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1 ***Klebsiella pneumoniae* survives within macrophages by avoiding delivery to lysosomes.**

2 Victoria Cano^{1,2†}, Catalina March^{1,2†}, Jose Luis Insua^{3†}, Nacho Aguiló^{2,5}, Enrique Llobet^{1,2,4}, David
3 Moranta^{1,2,4}, Verónica Regueiro^{1,2,4}, Gerry P Brenan⁶, Maria Isabel Millán-Lou^{2,5}, Carlos Martín^{2,5},
4 Junkal Garmendia^{2,7}, José A. Bengoechea^{3,8}.

5 Laboratory Infection and Immunity, Fundació d'Investigació Sanitària de les Illes Balears (FISIB),
6 Bunyola, Spain¹; Centro de Investigación Biomédica en Red Enfermedades Respiratorias
7 (CIBERES), Bunyola, Spain²; Centre for Infection and Immunity, Queen's University Belfast,
8 Belfast, United Kingdom³; Institut d'Investigació Sanitària de Palma (IdISPa), Palma, Spain⁴; Grupo
9 de Genética de Micobacterias, Dpto. Microbiología, Medicina Preventiva y Salud Pública,
10 Universidad de Zaragoza, Zaragoza, Spain⁵; School of Biological Sciences, Queen's University
11 Belfast, Belfast, United Kingdom⁶; Instituto de Agrobiotecnología, CSIC-Universidad Pública de
12 Navarra-Gobierno de Navarra, Mutilva, Spain⁷; Consejo Superior de Investigaciones Científicas
13 (CSIC), Madrid, Spain⁸

14 † These authors contributed equally to this work.

15

16 *Corresponding author:

17 Prof. José A. Bengoechea

18 Centre for Infection and Immunity,

19 Queen's University Belfast

20 Health Sciences Building,

21 97 Lisburn Rd. Belfast, UK BT9 7AE

22 Phone: +44 (0) 28 9097 2260; Fax: +44 (0) 28 9097 2671

23 E-mail: j.bengoechea@qub.ac.uk

24

25 Running title: *Klebsiella* intracellular survival

26

27 **SUMMARY**

28 *Klebsiella pneumoniae* is an important cause of community-acquired and nosocomial
29 pneumonia. Evidence indicates that *Klebsiella* might be able to persist intracellularly within a
30 vacuolar compartment. This study was designed to investigate the interaction between
31 *Klebsiella* and macrophages. Engulfment of *K. pneumoniae* was dependent on host
32 cytoskeleton, cell plasma membrane lipid rafts and the activation of PI 3-kinase (PI3K).
33 Microscopy studies revealed that *K. pneumoniae* resides within a vacuolar compartment, the
34 *Klebsiella* containing vacuolae (KCV), which traffics within vacuoles associated with the
35 endocytic pathway. In contrast to UV-killed bacteria, the majority of live bacteria did not
36 colocalize with markers of the lysosomal compartment. Our data suggest that *K. pneumoniae*
37 triggers a programmed cell death in macrophages displaying features of apoptosis. Our
38 efforts to identify the mechanism(s) whereby *K. pneumoniae* prevents the fusion of the
39 lysosomes to the KCV uncovered the central role of the PI3K-Akt-Rab14 axis to control the
40 phagosome maturation. Our data revealed that the capsule is dispensable for *Klebsiella*
41 intracellular survival if bacteria were not opsonized. Furthermore, the environment found by
42 *Klebsiella* within the KCV triggered the downregulation of the expression of *cps*. Altogether,
43 this study proves evidence that *K. pneumoniae* survives killing by macrophages by
44 manipulating phagosome maturation which may contribute to *Klebsiella* pathogenesis.

45

46 INTRODUCTION

47 In the late nineteenth century, Eli Metchnikoff appreciated phagocytosis as a key process in
48 the battle against pathogens. Phagocytosis can be conceptually divided into phagosome formation
49 and its subsequent evolution into a degradative compartment, a process termed phagosome
50 maturation. This is important because the nascent phagosome is not microbicidal. Maturation not
51 only aids clearing infection, but also generates and routes antigens for presentation on MHC
52 molecules in order to activate the adaptive immune system (Trombetta and Mellman. 2005).
53 Phagosome maturation involves the sequential acquisition of different proteins, many of them of the
54 endocytic pathway (Vieira *et al.* 2002, Flannagan *et al.* 2012). Thus, during and/or immediately after
55 phagosome closure, the phagosome fuses with early endosomes, acquiring Rab5 and early
56 endosome antigen 1 (EEA1). The phagosome rapidly loses the characteristics of early endosome
57 and acquires late endosome features. The late phagosome is positive for Rab7, the mannose-6-
58 phosphate receptor, lysobisphosphatidic acid, lysosome-associated membrane proteins (Lamps) and
59 CD63. Ultimately, the organelle fuses with lysosomes to form the phagolysosome, identified by the
60 presence of hydrolytic proteases, such as processed cathepsin D, cationic peptides and by an
61 extremely acidic luminal pH which is regulated primarily by the vacuolar (V-type) ATP-ase
62 complex. In the course of maturation, an oxidative system formed by the NADPH oxidase and
63 ancillary proteins is also activated.

64 Many pathogens have developed strategies to counteract the microbicidal action of
65 macrophages (Flannagan *et al.* 2009, Sarantis and Grinstein. 2012). Some pathogens inhibit
66 phagocytosis. For example, the role of capsule polysaccharides in preventing opsonophagocytosis
67 has been appreciated for many pathogens including *Neisseria meningitidis*, *Staphylococcus aureus*
68 and *streptococci*. Others, such as enteropathogenic *Escherichia coli*, inhibit engulfment by blocking
69 PI 3-kinase (PI3K) signaling whereas *Yersinia* species inhibits phagocytosis by injecting type III
70 secretion effectors. Conversely, *Salmonella typhimurium* induces its own uptake and, once inside a
71 modified phagosome, triggers macrophage death by a caspase-1 dependent process called pyroptosis

72 (Fink and Cookson. 2007). *Brucella* spp. resist an initial macrophage killing to replicate in a
73 compartment segregated from the endocytic pathway with endoplasmic reticulum properties (von
74 Bargen *et al.* 2012).

75 *Klebsiella pneumoniae* is a Gram negative capsulated pathogen which causes a wide range
76 of infections, from urinary tract infections to pneumonia, being particularly devastating among
77 immunocompromised patients with mortality rates between 25% and 60% (Sahly and Podschun.
78 1997). *K. pneumoniae* is an important cause of community-acquired pneumonia in individuals with
79 impaired pulmonary defences and is a major pathogen for nosocomial pneumonia. Pulmonary
80 infections are often characterized by a rapid clinical course thereby leaving very short time for an
81 effective antibiotic treatment. *K. pneumoniae* isolates are frequently resistant to multiple antibiotics
82 (Munoz-Price *et al.* 2013), which leads to a therapeutic dilemma. In turn, this stresses out the
83 importance of pulmonary innate defense systems to clear *K. pneumoniae* infections.

84 Resident alveolar macrophages play a critical role in the clearance of bacteria from the lung
85 by their capacity for phagocytosis and killing. It has been shown that depletion of alveolar
86 macrophages results in reduced killing of *K. pneumoniae in vivo* (Broug-Holub *et al.* 1997, Cheung
87 *et al.* 2000). This suggests that *Klebsiella* countermeasures against phagocytosis would be
88 important virulence factors. Supporting this notion, *K. pneumoniae* capsule (CPS) reduces
89 phagocytosis by neutrophils and macrophages (March *et al.* 2013, Cortes *et al.* 2002b, Regueiro *et al.*
90 2006, Alvarez *et al.* 2000) and CPS mutant strains are avirulent not being able to cause pneumonia
91 and urinary tract infections (Cortes *et al.* 2002b, Lawlor *et al.* 2005, Camprubi *et al.* 1993).

92 *K. pneumoniae* has been largely considered as an extracellular pathogen. However, there are
93 reports showing that *K. pneumoniae* is internalized *in vitro* by different cell types being able to
94 persist intracellularly for at least 48 h (Oelschlaeger and Tall. 1997). It has been also reported the
95 presence of intracellular *Klebsiella* spp. within a vacuolar compartment inside human macrophages,
96 mouse alveolar macrophages and lung epithelial cells *in vivo* (Cortes *et al.* 2002b, Fevre *et al.*
97 2013, Willingham *et al.* 2009, Greco *et al.* 2012). The present study was designed to investigate the

98 interaction between *K. pneumoniae* and macrophages. We report that *K. pneumoniae* survives
99 within macrophages by deviating from the canonical endocytic pathway and residing in a unique
100 intracellular compartment which does not fuse with lysosomes. Mechanistically, our results indicate
101 that *Klebsiella* targets the PI3K-Akt-Rab14 axis to control the phagosome maturation. Finally, we
102 present evidence indicating that *K. pneumoniae* has the potential to kill and escape from the
103 phagocyte.

104

105

106 **RESULTS**

107 ***K. pneumoniae* survives inside macrophages.**

108 To explore whether *K. pneumoniae* resides inside macrophages *in vivo*, macrophages were
109 isolated from the bronchoalveolar lavage of mice infected intranasally with *K. pneumoniae* strain
110 43816 (hereafter Kp43816R). Confocal microscopy experiments showed that 85 ± 4 % of the
111 intracellular bacteria did not colocalize with the lysosomal marker cathepsin D (Fig 1A).
112 Macrophages isolated obtained from the bronchoalveolar lavage were pulsed-chased with
113 tetramethylrhodamine-labelled dextran (TR-dextran) as described in the Experimental procedures.
114 Pulse-chase protocols with TR-dextran are extensively used in the literature to label lysosomes
115 (Morey *et al.* 2011, Eissenberg *et al.* 1988, Hmama *et al.* 2004, Lamothe *et al.* 2007). Confocal
116 microscopy revealed that 80 ± 3 % intracellular *Klebsiella* did not colocalize with TR-dextran (Fig
117 1A).

118 To assess the interaction of *K. pneumoniae* and macrophages in more detail, we standardized
119 the infection conditions of the mouse macrophage cell line MH-S with Kp43816R. We optimized
120 the time of bacteria-cell contact (30, 60 and 120 min), the multiplicity of infection (MOI) (100, 50
121 or 10 bacteria per cell), and the antibiotic treatment necessary to kill the remaining extracellular
122 bacteria after the contact. To synchronize infection, plates were centrifuged at 200 x g during 5 min
123 and intracellular bacteria were enumerated after macrophage lysis with 0.5% saponin in PBS. We
124 found that 90 min treatment with a combination of gentamicin (300 µg/ml) and polymyxin B (15

125 $\mu\text{g/ml}$) was necessary to kill 99.9% of the extracellular bacteria. The highest numbers of engulfed
126 bacteria were obtained after 120 min of bacteria-cell contact with a multiplicity of infection (MOI)
127 of 100:1. However, these conditions also triggered a significant decrease in cell viability as detected
128 by the trypan blue exclusion method. 30 min of contact and a MOI of 50:1 were the conditions in
129 which no decrease in cell viability was observed and, therefore, they were used in the subsequent
130 experiments described in this study.

