist within the udder
429 environment. This study has contributed to the overall understanding of persistent
430 bovine *E. coli* mastitis and takes us one step closer to unravelling the pathogenic
431 mechanisms used by these bacteria to establish such infections. Further
432 investigation to elucidate the nature of the factors that allow these bacteria to adhere
433 and invade is likely to yield important information about the bacterial contribution to
434 the invasion process and possibly allow the identification of novel therapeutic targets
435 to aid in the control and treatment of this problematic disease.

436

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- 543

544 **Figures**

545

546 **Figure 1.** Primary Bovine Mammary cell cultures consist of a co-culture of
547 myoepithelial cells and epithelial cells. Confocal micrographs of primary bovine
548 mammary cells isolated as described in Materials and Methods. Specific antibodies
549 (red) were used to identify the cell types present – cytokeratin (epithelial cells), α -
550 smooth muscle actin (myoepithelial cells) and vimentin (fibroblasts). Alexa 488-
551 conjugated phalloidin was used to visualise the actin cytoskeleton (green). Scale bar
552 = 10 μ m.

553

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

563

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

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

573

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

583

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

592

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

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

602

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

614

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

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

627

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

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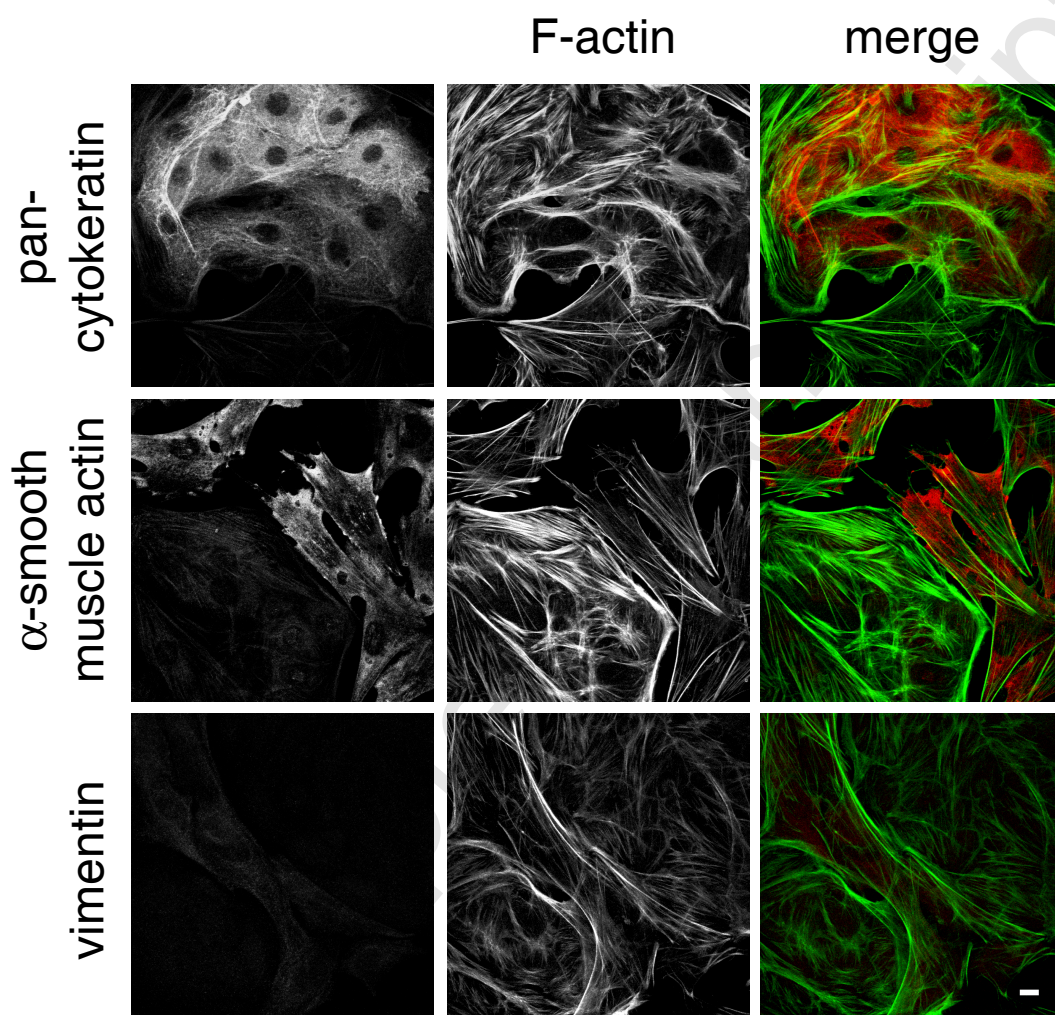
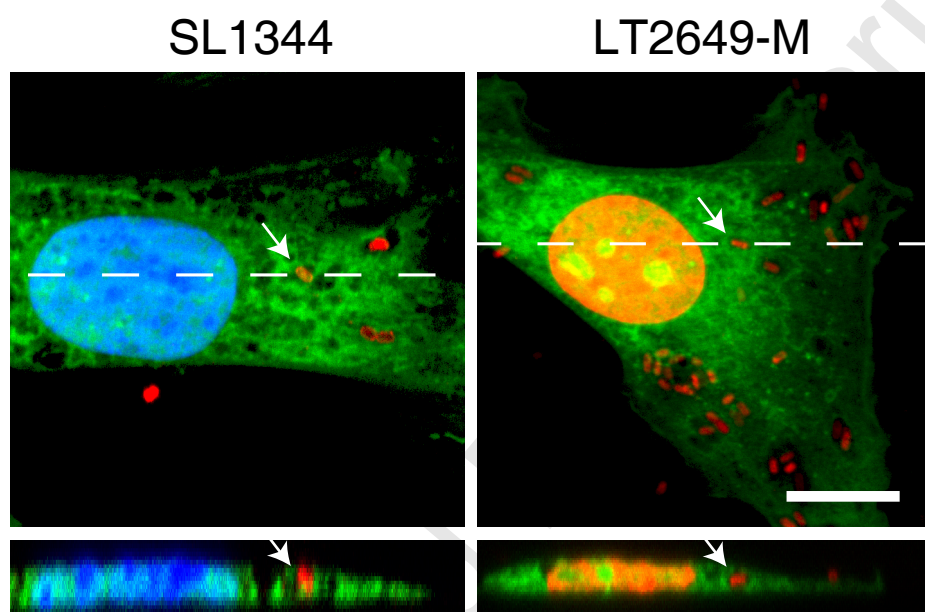
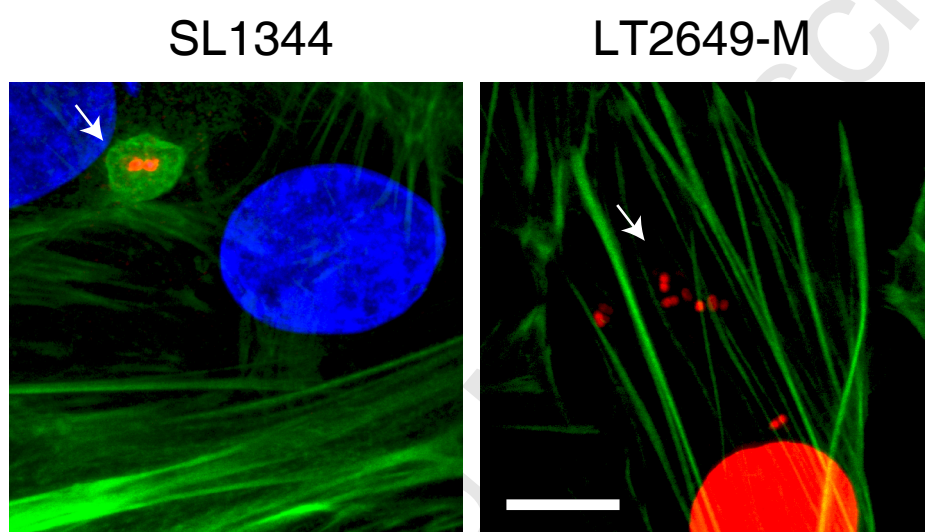
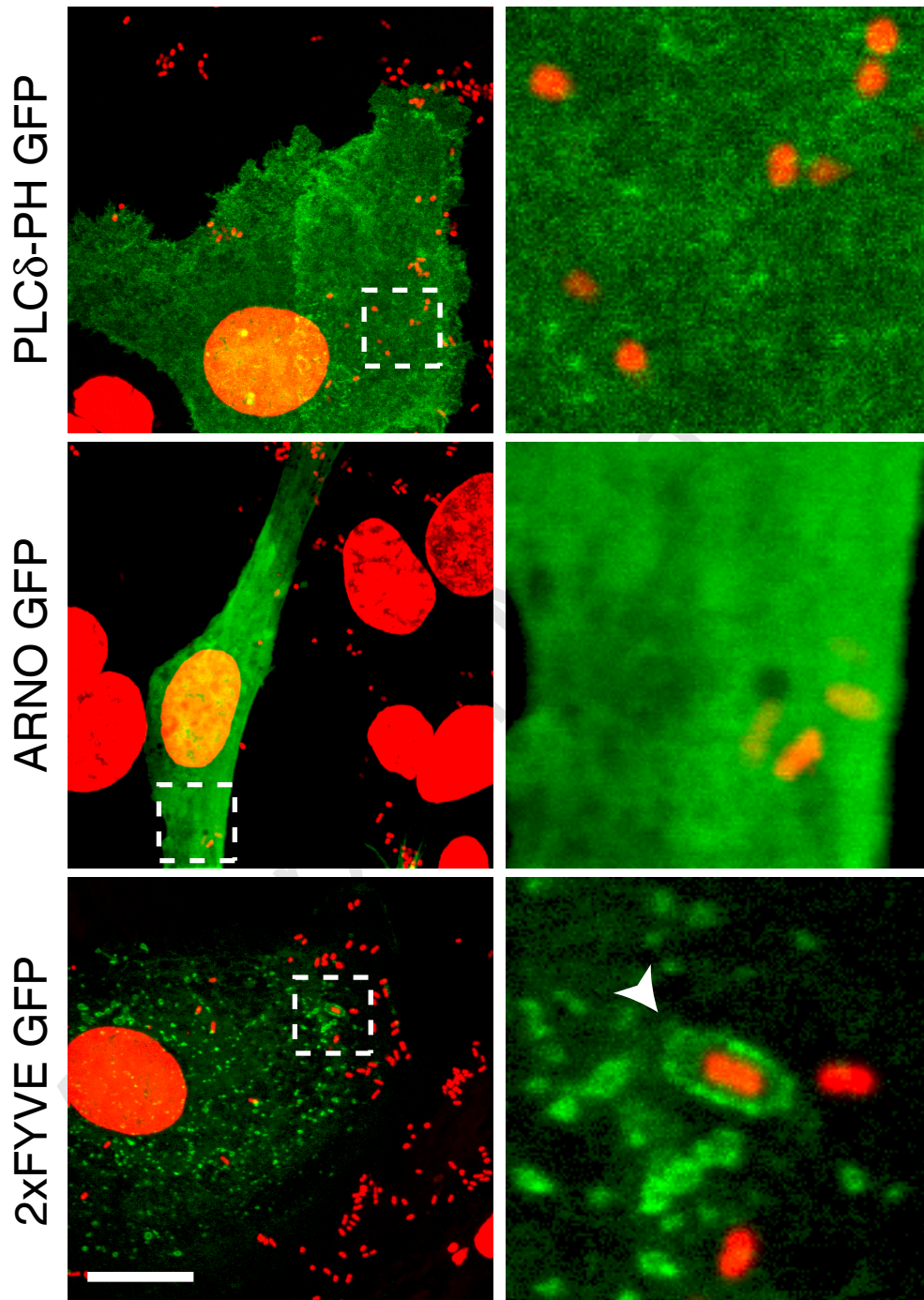
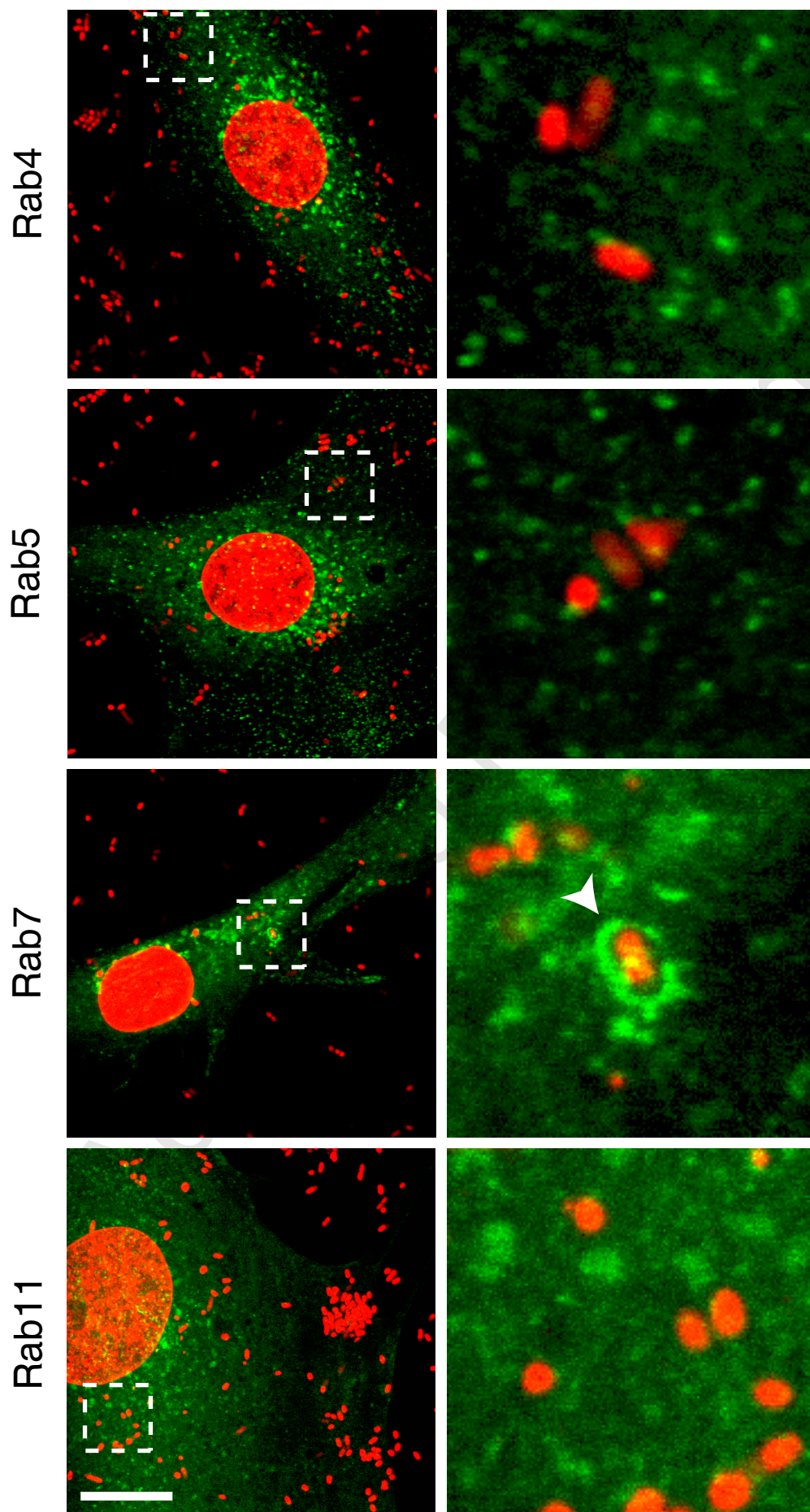