131 To investigate the molecular mechanisms used by mouse macrophages to engulf Kp43816R,
132 infections were carried out in the presence of inhibitors of host cell functions (Fig 1B). Cytochalasin
133 D and nocodazol reduced the engulfment of Kp43816R hence indicating that Kp43816R
134 phagocytosis requires the assembly of F-actin and the host microtubule network. Methyl- β -
135 cyclodextrin (M β CD), which depletes cholesterol from host cell membranes, was employed to
136 analyse the involvement of lipid rafts in Kp43816R phagocytosis. Cholesterol depletion impaired
137 *Klebsiella* engulfment by MH-S. Similar results were obtained when cells were treated with filipin
138 and nystatin (Fig. 1B). Since the generation of phosphoinositides is linked to phagosome formation
139 (Vieira *et al.* 2001), we assessed the contribution of the PI3K signalling pathway on Kp43816R
140 phagocytosis. Pre-treatment of MH-S cells with LY294002, a specific inhibitor of PI3K activity,
141 resulted in the blockage of Kp43816R phagocytosis (Fig. 1B). Immunofluorescence experiments
142 further confirmed that treatment of cells with LY294002 inhibited the engulfment of *Klebsiella* (Fig
143 S1). This was also true for UV-killed bacteria (Fig S1). Akt is a downstream effector of PI3K which
144 becomes phosphorylated upon activation of the PI3K signalling cascade. As expected, western blot
145 analysis revealed that Kp43816R induces the phosphorylation of Akt in a PI3K-dependent manner
146 since LY294002 inhibited *Klebsiella*-induced phosphorylation of Akt (Fig. 1C-D). UV-killed
147 bacteria also induced the phosphorylation of Akt although the levels were significantly lower than
148 those induced by live bacteria (Fig 1C). The PI3K-Akt cascade is also activated by Kp43816R in
149 human macrophages (THP-1 monocytes differentiated to macrophages by phorbol-12-myristate-13-
150 acetate [PMA] treatment; hereafter mTHP-1) (Fig. S2).

151 Bacterial intracellular location in MH-S cells was assessed 3 and 6 h post infection by
152 transmission electron microscopy (TEM). In good agreement with other published observations *in*
153 *vivo* (Cortes *et al.* 2002b, Fevre *et al.* 2013, Willingham *et al.* 2009, Greco *et al.* 2012), bacteria were
154 located in a vacuolar compartment (data not shown). To determine the fate of intracellular
155 Kp43816R, MH-S cells were infected with GFP-expressing Kp43816R and the number of
156 intracellular bacteria was assessed microscopically using differential (extracellular/intracellular)
157 staining and by plating after different incubation times. The number of intracellular bacteria in MH-
158 S cells decreased during the first 2 h of infection but then it remained constant until 7.5 h post
159 infection (Fig 2A). Immunofluorescence analysis revealed that the number of infected macrophages
160 decreased during the first 2 h hence suggesting that some cells are able to clear the infection.
161 However, after 2 h, the percentage of infected macrophages did not change until the end of the
162 experiment (Fig 2B). We did not observe any change of host cell morphology (data not shown). The
163 majority of infected macrophages contained less than three bacteria (Fig 2C). The fact that the
164 number of macrophages containing between three and five bacteria or more than five did not
165 change over time suggests that there is not significant bacteria replication. Similar results were
166 obtained when mTHP-1 cells were infected (Fig S3).

167 To elucidate whether those intracellular bacteria assessed by microscopy were indeed viable,
168 cells were infected with *Klebsiella* harbouring two plasmids, one conferring constitutive expression
169 of mCherry (pJT04mCherry) and another one (pMMB207gfp3.1) expressing *gfp* under the control
170 of an IPTG inducible promoter. Therefore, only metabolically active bacteria will be mCherry-GFP
171 positive. Microscopy analysis using differential (extracellular/intracellular) staining showed that
172 more than 75% of intracellular bacteria were mCherry-GFP positive 3.5 h post infection (Fig 2D-E).
173 This percentage did not change over time. To further confirm that intracellular *Klebsiella* are
174 metabolically active, fluorescent *in situ* hybridisation (FISH) was carried out by using the
175 oligonucleotide probes EUB338 and GAM42a (see Experimental procedures). The detection of
176 bacteria by these oligonucleotide probes is dependent on the presence of sufficient ribosomes per

177 cell, hence providing qualitative information on the physiological state of the bacteria (Christensen
178 *et al.* 1999, Morey *et al.* 2011). Microscopy analysis revealed that the number of bacteria
179 metabolically active (FISH positive) *versus* the total number of intracellular bacteria (GFP positive)
180 was maintained through the infection (Fig. S4).

181 Collectively, these results showed that Kp43816R phagocytosis by macrophages is an event
182 dependent on host cytoskeleton and cell plasma membrane lipid rafts. Moreover, the PI3K/Akt host
183 signalling pathway is activated by Kp43816R infection and it is required for bacterial phagocytosis.
184 Our data demonstrate that Kp43816R survives within macrophages through the course of infection
185 and the TEM experiments may suggest that Kp43816R may reside in a specific compartment that
186 we named the *Klebsiella* containing vacuole (KCV).

187

188 ***K. pneumoniae* elicits a cytotoxic effect on macrophages.**

189 Examination of the infected monolayers by immunofluorescence at different time points
190 revealed a decreased in the overall monolayer density at 10 h post infection which became more
191 evident 20 h post infection (Fig S5A). This observation prompted us to study whether Kp43816R
192 exerts a cytotoxic effect on macrophages. We assessed the viability of infected MH-S cells by
193 measuring the levels of LDH release. Kp43816R infection was associated with a 35% decrease in
194 cell viability after 20 h of infection. Kp43816R-triggered cytotoxic effect on macrophages was also
195 evident when cell viability was estimated by the neutral red uptake assay (Fig S5B).

196 The induction of host cell apoptosis is one mechanism used by some pathogens to augment
197 infection (Navarre and Zychlinsky. 2000). To test whether Kp43816R causes apoptosis of MH-S
198 cells, apoptosis was measured with annexin V, to analyze phosphatidylserine translocation to the
199 outer leaflet of the plasma membrane, and 7-actinomycin D (AAD) to evaluate plasma membrane
200 integrity. Flow cytometry analysis of infected cells showed a significant increased in annexin
201 V⁺AAD⁻ cells over time (Fig. 3). The amount of double-positive annexinV⁺AAD⁺ cells, which
202 corresponds to a necrotic-like phenotype, was markedly lower than the amount of cells annexin

203 V⁺AAD⁻ at all times analyzed. These results indicate phosphatidylserine translocation and intact
204 membrane integrity, a classical apoptotic phenotype, hence suggesting that Kp43816R triggers
205 apoptosis in macrophages.

206

207 ***K. pneumoniae* prevents phagosome fusion with lysosomes.**

208 Because Kp43816R is able to survive within macrophages, we hypothesized that *Klebsiella*
209 must either divert the normal process of phagosome maturation or withstand the hostile
210 environment of the mature phagolysosome. Therefore, we analyzed the maturation of the KCV
211 during the course of an infection by unravelling the association of the KCV with compartments of
212 the exocytic and endocytic pathways. Bacteria did not colocalize with either markers of the
213 endoplasmic reticulum (calnexin) or markers of the Golgi network (GM 130) at any time point
214 analyzed (Fig S6). EEA1 is an early endosome-specific peripheral membrane protein which
215 colocalizes with the small GTP binding protein Rab5 (Vieira *et al.* 2002, Flannagan *et al.* 2012). As
216 shown in Figure 4, we could detect the presence of EEA1 on 22 ± 4% of KCVs at 15 min post
217 infection. The percentage of vacuoles positive for this marker dropped to 15 ± 9% and to 5 ± 1% at
218 60 and 90 min post infection, respectively (Fig 4). We next sought to determine whether the KCV
219 acquires the late endosomal markers Lamp1 and Rab7 (Vieira *et al.* 2002, Flannagan *et al.* 2012).
220 KCVs were positive for Lamp1 already at 15 min post infection and the percentage of positive
221 KCVs increased over time (Fig 4). KCVs remained positive for Lamp1 until 7.5 h post infection.
222 Rab7 is a small GTPase that controls vesicular transport to late endosomes and lysosomes in the
223 endocytic pathway (Rink *et al.* 2005). To assess the presence of Rab7 on KCVs, macrophages were
224 transfected with GFP-Rab7 and then infected with Kp43816R. The majority of the vacuoles
225 containing Kp43816R were positive for both Rab7 and Lamp1 (Fig 4). To determine the activation
226 status of Rab7 we asked whether RILP, a Rab7 effector protein that exclusively recognizes the
227 active (GTP bound) conformation of Rab7 (Cantalupo *et al.* 2001, Jordens *et al.* 2001), labels the
228 KCV. Before infection, cells were transfected with a plasmid containing GFP fused to the C-

229 terminal Rab7-binding domain of RILP, called “RILP-C33”, which can be used as a reliable index
230 of the presence and distribution of active Rab7 (Cantalupo *et al.* 2001, Jordens *et al.* 2001). As
231 shown in Figure 4 RILP-C33-EGFP colocalized with the majority of KCVs. These vacuoles were
232 also positive for Lamp1.

233 Since the interaction of Rab7 with RILP drives fusion with lysosomes (Cantalupo *et al.*
234 2001, Jordens *et al.* 2001), we sought to determine whether KCV colocalizes with lysosomal
235 markers. Although there are not markers that unambiguously distinguish late endosomes from
236 lysosomes, mounting evidence indicates that an acidic luminal pH and the presence of hydrolytic
237 proteases, such as processed cathepsin D, are characteristics of the phagolysosomal fusion (Vieira *et*
238 *al.* 2002, Flannagan *et al.* 2012). We used the fixable acidotropic probe LysoTracker to monitor
239 acidic organelles in infected macrophages. We found a major overlap between the dye and the
240 KCVs (Fig 5), hence indicating that the KCV is acidic. We next examined the presence in the
241 vacuole of cathepsin D as a marker for the lysosomal soluble content. The majority of the KCVs did
242 not colocalize with cathepsin D (Fig 5), thereby suggesting that the KCV does not fuse with
243 lysosomes. To further sustain this notion, we assessed KCV colocalization with TR-dextran. Prior
244 to bacterial infection macrophages were pulsed with TR-dextran for 2 h followed by a 1 h chase in
245 dye-free medium to ensure that the probe is delivered from early and recycling endosomes to
246 phagolysosomes (Morey *et al.* 2011, Eissenberg *et al.* 1988, Hmama *et al.* 2004, Lamothe *et al.*
247 2007). Confocal immunofluorescence showed that the majority of the KCVs did not colocalize with
248 TR-dextran (Fig 5B). In contrast, when macrophages were infected with UV-killed Kp43816R more
249 than 70% of the KCVs did colocalize with cathepsin D and TR-dextran 1.5 h post infection (Fig
250 S7). Collectively, these results strongly support the notion that the majority of KCVs containing
251 live bacteria prevent the fusion of the vacuole with lysosomes.

252 Similar findings were obtained when mTHP-1 cells were infected. KCV was not associated
253 with compartments of the exocytic pathway, either Golgi network or endoplasmic reticulum, but
254 acquired markers of the endocytic pathway, EEA1 and Lamp1 (Fig S8A). The majority of KCVs

255 colocalized with LysoTracker (Fig S8A) but they were negative for cathepsin D (Fig S8B). In
256 contrast, nearly 70% of UV-killed Kp43816R colocalized with cathepsin D after 2 h post infection
257 (Fig S8B). Altogether, these results indicate that only phagosomes containing UV-killed *Klebsiella*
258 bacteria fuse with lysosomes in human macrophages.

259 In summary, these findings suggest that *K. pneumoniae* trafficks inside macrophages within
260 vacuoles associated to the endocytic pathway, and that live bacteria perturb the fusion of the KCV
261 with the hydrolases-rich lysosomal compartment.

262

263 **Inhibition of compartment acidification affects *K. pneumoniae* intracellular survival.**

264 Phagosome acidification has been shown to be essential for the intracellular survival of
265 several pathogens (Morey *et al.* 2011, Ghigo *et al.* 2002, Porte *et al.* 1999). Therefore, we
266 investigated the effect of inhibiting KCV acidification on *K. pneumoniae* survival. Bafilomycin A₁
267 is a specific inhibitor of the vacuolar type H⁺-ATPase in cells, and inhibits the acidification of
268 organelles containing this enzyme, such as lysosomes and endosomes. As expected,
269 phagolysosomal acidification was sensitive to bafilomycin A₁ treatment (Fig 6A), hence confirming
270 dependence on the vacuolar H⁺-ATPase. Moreover, bafilomycin A₁ treatment also abrogated the
271 overlap between Kp43816R and the probe LysoTracker (Fig 6A). To assess the effect of vacuolar
272 acidification on Kp43816R survival, cells were treated with bafilomycin A₁ at the onset of the
273 gentamicin treatment and bacteria were enumerated by plating at different time points. Data shown
274 in Figure 6C revealed that the number of intracellular Kp43816R decreased in bafilomycin A₁
275 treated cells over time compared to infected untreated cells. Control experiments revealed that
276 bafilomycin A₁ has no toxic effect on *K. pneumoniae* (our control experiments [data not shown]) or
277 on other Gram-negative bacteria (Morey *et al.* 2011, Porte *et al.* 1999). Microscopy analysis
278 revealed that the percentages of Kp43186R colocalization with TR-dextran in bafilomycin A₁
279 treated cells either at 3.5 or 5.5 h post infection (19 ± 4 and 20 ± 5%, respectively) were similar to
280 those in DMSO (vehicle solution)-treated cells (20 ± 4 and 24 ± 6 %, respectively). In turn, the

281 percentage of mCherry-GFP positive intracellular bacteria dropped from 85 ± 7 % in DMSO-treated
282 cells to 25 ± 4 % in bafilomycin A₁ treated cells already at 2.5 h post infection ($P < 0.05$ Mann-
283 Whitney U test). Altogether, these observations suggest that Kp43816R intracellular survival
284 requires KCV acidification.

285

286 **PI3K-AKT and Rab14 contribute to *K. pneumoniae* intracellular survival.**

287 *S. enterica* serovar *typhimurium* perturbs the fusion of the phagosomes with lysosomes by
288 activating the host kinase Akt (Kuijl *et al.* 2007). In turn, inhibition of Akt activation reduces
289 *Salmonella* intracellular survival (Kuijl *et al.* 2007, Chiu *et al.* 2009). Several pathogens also target
290 the PI3K-Akt axis to manipulate cell biology for their own benefit (Krachler *et al.* 2011). Since
291 Kp43816R induced the activation of Akt in a PI3K-dependent manner we sought to determine the
292 contribution of the PI3K-Akt axis to the intracellular survival of *K. pneumoniae*. Treatment of cells
293 with the PI3K inhibitor LY294002 or the Akt inhibitor AKT X at the onset of the gentamicin
294 treatment reduced the number of intracellular bacteria in MH-S cells (Fig 7A). Moreover,
295 microscopy analysis revealed that more than 70% bacteria colocalized with either TR-dextran or
296 cathepsin D in cells treated with AKT X (Fig 7B and Fig S9). Collectively, these results support the
297 notion that Kp43816R targets the PI3K-Akt axis to survive intracellularly.

298 At least 18 Rab GTPases are implicated in phagosomal maturation (Smith *et al.* 2007).
299 Interestingly, *Salmonella* targets Rab14 to prevent phagosomal maturation in an Akt dependent
300 manner (Kuijl *et al.* 2007). We speculated that Kp43816R may also target Rab14 to control the
301 maturation of the phagosome. Immunofluorescence experiments revealed that GFP-Rab14
302 colocalized with the KCVs (Fig 7C-D). To determine whether Rab14 recruitment is required for
303 intracellular survival, cells were transfected with a Rab14 dominant-negative construct (DN-Rab14)
304 or control vector and then infected with Kp43816R. As shown in figure 7E, we found a 60%
305 decrease in the number of intracellular bacteria in cells transfected with DN-Rab14. Supporting that
306 *Klebsiella* recruited Rab14 to the KVC in an Akt-dependent manner, GFP-Rab14 did not colocalize

307 with the KCV in AKT X treated cells (7 ± 2 % percentage of colocalization at 2.5 h post infection)
308 (Fig 7F).

309 In summary, our results are consistent with a model where Kp43816R targets the PI3K-Akt-
310 Rab14 axis to control the phagosome maturation to survive inside macrophages.

311

312 ***K. pneumoniae* capsule polysaccharide is dispensable for intracellular survival.**

313 We were keen to identify *K. pneumoniae* factors necessary for intracellular survival. Given
314 the importance of *K. pneumoniae* CPS on host-pathogen interactions, we explored whether CPS is
315 also necessary for *K. pneumoniae* intracellular survival. As anticipated, a CPS mutant was engulfed
316 by MH-S and mTHP1 macrophages in higher numbers than Kp43816R (data not shown). For the
317 sake of comparison with the wild-type strain in time-course experiments, we adjusted the MOI of
318 the CPS mutant to get comparable numbers of intracellular bacteria at the beginning of the
319 infection. Time course experiments showed no differences between the number of intracellular
320 bacteria of both strains in MH-S and mTHP1 cells (Fig 8A).

321 Given the critical role of CPS in preventing complement-mediated opsonophagocytosis
322 (Alvarez *et al.* 2000, de Astorza *et al.* 2004, Cortes *et al.* 2002a), we evaluated whether the
323 intracellular fate of the CPS mutant could be modified by bacterial opsonization with human serum.
324 In agreement with previous reports (de Astorza *et al.* 2004, Cortes *et al.* 2002a), opsonization of the
325 CPS mutant resulted in an increase in the number of ingested bacteria by mTHP1 cells compared to
326 nonopsonized bacteria (Fig 8B). For the sake of comparison, the MOI was adjusted to get
327 comparable numbers of intracellular bacteria at the beginning of the infection. The number of CFU
328 recovered from cells infected with the opsonized CPS mutant was significantly lower than the
329 number of CFU recovered from cells infected with non-opsonized bacteria (100 fold lower at 8 h
330 post infection; Fig 8C). These data indicate that internalization via the C3 receptor results in a
331 significant loss of intracellular viability, presumably because these bacteria are ultimately delivered
332 to lysosomes.

333 The lack of contribution of CPS to intracellular survival prompted us to analyze the
334 expression of *cps* in the KCV. To monitor *cps* expression over time, we generated a transcriptional
335 fusion in which the *cps* promoter region was cloned upstream a promoterless *gfp* that encodes a
336 protein tagged at the C terminus with the (LVA) peptide. The GFP(LVA) protein is targeted for Tsp
337 protease degradation within the bacteria and has been reported to have 40-min half-life, while
338 untagged GFP is very stable (estimated *in vivo* half-life, 24 h) (Miller *et al.* 2000). We assessed
339 GFP fluorescence in Kp43816R containing the unstable GFP reporter grown in LB. *Klebsiella* was
340 stained using rabbit anti-*Klebsiella* serum followed by Rhodamine-conjugated donkey anti-rabbit
341 secondary antibody. FACS analysis revealed an overlap between GFP fluorescence (green
342 histogram) and Rhodamine fluorescence (red histogram) in bacteria grown in LB (Fig 8D, panel
343 label as inoculum) which is in perfect agreement with the constitutive expression of *cps* by bacteria
344 grown in LB. To investigate *cps* expression in intracellular bacteria, MH-S cells were infected with
345 Kp43816R containing the GFP reporter. Cells were processed as described in Experimental
346 procedures, and fluorescence analysed by FACS at different time points post infection. GFP
347 fluorescence (green histograms) was measured in the gated Rhodamine positive population (red
348 histograms). Data in Figure 8D shows that GFP fluorescence decreased over time in the
349 intracellular bacteria reaching the levels of the control strain carrying the empty vector (grey
350 histogram), which is considered negative for GFP fluorescence.

351 To explore whether the acidic pH of the KCV might be responsible for the downregulation
352 of *cps* expression, bacteria were grown in M9 minimal medium, with 8 μ M magnesium sulfate,
353 buffered to different pHs. The expression of the *cps::gfp* fusion was 5-fold lower when bacteria
354 were grown at pH 5.5 than at pH 7.5 (Fig 8E). Similar results were obtained when the mRNA levels
355 of *wzi*, *orf7* and *gnd*, three genes of the *cps* operon (Arakawa *et al.* 1995), were assessed by real
356 time quantitative PCR (RT-qPCR) (Fig 8F).

357 Collectively, these findings show that *K. pneumoniae* CPS is dispensable for intracellular
358 survival. In fact, the environment found by *Klebsiella* within the KCV triggers the downregulation

359 of the expression of *cps*. The fact that opsonization affects the intracellular survival of the CPS
360 mutant indicates that the mechanism of bacteria entry into macrophages has a major impact in the
361 ability of *K. pneumoniae* to survive intracellularly.

362

363 **DISCUSSION**

364 In this work, we present compelling evidence demonstrating that *K. pneumoniae* survives
365 killing by macrophages by manipulating phagosome maturation. Our data sustain that *K.*
366 *pneumoniae* traffics within vacuoles associated with the endocytic pathway in mouse and human
367 macrophages. In contrast to UV-killed bacteria, which colocalize with lysosomal markers, live
368 bacteria modify the vacuole biogenesis preventing the fusion of the KCV with the hydrolases-rich
369 lysosomal compartment. *K. pneumoniae* thus increases the list of pathogens able to alter phagosome
370 maturation.

371 Engulfment of *K. pneumoniae* by mouse and human macrophages was dependent on host
372 cytoskeleton, cell plasma membrane lipid rafts and the activation of PI3K which are all commonly
373 needed to engulf pathogens and inert particles such as latex beads (Vieira *et al.* 2002, Flannagan *et*
374 *al.* 2012). TEM analysis suggested that *K. pneumoniae* resides inside a vacuolar compartment and,
375 by using FISH and two fluorescent markers tagging, we confirmed that intracellular bacteria are
376 metabolically active. Several lines of evidence indicate that *K. pneumoniae* infections are associated
377 with cell death (Willingham *et al.* 2009, Cano *et al.* 2009, Cai *et al.* 2012). In good agreement, in this
378 study we show that *K. pneumoniae* triggers a programmed cell death in macrophages displaying
379 features of apoptosis. Of note, kinase activity profiling in whole lungs during *K. pneumoniae*
380 infection showed the activation of kinases associated to induction of apoptosis (Hoogendijk *et al.*
381 2011). However, Willingham and co-workers reported that *K. pneumoniae* activates the NLRP3-
382 dependent cell death programme termed pyronecrosis (Willingham *et al.* 2009). Similar apparently
383 contradictory findings have been reported for *Shigella flexneri* infections. *Shigella* triggers
384 apoptotic and pyroptotic cell death in macrophages depending on the bacterial dosage and time of

385 infection (Willingham *et al.* 2007,Hilbi *et al.* 1998). In that case, short time of bacteria-cell contact
386 and low MOI are associated to induction of apoptosis (Willingham *et al.* 2007,Hilbi *et al.* 1998).
387 Notably, the infection conditions in our study are different to those used by Willingham and co-
388 workers who used a MOI four times higher than ours (Willingham *et al.* 2009). Future studies are
389 warranted to carefully assess the influence of infection conditions on *Klebsiella*-induced cell death.