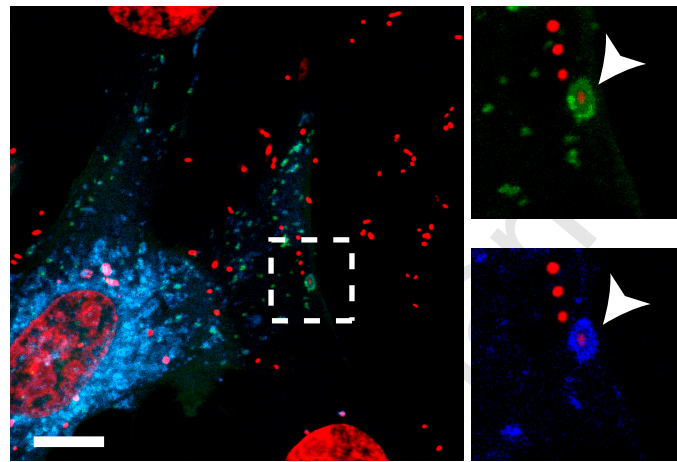
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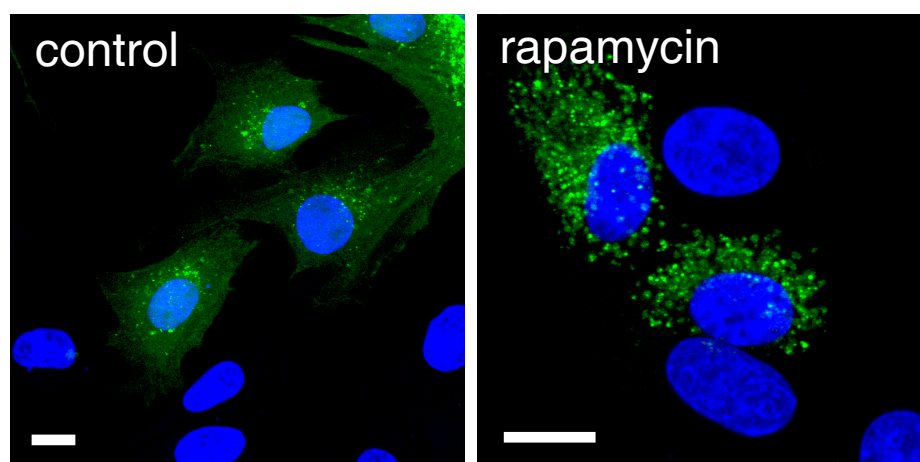