390 Manipulation of cell death is a common pathogenic strategy not only for bacteria but also for
391 viruses (Finlay and McFadden. 2006). In general, viruses either accelerate or inhibit apoptosis of
392 the infected cell, depending on the biology of the specific virus. Like viruses, obligate intracellular
393 bacteria generally suppress apoptotic death. Because apoptosis is a less inflammatory process than
394 necrotic death, many nonobligate intracellular pathogens trigger apoptotic death to avoid cell to cell
395 communications. Thus, *Klebsiella*-induced macrophage death by apoptosis could be considered a
396 central aspect of *Klebsiella* infection biology taken into account the evidence demonstrating that
397 alveolar macrophages play a critical role in the clearance of *Klebsiella* (Broug-Holub *et al.*
398 1997,Cheung *et al.* 2000) and the importance of an early inflammatory responses to control the
399 infection (Greenberger *et al.* 1996a,Greenberger *et al.* 1996b,Happel *et al.* 2005,Happel *et al.*
400 2003).

401 The vacuole of *K. pneumoniae* and its biogenesis was studied by immunofluorescence. The
402 presence of EEA1 on the KCV indicates that internalized bacteria are initially present in a vacuole
403 related to the endocytic pathway. However, *K. pneumoniae* does not remain in early endosomes as
404 demonstrated by the acquisition of Lamp1 and Rab7. A hallmark of the maturation is the exclusion
405 of lysosomal hydrolases in the majority of KCVs containing live bacteria. In contrast, more than
406 50% of the KCVs containing UV-killed bacteria were positive for lysosomal markers already 90
407 min post infection. The KCV is acidic most likely due to the activity of vacuolar proton-ATPases.
408 Notably, inhibition of these pumps by bafilomycin A₁ resulted in a decrease in intracellular
409 bacterial numbers. Similar findings have been reported for non typable *H. influenzae*, *Tropheryma*
410 *whipplei*, and *Brucella suis* (Morey *et al.* 2011,Ghigo *et al.* 2002,Porte *et al.* 1999). The reduction

411 of intracellular viability may have several explanations. Bafilomycin A₁ might affect other
412 macrophage functions necessary for *K. pneumoniae* survival. An alternative hypothesis, and more
413 appealing to us, is that *K. pneumoniae* requires a low pH environment for survival within the KCV
414 which is in agreement with our data showing a significant decrease in the number of metabolic
415 active intracellular bacteria in bafilomycin A₁-treated cells. For example, the acidic environment
416 may facilitate the uptake of nutrients by *Klebsiella*. Acidic pH is required for the transport of
417 glucose in *Coxiella burnetii* (Howe and Mallavia. 2000) and localization in an acidic environment
418 facilitates the availability of iron for the growth of *Francisella tularensis* (Fortier *et al.* 1995). In
419 addition, low pH may regulate the expression of factors essential for intracellular survival. This has
420 been shown to be true for virulence gene transcription in *S. typhimurium* (Yu *et al.* 2010). In this
421 context, our data revealed that *Klebsiella* downregulates the expression of *cps* when residing within
422 the KCV. Interestingly, when *Klebsiella* was cultured in low magnesium and acidic pH we also
423 found a downregulation of *cps* expression. It is tempting to speculate that these signals could trigger
424 *cps* downregulation within the KCV. In fact, we show here that the KCV is acidic and there are
425 reports suggesting that the magnesium concentration in pathogen-containing vacuoles is in the
426 micromolar range (Garcia-del Portillo *et al.* 1992). Future efforts will be devoted to characterize the
427 chemical composition of the KCV as well as the transcriptional landscape of intracellular *K.*
428 *pneumoniae*.

429 It was interesting to consider the mechanism(s) whereby *K. pneumoniae* prevents the fusion
430 of the lysosomes to the KCV. The overall resemblance between the KCV and the *Salmonella*
431 containing vacuole (acidic Lamp-1-positive cathepsin-negative vacuole) prompted us to explore
432 whether *K. pneumoniae* employs similar strategies as *Salmonella* to subvert phagosome maturation.
433 Kuijl and coworkers (Kuijl *et al.* 2007) demonstrated that *S. typhimurium* activates Akt to prevent
434 phagosome-lysosome fusion. Since *K. pneumoniae* activates Akt *in vitro* (this work and (Frank *et*
435 *al.* 2013)) and *in vivo* (Hoogendijk *et al.* 2011) we speculated that activated Akt may also promote
436 *Klebsiella* intracellular survival. Indeed this was the case. Akt inhibition resulted in a significant

437 decrease in bacterial intracellular survival associated with an increased colocalization of the KCV
438 with lysosomal markers. The fact that Akt is implicated in the intracellular survival of other
439 pathogens, including *M. tuberculosis* (Kuijl *et al.* 2007), strongly suggests that this kinase is a
440 central host node targeted by pathogens to take control over cellular functions.

441 PI3K/Akt governs phagosome maturation by controlling, at least, the activation of Rab
442 GTPases (Thi and Reiner. 2012), although Rab14 is emerging as a central Rab in this process.
443 Previous data indicate that pathogens hijack Rab14 to manipulate phagosome maturation. The *M.*
444 *tuberculosis* vacuole recruits and retains Rab14 to maintain early endosomal characteristics (Kyei *et*
445 *al.* 2006) whereas *S. typhimurium* containing vacuole retains Rab14 in an Akt-dependent manner to
446 arrest phagosome maturation (Kuijl *et al.* 2007). Immunofluorescence confirmed that the KCV is
447 positive for Rab14 in an Akt-dependent manner whereas transient transfection of the dominant-
448 negative Rab14 resulted in a decrease in bacteria intracellular survival. In aggregate, this evidence
449 supports a scenario in which *K. pneumoniae* manipulates phagosome maturation by targeting a
450 PI3K-Akt-Rab14 pathway. Nevertheless, we do not rule out that there are additional pathways
451 necessary for *Klebsiella* intracellular survival.

452 We were keen to identify the bacterial factors interfering with the phagosomal maturation
453 pathway. Given the critical role of *K. pneumoniae* CPS in preventing host defense responses (March
454 *et al.* 2013, Rgueiro *et al.* 2006, Lawlor *et al.* 2005, Frank *et al.* 2013, Moranta *et al.* 2010, Campos *et*
455 *al.* 2004, Lawlor *et al.* 2006), we hypothesized that CPS is necessary for intracellular survival. To
456 our initial surprise, CPS does not play a large role, if any, in intracellular survival of *Klebsiella*
457 since a *cps* mutant did not display any loss of viability upon phagocytosis. Furthermore, the *cps*
458 mutant also triggered a programmed cell death in macrophages (data not shown). At first glance,
459 these findings may seem contradictory with the well-established role of CPS in *K. pneumoniae*
460 virulence. However, considering the presence of complement in the bronchoalveolar fluid (Wu *et*
461 *al.* 2005), the fact that opsonization results in more efficient internalization of pathogens and
462 maturation of phagosomes (Aderem and Underhill. 1999), and the well-known role of CPS in

463 preventing complement opsonization (de Astorza *et al.* 2004,Cortes *et al.* 2002a), we hypothesized
464 that the *cps* mutant opsonization is deleterious to its intracellular fate. Indeed, this was the case
465 hence revealing the critical role of CPS on *Klebsiella*-macrophage interplay. These results also
466 illustrate how the mode of entry of a pathogen influences its intracellular outcome. Similar findings
467 have been reported for other pathogens (Geier and Celli. 2011,Gordon *et al.* 2000,Drevets *et al.*
468 1993) but it cannot be considered a general feature since complement opsonization does not affect
469 the intracellular fate of *Salmonella* and *M. tuberculosis* (Drecktrah *et al.* 2006,Zimmerli *et al.*
470 1996).

471 At present we can only speculate why *Klebsiella* downregulates the expression of *cps* once
472 inside the KCV. Since CPS biosynthesis is a metabolically demanding process, *Klebsiella* may
473 downregulate *cps* expression to better survive in the intracellular environment poor in nutrients. It
474 is also plausible that CPS may interfere with *Klebsiella* factors implicated in the intracellular
475 survival. Current efforts of the laboratory are devoted to identify these factors.

476 Finally, it is worthwhile commenting on the clinical implications of this study. The
477 antibiotics commonly used to treat *Klebsiella* infections are not very efficient against intracellular
478 bacteria. In turn, our findings provide rationale for the use of inhibitors targeting the PI3K-Akt
479 signaling cascade to eliminate intracellular *K. pneumoniae*. The concept of eradicating pathogens
480 through targeting host factors modulated by pathogens has received wide attention in the infectious
481 disease arena. Several promising drugs have been developed or are being developed to antagonize
482 PI3K/Akt due to its relevance for many human cancers. Of note, there are *in vitro* and *in vivo*
483 studies supporting the use of Akt inhibitors to eliminate intracellular *Salmonella* and *M.*
484 *tuberculosis* (Kuijl *et al.* 2007,Chiu *et al.* 2009). Therefore, we propose that agents targeting
485 PI3K/Akt might provide selective alternatives to manage *K. pneumoniae* pneumonias. Careful
486 designed preclinical trials using the well establish mouse pneumonia model are warranted to test
487 this hypothesis.

488

489 **EXPERIMENTAL PROCEDURES**

490 **Bacterial strains and growth conditions.**

491 Kp43816R is a rifampicin-resistant derivative of *K. pneumoniae* pneumonia clinical isolate
492 [ATCC 43816; (Bakker-Woudenberg *et al.* 1985)]. This strain has been widely used to study the
493 host response to Gram-negative pneumonia because it recapitulates acute pneumonia with fatal
494 systemic spread at a relatively low infectious dose. Kp43816R expresses a type 1 O-polysaccharide
495 and a type 2 capsule. Bacteria were grown in lysogeny broth (LB) at 37°C on an orbital shaker (180
496 rpm). To UV kill bacteria, samples were UV irradiated (1 joule for 15 min) in a BIO-LINK BLX
497 crosslinker (Vilber Lourmat). When appropriate, antibiotics were added to the growth medium at
498 the following concentrations: rifampicin (Rif) 50 µg/ml, ampicillin (Amp), 100 µg/ml for *K.*
499 *pneumoniae* and 50 µg/ml for *E. coli*; kanamycin (Km) 100 µg/ml; chloramphenicol (Cm) 12.5
500 µg/ml.

501 **Construction of a *K. pneumoniae* *cps* mutant.**

502 Primers for *manC* mutant construction were designed from the known *K. pneumoniae* K2
503 gene cluster sequence (Arakawa *et al.* 1995). Primer pairs ManCUPF (5'-
504 CGCTTAAAGACCAGCGTGTCG -3'), ManCUPR (5'-
505 CGGATCCGATCAGCGGGTCGTCGCCGTG____-3'), and ManCDOWNF (5'-
506 CGGATCCGCAGCGACGAGAAGCTGGTGG-3' *Bam*HI site underlined), ManCDOWNR (5'-
507 GGATATCCCGCAGGCCGGTG -3') were used in two sets of asymmetric PCRs to obtain DNA
508 fragments ManCUP and ManCDown, respectively. DNA fragments ManCUP and ManCDOWN
509 were annealed at their overlapping region and amplified by PCR as a single fragment using primers
510 ManCUPF and ManCDOWNR. This PCR fragment was cloned into pGEM-T Easy to obtain
511 pGEMTΔ*manC*. A kanamycin cassette, obtained as a 1.5 kb PCR fragment from pKD4 (Datsenko
512 and Wanner. 2000) using primers cassette-F1 (5'-
513 CGCGGATCCGTGTAGGCTGGAGCTGCTTCG-3' *Bam*HI site underlined) and cassette-R1 (5'-
514 CGCGGATCCCATGGGAATTAGCCATGGTCC -3' *Bam*HI site underlined), was BamHI-

515 digested and cloned into BamHI-digested pGEMT Δ manC to obtain pGEMT Δ manCKm. Primers
516 ManCUPF and ManCDOWNR were used to amplify a 3.5 kb fragment which was electroprated
517 into Kp43816R containing pKOBEG-sacB plasmid (Derbise *et al.* 2003). The vector pKOBEG-
518 sacB contains the Red operon expressed under the control of the arabinose inducible pBAD
519 promoter and the sacB gene that is necessary to cure the plasmid. A recombinant in which the wild-
520 type allele was replaced by Δ man::Km was verified by PCR and named 43 Δ manCKm. The mutant
521 was resistant to the CPS-specific phage ϕ 2.

522 **Eukaryotic cells culture.**

523 Murine alveolar macrophages MH-S (ATCC, CRL-2019) and human monocytes THP-1
524 (ATCC, TIB-202) were grown in RPMI 1640 tissue culture medium supplemented with 10% heat-
525 inactivated fetal calf serum (FCS) and 10 mM Hepes at 37°C in an humidified 5% CO₂ atmosphere.
526 THP-1 cells were differentiated to macrophages by PMA-treatment (10 ng/ml for 12 h).

527 **Infection of macrophages.**

528 Macrophages were seeded in 24-well tissue culture plates at a density of 7×10^5 cells per
529 well 15 h before the experiment. Bacteria were grown in 5-ml LB, harvested in the exponential
530 phase ($2500 \times g$, 20 min, 24°C), washed once with PBS and a suspension containing approximately
531 1×10^9 cfu/ml was prepared in 10 mM PBS (pH 6.5). Cells were infected with 35 μ l of this
532 suspension to get a multiplicity of infection of 50:1 in a final volume of 500 μ l RPMI 1640 tissue
533 culture medium supplemented with 10% heat-inactivated FCS and 10 mM Hepes. To synchronize
534 infection, plates were centrifuged at $200 \times g$ during 5 min. Plates were incubated at 37°C in a
535 humidified 5% CO₂ atmosphere. After 30 min of contact, cells were washed twice with PBS and
536 incubated for additional 90 min with 500 μ l RPMI 1640 containing 10% FCS, 10 mM Hepes,
537 gentamicin (300 μ g/ml) and polymyxin B (15 μ g/ml) to eliminate extracellular bacteria. This
538 treatment did not induce any cytotoxic effect which was verified measuring the release of lactate
539 dehydrogenase (LDH) and by immunofluorescence microscopy (data not shown). For time course

540 experiments, after the 90 min treatment period, cells were washed three times with PBS and
541 incubated with 500 μ l RPMI 1640 containing 10% FCS, 10 mM Hepes, gentamicin (100 μ g/ml).

542 To determine intracellular bacterial load, cells were washed three times with PBS and lysed
543 with 300 μ l of 0.5% saponin in PBS for 10 min at room temperature. Serial dilutions were plated on
544 LB to quantify the number of intracellular bacteria. Intracellular bacterial load is represented as cfu
545 per well. All experiments were done with triplicate samples on at least three independent occasions.

546 When indicated, cells were pre-incubated for 1 h with nocodazole (50 μ g/ml), filipin (5
547 μ g/ml), nystatin (25 μ g/ml), LY294002 hydrochloride (75 μ M), or for 30 min with cytochalasin D
548 (5 μ g/ml) before carrying out infections as described above. Cells were also pre-incubated for 1 h
549 with 1 mM methyl- β -cyclodextrin (M β CD), washed twice with PBS to remove cholesterol and
550 infected. In other experiments, LY294002 hydrochloride (75 μ M), AKT X (10 μ M), or 100 nM
551 bafilomycin A₁ were added to the cells during the gentamicin treatment and kept until the end of
552 experiment. Exposure to these drugs had no effect on cell and bacterial viability under the
553 conditions tested. All drugs were purchased from Sigma.

554 **Immunofluorescence and transmission electron microscopy.**

555 Cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates. Infections
556 were carried out as described before with *K. pneumoniae* strains harbouring pFPV25.1Cm (March
557 *et al.* 2013). Control experiments showed that there were no differences in the number of
558 intracellular bacteria recovered over time from cells infected with bacteria containing pFPV25.1Cm
559 or no plasmid (data not shown). When indicated, cells were washed three times with PBS and fixed
560 with 3% paraformaldehyde (PFA) in PBS pH 7.4 for 15 min at room temperature. For EEA1
561 staining, cells were fixed with 2.5% PFA for 10 min at room temperature followed by 5% PFA +
562 methanol (1:4 v/v) at -20°C for 5 min. Methanol fixation (3% PFA for 20 min at room temperature
563 followed by 1 min cold methanol) was used for cathepsin D whereas periodate-lysine-
564 paraformaldehyde fixation (0.01 M NaIO₄, 0.075 M L-lysine, 0.0375 M NaPO₄ buffer pH 7.4, 2%
565 paraformaldehyde: 20 min room temperature) was used for calnexin. The actin cytoskeleton was

566 stained with Rhodamine-Phalloidin (Invitrogen) diluted 1:100, DNA was stained with Hoechst
567 33342 (Invitrogen) diluted 1:2500. *Klebsiella* was stained with rabbit anti-*Klebsiella* serum diluted
568 1:5000. Early endosomes were stained with goat anti-EEA1 (N-19) antibody (Santa Cruz
569 Biotechnology) diluted 1:50. Late endosomes were stained with rat anti-Lamp-1 (1D4B) antibody
570 (Developmental Studies Hybridoma Bank). Lysosomes were labelled with goat anti-human
571 cathepsin D (G19) or rabbit anti-human cathepsin D (H-75) antibodies (Santa Cruz Biotechnology)
572 diluted 1:100. Golgi network was stained with mouse anti-GM130 (BD Laboratories) diluted 1:400.
573 Endoplasmic reticulum was stained with rabbit anti-calnexin (SPA-860; Enzo Life Sciences) diluted
574 1:400. Donkey anti-rabbit, donkey anti-mouse, donkey anti-rat and donkey anti-goat conjugated to
575 Rhodamine, Cy5 or Cy2 secondary antibodies were purchased from Jackson Immunological and
576 diluted 1:200. Donkey anti-rabbit conjugated to AlexFluor 595 and goat anti-rabbit conjugated to
577 Cascade blue antibodies (Life technologies) were diluted 1:200.

578 Fixable dextran 70,000 (molecular weight) labelled with Texas red (TR-dextran) (Molecular
579 Probes) was used to label lysosomes in a pulse-chase assay. Briefly, macrophages seeded on glass
580 coverslips were labelled by pulsing with 250 µg/ml of TR-dextran for 2 h at 37°C in 5% CO₂ in
581 RPMI 1640 medium. To allow TR-dextran to accumulate in lysosomes, medium was removed; cells
582 were washed three times with PBS, and incubated for 1 h in dye-free medium (chase). After the
583 chase period, cells were infected.

584 LysoTracker Red DND-99 (Invitrogen) was used to label acidic organelles following the
585 instructions of the manufacturer. 0.5 µM LysoTracker RedDN99 was added to the tissue culture
586 medium 30 min before fixing the cells. The residual fluid marker was removed by washing the cells
587 three times with PBS, followed by fixation.

588 Staining was carried out in 10% horse serum, 0.1% saponin in PBS. Coverslips were washed
589 twice in PBS containing 0.1% saponin, once in PBS, and incubated for 30 minutes with primary
590 antibodies. Coverslips were then washed twice in 0.1% saponin in PBS and once in PBS and
591 incubated for 30 minutes with secondary antibodies. Finally, coverslips were washed twice in 0.1%

592 saponin in PBS, once in PBS and once in H₂O, mounted on Aqua Poly/Mount (Polysciences).
593 Immunofluorescence was analysed with a Leica CTR6000 fluorescence microscope. Images were
594 taken with a Leica DFC350FX monochrome camera. Confocal microscopy was carried out with a
595 Leica TCS SP5 confocal microscope. Depending of the marker, a KCV was considered positive
596 when it fulfilled these criteria: (i) the marker was detected throughout the area occupied by the
597 bacterium; (ii) the marker was detected around/enclosing the bacterium, (iii) the marker was
598 concentrated in this area, compared to the immediate surroundings. To determine the percentage of
599 bacteria that colocalized with each marker, all bacteria located inside a minimum of 100 infected
600 cells were analysed in each experiment. Experiments were carried out by triplicate in three
601 independent occasions.

602 For extra-/intracellular bacteria differential staining, PFA fixed cells were incubated with
603 PBS containing 10% horse serum, Hoechst 33342 and rabbit anti-*Klebsiella* for 20 min. Coverslips
604 were washed three times with PBS and stained as described above with donkey anti-rabbit
605 conjugated to Rhodamine secondary antibody. Coverslips were washed three times in PBS and once
606 in distilled water before mounting onto glass slides using Prolong Gold antifade mounting gel
607 (Invitrogen).

608 For transmission electron microscopy (TEM), cells were seeded in 24-well tissue culture
609 plates. Infections were carried out as described before, fixed with glutaraldehyde and processed for
610 TEM as described previously (Kruskal *et al.* 1992).

611 **Assessment of intracellular bacteria viability**

612 **(i) Fluorescent *in situ* hybridisation**

613 We carried out hybridization of PFA fixed infected cells with fluorescently labelled
614 oligonucleotides as described before (Morey *et al.* 2011). Alexa488 conjugated DNA probes
615 EUB338 (5'-GCTGCCTCCCGTAGGAGT-3') and GAM42a (5'-GCCTTCCCACATCGTTT-3')
616 were designed for specific labelling of rRNA of eubacteria and gamma subclass of Proteobacteria,
617 respectively (Manz *et al.* 1993). A DNA probe non-EUB338, complementary to EUB338 was used

618 as a negative control. The detectability of bacteria by such oligonucleotide probes is dependent on
619 the presence of sufficient ribosomes per cell, hence providing qualitative information on the
620 physiological state of the bacteria on the basis of the number of ribosomes per cell. These probes
621 were used together to obtain a stronger signal, added to a final concentration of 5 nM each in the
622 hybridization buffer. The hybridization buffer contained 0.9M NaCl, 20mM Tris-HCl (pH 7.4),
623 0.01% sodium dodecyl sulfate (SDS) and 35% formamide. Coverslips were first washed with
624 deionized water. Hybridization was carried out for 1.5 h at 46°C in a humid chamber; followed by a
625 30 min wash at 48°C. Washing buffer contained 80 mM NaCl, 20 mM Tris-HCl (pH 7.4), 0.01%
626 sodium dodecyl sulfate (SDS) and 5 mM EDTA (pH 8). After washing, DNA staining for total
627 bacteria was carried out by incubating the coverslips in PBS containing Hoechst 33342 for 20 min.
628 Coverslips were then washed three times in PBS and once in distilled water before mounting onto
629 glass slides using Prolong Gold antifade mounting gel.