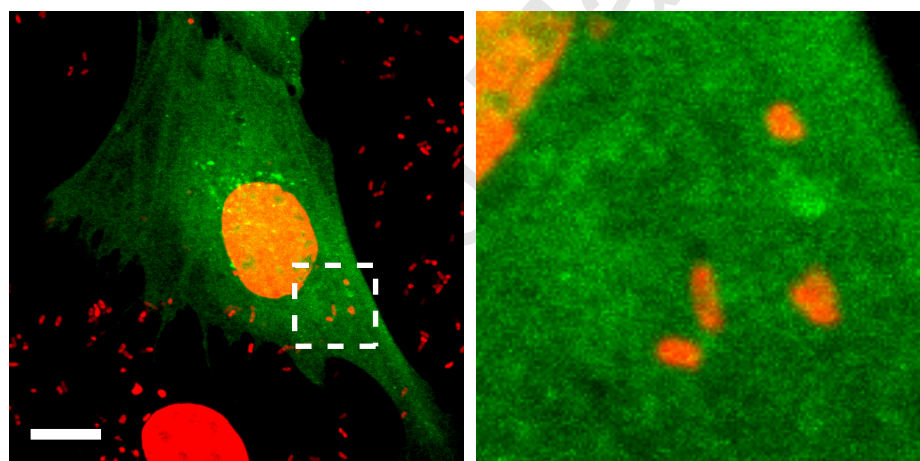




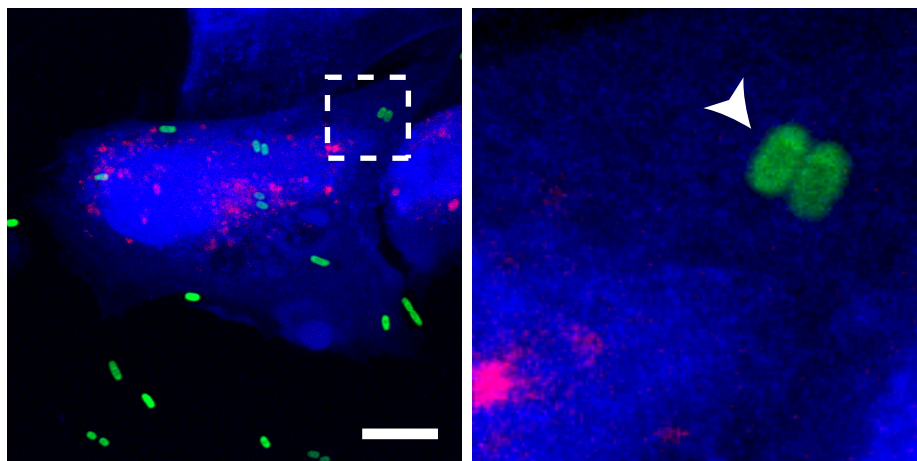
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