630 **(ii) Two fluorescent markers tagging**

631 pJT04mCherry, expressing mCherry constitutively (kindly donated by Miguel Valvano, to
632 be described elsewhere), and pMMB207gfp3.1 (Pujol *et al.* 2005), expressing *gfpmut3.1* under the
633 control of an IPTG-inducible promoter, were conjugated into Kp43816R. Control experiments
634 confirmed that UV-killed *Klebsiella* was always mCherry positive and GFP negative whereas live
635 *Klebsiella* was mCherry positive and only GFP positive if IPTG was added (1 mM, 1.5 h) to the
636 medium. Cells were infected with Kp43816R harbouring both plasmids and IPTG was added to the
637 medium 1.5 h before fixing the cells with PFA. To stain extracellular bacteria, PFA fixed cells were
638 incubated with PBS containing 10% horse serum, and rabbit anti-*Klebsiella* for 20 min. Coverslips
639 were washed three times with PBS and stained as described above with goat anti-rabbit antibodies
640 conjugated to Cascade Blue (C2764, Life Technologies). Immunofluorescence was analysed with a
641 Leica CTR6000 fluorescence microscope. Images were taken with a Leica DFC350FX
642 monochrome camera.

643 **Isolation of *in vivo* infected macrophages**

644 Mice were treated in accordance with the Directive of the European Parliament and of the
645 Council on the protection of animals used for scientific purposes (Directive 2010/63/EU) and in
646 agreement with the UK Home Office (licence PLZ 2700) and the Bioethical Committee of the
647 University of the Balearic Islands (authorisation number 1748).

648 Infections were performed as previously described (Insua *et al.* 2013). Briefly, five- to
649 seven-week-old male C57BL/6 mice (Harlan) were anesthetized by intraperitoneal injection with a
650 mixture containing ketamine (50 mg/kg) and xylazine (5 mg/kg). Overnight bacterial cultures were
651 centrifuged (2500 x g, 20 min, 22°C), resuspended in PBS and adjusted to 5×10^4 CFU/ml for
652 determination of bacterial loads. 20 μ l of the bacterial suspension were inoculated intranasally in
653 four 5 μ l aliquots. To facilitate consistent inoculations, mice were held vertically during inoculation
654 and placed on a 45° incline while recovering from anaesthesia. 24 h post infection, mice were
655 euthanized by cervical dislocation and bronchoalveolar lavage was performed as previously
656 described (Cai *et al.* 2012). The lavage fluid from four mice was pooled together and spun at 300 x
657 g for 10 min to pellet alveolar macrophages. Cells were cultured on 12 mm circular coverslips in
658 24-well tissue culture plates at a concentration of 0.5×10^6 cells/well in 1 ml RPMI 1640 tissue
659 culture medium supplemented with 10% heat-inactivated FCS and 10 mM HEPES and gentamicin
660 (100 μ g/ml). After 2 h of incubation, nonadherent cells were washed off with PBS, and cells were
661 fixed. Cathepsin D staining was performed as previously described. To label lysosomes using TR-
662 dextran, after washing off the nonadherent cells, the attached macrophages were pulsed with TR-
663 dextran (250 μ g/ml) for 2 h in RPMI 1640 medium containing gentamicin (100 μ g/ml). Cells were
664 washed three times with PBS, and incubated for 1 h in dye-free medium (chase). After the chase
665 period, cells were fixed. Immunofluorescence was analysed with a Leica TCS SP5 confocal
666 microscope.

667 **Neutral red uptake assay for the estimation of cell viability.**

668 Cell viability was determined by assessing the ability of viable cells to incorporate and bind
669 the supravital dye neutral red in the lysosomes. The protocol described by Repetto and coworkers

670 (Repetto *et al.* 2008) was followed with minor modifications. Macrophages were seeded on 96-well
671 tissue culture plates at 5×10^5 cells/well 18 h before the experiment. Cells were infected to get a
672 multiplicity of infection of 50:1 in a final volume of 200 μ l RPMI 1640 tissue culture medium
673 supplemented with 10% heat-inactivated FCS and 10 mM Hepes. To synchronize infection, plates
674 were centrifuged at 200 x g during 5 min. Plates were incubated at 37°C in a humidified 5% CO₂
675 atmosphere. After 90 min of contact, cells were washed twice with PBS and incubated overnight
676 with 200 μ l RPMI 1640 containing 10% FCS, 10 mM Hepes, gentamicin (100 μ g/ml). Cells were
677 washed twice with PBS and incubated with 100 μ l of freshly prepared neutral red medium (final
678 concentration 40 μ g/ml neutral red [Sigma] in tissue culture medium) for 2 h. Wells were washed
679 once with PBS and the remaining biomass-adsorbed neutral red was solubilized with 150 μ l neutral
680 red destaining solution (50% ethanol 96%; 49% deionised water, 1% glacial acetic acid). Staining
681 was then quantified by determining the OD₅₄₀ in a 96-well microplate reader, and used to compare
682 relative neutral red staining of uninfected cells and cells that were lysed completely with 1% Triton
683 X-100. Experiments were carried out by triplicate in six independent occasions.

684 **Detection of Akt phosphorylation by Western blotting**

685 Macrophages were seeded on 6-well tissue culture plates at 10^6 cells/well. Cells were
686 infected with Kp43816R , washed 3 times with cold PBS, scraped and lysed with 100 μ l lysis buffer
687 (1x SDS Sample Buffer, 62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT,
688 0.01% w/v bromophenol blue) on ice. Samples were sonicated, boiled at 100°C for 10 min and
689 cooled on ice before polyacrylamide gel electrophoresis and Western Blotting. Akt phosphorylation
690 was detected with primary rabbit anti-phospho Ser473 Akt (Cell Signaling Technology) antibody
691 diluted 1:1,000 and secondary goat anti-rabbit antibody conjugated to horseradish peroxidase
692 (Thermo Scientific) diluted 1:10,000. Tubulin was detected with primary mouse anti-tubulin
693 antibody (Sigma) diluted 1:3,000 and secondary goat anti-mouse antibody (Pierce) conjugated to
694 horseradish peroxidase diluted 1:1,000. To detect tubulin, membranes were reprobbed after stripping

695 of previously used antibodies using Western Blot Stripping Buffer (Thermo Scientific). Images
696 were recorded with a GeneGnome HR imaging system (Syngene).

697 **Apoptosis analysis *in vitro*.**

698 Apoptosis of macrophages was analysed as previously described (Aguilo *et al.* 2013).
699 Briefly, phosphatidylserine exposure and membrane integrity were analyzed by using Annexin-V
700 and 7-AAD (BD Biosciences) and FACS according to manufacturer instructions. Cells were
701 washed with PBS and incubated with APC-conjugated Annexin-V and 7-AAD in Annexin-binding
702 buffer for 15 min. After that, cells were washed twice with PBS, fixed with 4% PFA during 30 min
703 and washed again with PBS. Both PBS and PFA contained 2.5 mM CaCl₂.

704 **Bacterial opsonisation.**

705 Normal human serum (NHS), kindly donated by the Balearics Blood Centre, was obtained
706 from five different donors (blood type O negative) and kept frozen at -80°C. 35 µl from a
707 suspension containing approximately 1x10⁹ cfu/ml in 10 mM PBS (pH 6.5) were added to 500 µl
708 RPMI 1640 tissue culture medium supplemented with 10 mM HEPES and 1% NHS. The suspension
709 was incubated at 37°C shaking (180 rpm) for 45 min. The suspension was used to infect mTHP1
710 cells as previously described.

711 **Plasmids and transient transfections**

712 For transient transfections with GFP-Rab7 (Addgene plasmid #28047) (Sun *et al.* 2010),
713 GFP-Rab14 (Kuijl *et al.* 2007), and RILP-C33-EGFP (Cantalupo *et al.* 2001), the Neon transfection
714 system was used (Life Technologies). 8 x 10⁶ cells were transfected (1400 v, 30 ms and 1 pulse)
715 with 2 µg of plasmid DNA. After, cells were seeded on 12 mm circular coverslips in 24-well tissue
716 culture plates and 24 h later were infected. In all cases, samples were fixed, stained and analysed by
717 immunofluorescence microscopy. pcDNA3 and DN-Rab14 (Seto *et al.* 2011) were transfected
718 using jetPEI-macrophage (Polyplus) following manufacturer's instructions. After 24 h, cells were
719 washed twice with PBS, infected, and intracellular bacterial load determined as previously
720 described.

721 **Construction of *cps* reporter strain**

722 DNA fragment containing the promoter region of the Kp43816R capsule operon was
723 amplified by PCR using *Vent* polymerase (NewEngland Biolabs) and primers K2ProcpsF (5'-
724 gaattcTGCTGGGACAAATTGCCACC-3') and K2ProcpsR (5'-
725 AGATGGATGACCCCGCGATC-3'). To construct a green fluorescent protein (GFP) reporter, the
726 PCR product was EcoRI-digested and cloned into the EcoRI-SmaI digested low-copy-number
727 vector pPROBE'-gfp[LVA] (Miller *et al.* 2000) to obtain pPROBE'43Procps. The plasmid was
728 introduced into Kp43816R by electroporation.

729 **Analysis of *cps* expression**

730 The reporter strain was grown at 37°C on an orbital incubator shaker (180 r.p.m.) until
731 OD₅₄₀ 1.2. The cultures were harvested (2500 x g, 20 min, 24°C) and resuspended to an OD₅₄₀ of
732 0.6 in PBS. 0.8-ml aliquot of this suspension was transferred to 1-cm fluorimetric cuvette and
733 fluorescence was measured with a spectrofluorophotometer (Perkin Elmer LS55) set as follows:
734 excitation, 485 nm; emission, 528 nm; slit width 5 nm; integration time 5 seconds. Results were
735 expressed as relative fluorescence units (RFU). All measurements were carried out in quintuplicate
736 on at least three separate occasions.

737 To obtain RNA, bacteria were grown at 37°C in 5 ml of medium on an orbital incubator
738 shaker (180 r.p.m.) until an OD₆₀₀ of 0.3. 3 ml of RNA later solution were added to the culture and
739 the mixture was incubated for 20 min to prevent RNA degradation. Total RNA was extracted using
740 Trizol as recommended by the manufacturer (Life Technology). The purification included a
741 DNAase treatment step. cDNA was obtained by retrotranscription of 1 µg of total RNA using a
742 commercial M-MLV Reverse Transcriptase (Sigma), and random primers mixture (Invitrogen). 20
743 ng of cDNA were used as a template in a 10-µl reaction. RT-PCR analyses were performed with a
744 Mx3005P qPCR System (Agilent Technologies) and using a KapaSYBR Fast qPCR Kit as
745 recommended by the manufacturer (Kapa biosystems). The thermocycling protocol was as follows;
746 95°C for 3 min for hot-start polymerase activation, followed by 40 cycles of 95°C for 10 s, and

747 56°C for 20 s. SYBR green dye fluorescence was measured at 521 nm. cDNAs were obtained from
748 two independent extractions of mRNA and each one amplified by RT-qPCR in two independent
749 occasions. Relative quantities of *wzi*, *orf7* and *gnd* mRNAs were obtained using the comparative
750 threshold cycle ($\Delta\Delta CT$) method by normalizing to *rpoD* gene. Primers used were: Kpn_RpoD_F1
751 (5'-CCGGAAGACAAAATCCGTAA-3') and Kpn_RpoD_R1 (5'-
752 CGGGTAACGTCGAACTGTTT-3'); Kp43/52_wzi_F2 (5'-TCGACCGCAATCATTGAGCA-3')
753 and Kp43/52_wzi_R2 (5'-CATCCTTACCCAGCCGTG-3'); Kp43/52_orf7_F1 (5'-
754 ATCAAGATTGCCGACGTTTCT-3') and Kp43/52_orf7_R1 (5'-
755 GCCTCTACCGCAACTAATCCA-3'); Kp43/52_gnd_F1 (5'-GGATC CGGCGAACCTCTTT-3')
756 and Kp43/52_gnd_R1 (5'-GCCCTGAGCATAGGAAACGA-3').

757 For analysis of *cps* expression from intracellular bacteria, macrophages were seeded in 6-
758 well plates and infected with Kp43816R containing pPROBE'43Procps or pPROBE'-gfp[LVA]
759 control vector at a MOI of 150:1. After 40 min, cells were washed twice with PBS and incubated
760 with 500 μ l RPMI 1640 containing 10% FCS, 10 mM Hepes, gentamicin (300 μ g/ml) and
761 polymyxin B (15 μ g/ml) to eliminate extracellular bacteria. At the indicated time points, cells were
762 lysed with 900 μ l 0.5 % saponin in PBS. The samples from two wells were combined and serial
763 dilutions were plated on LB to quantify the number of intracellular bacteria. Control experiments
764 showed that there were no differences in the number of intracellular bacteria recovered over time
765 from cells infected with bacteria containing pPROBE'-gfp[LVA] derivatives or no plasmid (data
766 not shown). By replica plating on plates containing kanamycin, it was determined that 85-100% of
767 the bacteria contained the reporter plasmid at any time point analysed. The rest of the lysate was
768 centrifuged (13 000 rpm, 1 min, room temperature) and resuspended in 1 ml 1 % BSA in PBS for
769 staining. Bacteria were stained with rabbit anti-*Klebsiella* serum diluted 1:5000 for 20 min, washed
770 twice with PBS, and incubated for 20 min with a 1:200 dilution of Rhodamine-conjugated donkey
771 anti-rabbit secondary antibody. Flow cytometry analyses were performed using a Cultek Epics XL
772 flow cytometer. Samples were gated for bacteria-like particles by using the rhodamine fluorescence

773 of the anti-*Klebsiella* labelling to identify bacterial cells and to exclude mammalian cell debris and
774 background noise. Lysed and stained uninfected macrophages were not rhodamine positive,
775 indicating that there was no cross-reactivity of the primary or secondary antibodies with MH-S
776 cells. Fluorescence compensation settings were determined in parallel under identical conditions by
777 using the constitutively GFP-expressing Kp43816R strain or the non-expressing strain, with and
778 without anti-*Klebsiella* antibody labelling. Approximately 10,000 events identified as *Klebsiella*
779 cells were collected per sample. A histogram of GFP fluorescence for the negative-control sample
780 (bacteria containing pPROBE'-gfp[LVA]) was created, and the area of the histogram containing
781 the bacterial population was considered to be negative for GFP fluorescence. All experiments were
782 done with triplicate samples on at least three independent occasions.

783 **Statistical analysis.**

784 Statistical analyses were performed using the one-tailed *t* test or, when the requirements
785 were not met, by the Mann-Whitney U test. $P < 0.05$ was considered statistically significant. The
786 analyses were performed using Prism4 for PC (GraphPad Software).

787

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1019 **FIGURE LEGENDS**

1020 **FIGURE 1. Phagocytosis of *K. pneumoniae* by macrophages.**

1021 (A) Immunofluorescence confocal microscopy showing the lack of colocalisation between *K.*
1022 *pneumoniae* and the lysosome marker cathepsin D or TR-dextran (pulse-chase experiment
1023 described in Experimental procedures) in macrophages isolated from the BALF of infected mice
1024 with Kp43816R harbouring pFPV25.1Cm. Methanol fixation was used for cathepsin D staining. (B)
1025 Involvement of PI3K, cytoskeleton and lipid rafts on Kp43816R phagocytosis by MH-S cells. (C)
1026 Immunoblot analysis of Akt phosphorylation (P-Akt) in lysates of MH-S cells infected with live or
1027 UV-killed Kp43816R for the indicated times (in minutes). Membranes were probed for tubulin as a
1028 loading control. Images are representative of three independent experiments. (D) Immunoblot
1029 analysis of Akt phosphorylation (P-Akt) in lysates of PI3K inhibitor (LY294002) or DMSO
1030 (vehicle solution)-treated MH-S cells infected with Kp43816R for 20 min. Membranes were probed
1031 for tubulin as a loading control. Images are representative of three independent experiments.

1032 **FIGURE 2. Dynamics of *K. pneumoniae* survival in MH-S cells.**

1033 (A) MH-S cells were infected with Kp43816R for 30 min (MOI 50:1). Wells were washed and
1034 incubated with medium containing gentamicin (300 µg/ml) and polymyxin B (15 µg/ml) for 90 min
1035 to eliminate extracellular bacteria, and then with medium containing gentamicin 100 µg/ml for up to
1036 7.5 h. Intracellular bacteria were quantified by lysis, serial dilution and viable counting on LB agar
1037 plates. (B) MH-S cells were infected with Kp43816R harboring pFPV25.1Cm and the percentage of
1038 macrophages containing intracellular bacteria (determined by extra-/intracellular differential
1039 staining) assessed over time. Extracellular bacteria were stained using rabbit anti-*Klebsiella*
1040 antibodies detected using donkey anti-rabbit conjugated to Rhodamine secondary antibodies. (C)
1041 Percentage of infected macrophages containing 1-2; 3-5, or more than 5 intracellular bacteria
1042 (determined by extra-/intracellular differential staining) over time. (D) MH-S cells were infected
1043 with Kp43816R harbouring pJT04mCherry, expressing mCherry constitutively, and
1044 pMMB207gfp3.1, expressing *gfpmut3.1* under the control of an IPTG-inducible promoter. IPTG (1

1045 mM) was added 1.5 h before fixation. Images were taken 3.5 h post infection. Images are
1046 representative of duplicate coverslips in three independent experiments. (E) Percentage of
1047 intracellular bacteria (determined by extra-/intracellular differential staining; *Klebsiella* antibodies
1048 were detected using goat anti-rabbit conjugated to Cascade blue antibodies) mCherry-GFP positive
1049 over time. In panel A, data, shown as $\text{Log}_{10}\text{CFU/well}$, are the average of three independent
1050 experiments. In panel B, at least 500 cells belonging to three independent experiments were counted
1051 per time point whereas in panels C and E, at least 300 infected cells from three independent
1052 experiments were counted per time point.

1053 **FIGURE 3. Apoptosis of MH-S cells.**

1054 (A) MH-S cells were mock-treated or infected with Kp43816R harboring pFPV25.1Cm. 6 h post
1055 infection, cells were stained with Annexin V and 7-AAD and analysed by flow cytometry. A
1056 representative experiment of three is shown. (B) Data from three independent experiments are
1057 represented as mean \pm SD.

1058 **FIGURE 4. Phagosome maturation during *K. pneumoniae* infection of MH-S cells.**

1059 (A) Upper and middle rows show the colocalization of Kp43816R harboring pFPV25.1Cm and
1060 EEA1 (images were taken 30 min post infection) and Lamp1 (images were taken 4 h post infection)
1061 using goat anti-EEA1 and donkey anti-goat conjugated to Rhodamine, and rat anti-Lamp-1 and
1062 donkey anti-rat conjugated to Rhodamine antibodies, respectively. Images are representative of
1063 triplicate coverslips in three independent experiments. (B) Panels show the colocalization of
1064 Kp431816R and Lamp1 and EGFP-Rab7 or RILP-C33-EGFP (images were taken 3.5 h post
1065 infection). Bacteria were stained using rabbit anti-*Klebsiella* and goat anti-rabbit conjugated to
1066 Cascade blue antibodies. Images are representative of triplicate coverslips in three independent
1067 experiments. (C) Percentage of Kp43816R colocalization with EEA1, Lamp1, and EGFP-Rab7 and
1068 RILP-C33-EGFP over a time course. Cells were infected, coverslips were fixed and stained at the
1069 indicated times. Values are given as mean percentage of Kp43816R colocalizing with the marker \pm

1070 SE. At least 300 infected cells belonging to three independent experiments were counted per time
1071 point.

1072 **FIGURE 5. Colocalization of *K. pneumoniae* with phagolysosomal markers.**

1073 (A) Upper row shows the colocalization of Kp43816R harboring pFPV25.1Cm and the dye
1074 LysoTracker at 4 h post infection. Middle row shows the colocalization of Kp43816R harboring
1075 pFPV25.1Cm and cathepsin D at 2 h post infection. Cathepsin D was stained using goat anti-human
1076 cathepsin D (G19) and donkey anti-goat conjugated to Rhodamine antibodies. Lower row displays
1077 the colocalization of Kp43816R harboring pFPV25.1Cm and TR-dextran at 2 h post infection.
1078 Images are representative of three independent experiments. (B) Percentage of Kp43816R
1079 colocalization with LysoTracker, cathepsin D and TR-dextran over a time course. Cells were
1080 infected, coverslips were fixed and stained at the indicated times. Values are given as mean
1081 percentage of Kp43816R colocalizing with the marker \pm SE. At least 300 infected cells belonging
1082 to three independent experiments were counted per time point.

1083 **FIGURE 6. Effect of vacuolar acidification on *K. pneumoniae* survival.**

1084 (A) Microscopy analysis showing that bafilomycin A₁ (100 nM) treatment abrogates LysoTracker
1085 staining of the KCV (images were taken at 4 h post infection). MH-S cells were infected with
1086 Kp43816R harboring pFPV25.1Cm. Images are representative of triplicate coverslips in two
1087 independent experiments. (B) Experimental outline to investigate the effect of vacuolar acidification
1088 on the intracellular survival of Kp43816R. (C) Intracellular bacteria in MH-S cells, treated (white
1089 symbols) or not (black symbols) with bafilomycin A₁, were quantified by lysis, serial dilution and
1090 viable counting on LB agar plates. Data, shown as CFU/well, are the average of three independent
1091 experiments. Significance testing performed by Log Rank test. *, $P < 0.05$.