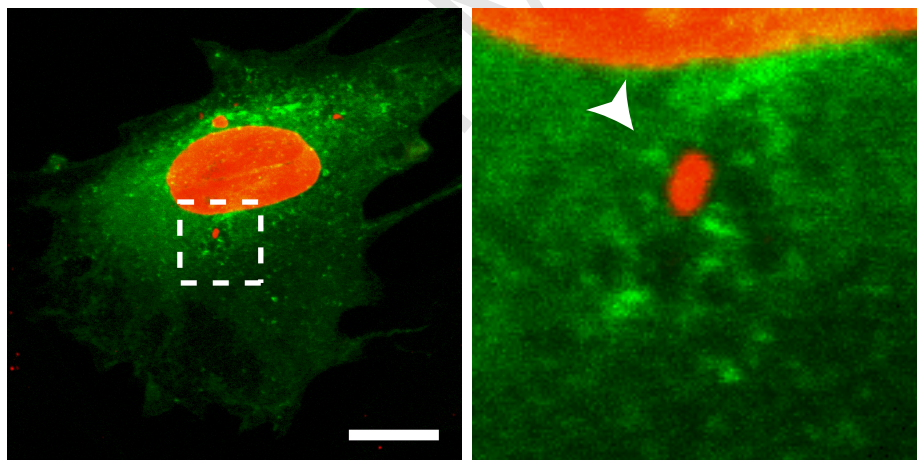
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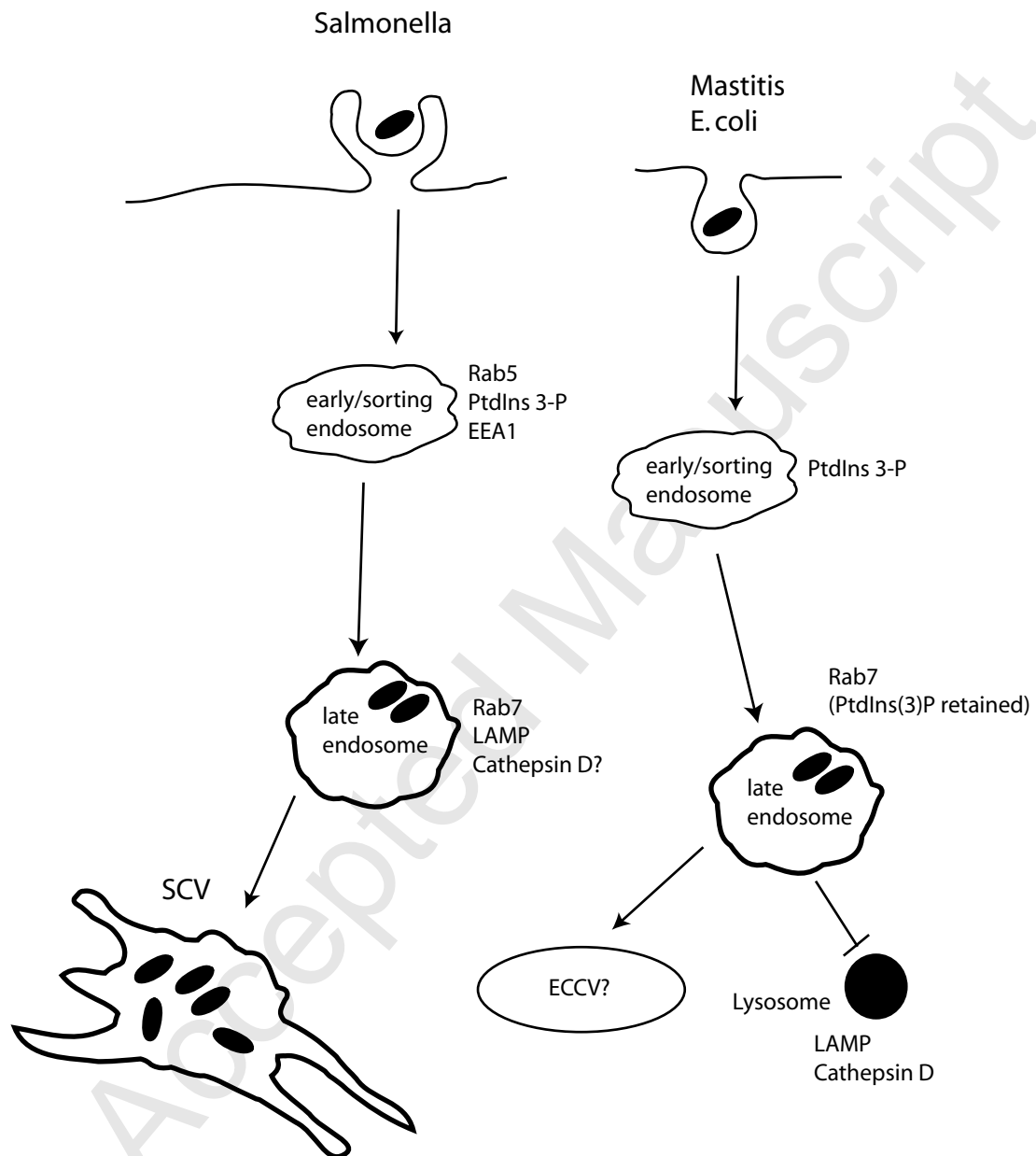


Figure 9