1092 **FIGURE 7. PI3K-AKT and Rab14 aid intracellular survival of *K. pneumoniae*.**

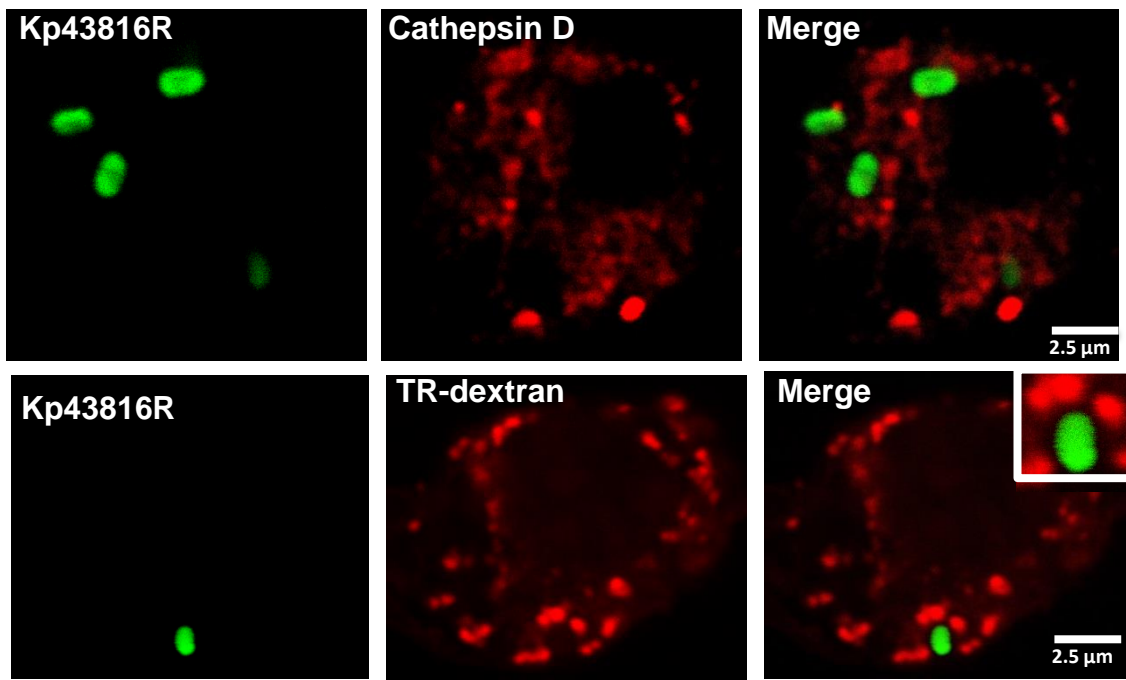
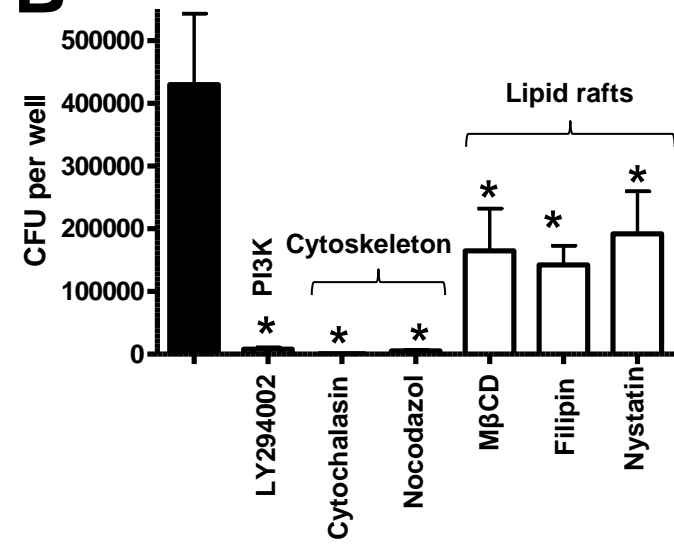
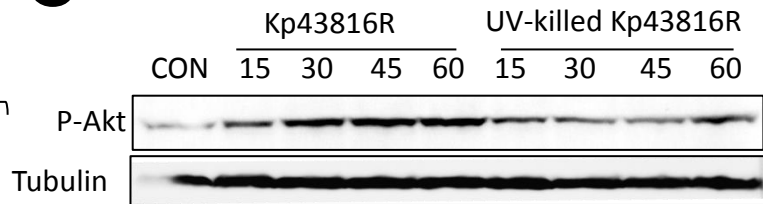
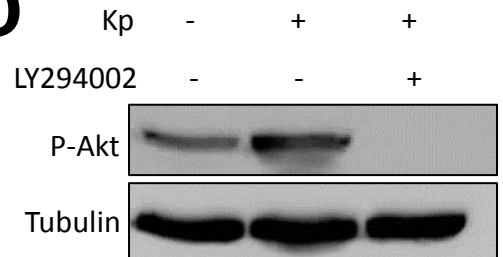
1093 (A) Quantification of intracellular bacteria in MH-S cells infected with Kp43816R which were
1094 mock-treated (black bar) or treated with LY294002 hydrochloride (75 μ M) or with AKT X (10
1095 μ M). Treatments were added after the time of contact and kept until cells were lysed for bacterial

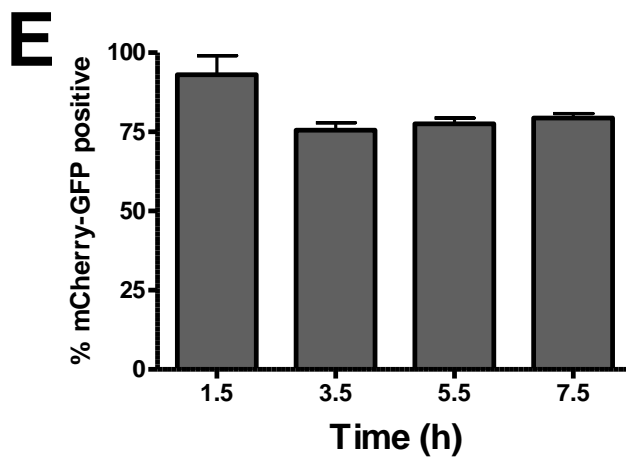
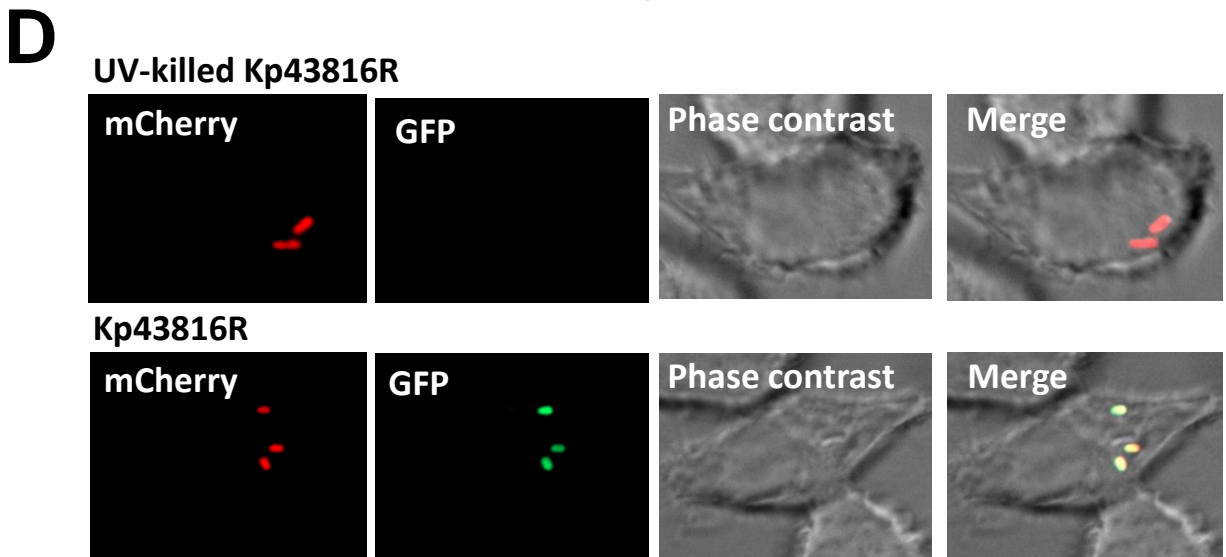
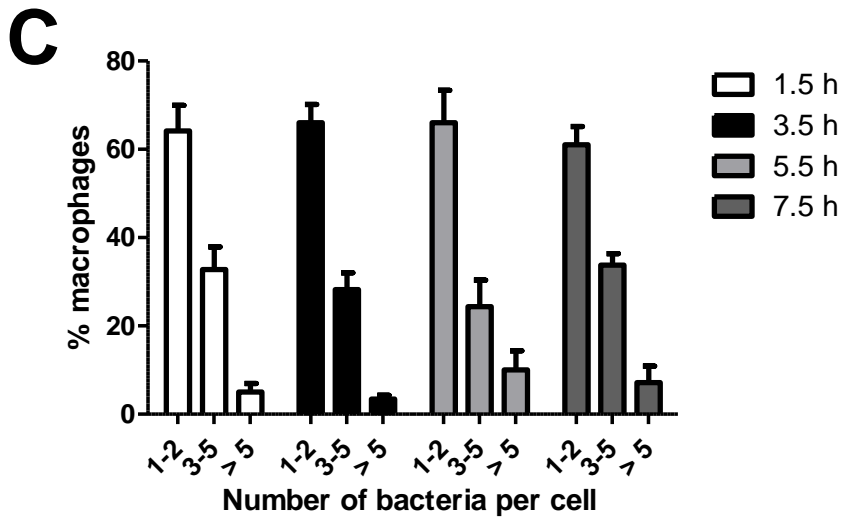
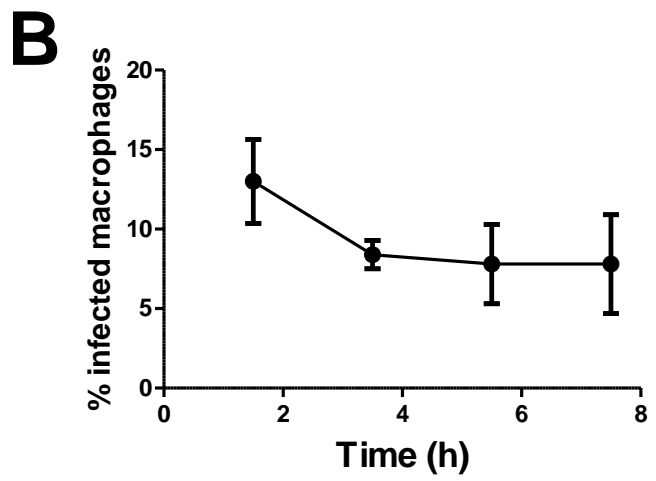
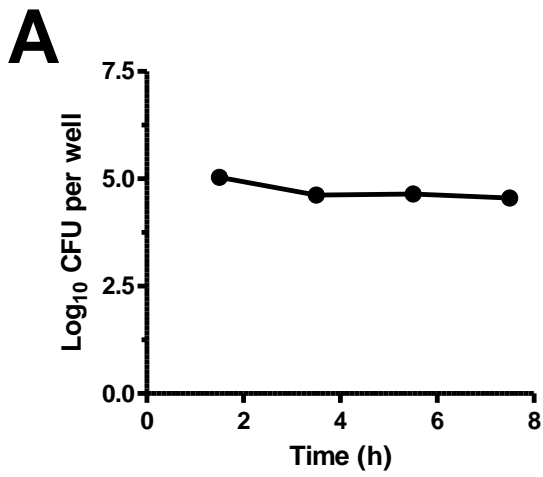
1096 enumeration. Data, shown as CFU/well, are the average of three independent experiments. *, $P <$
1097 0.05 (results are significantly different from the results for untreated cells; Mann-Whitney U test).
1098 (B) Percentage of Kp43816R colocalization with TR-dextran or cathepsin D in cells mock-treated
1099 or treated with the Akt inhibitor AKT X over a time course. Cells were infected, coverslips were
1100 fixed and stained at the indicated times. AKT X was added after the time of contact and kept until
1101 cells were fixed. Values are given as mean percentage of Kp43816R colocalizing with the marker \pm
1102 SE. At least 300 infected cells belonging to three independent experiments were counted per time
1103 point. *, $P <$ 0.05 (results are significantly different from the results for untreated cells; Mann-
1104 Whitney U test). (C) Colocalization of Kp431816R and Lamp1 and EGFP-Rab14 (images were
1105 taken 3.5 h post infection). Bacteria were stained using rabbit anti-*Klebsiella* and goat anti-rabbit
1106 conjugated to Cascade blue antibodies. Images are representative of triplicate coverslips in three
1107 independent experiments. (D) Percentage of Kp43816R colocalization with EGFP-Rab14 over a
1108 time course. Cells were infected, coverslips were fixed and stained at the indicated times. Values
1109 are given as mean percentage of Kp43816R colocalizing with the marker \pm SE. At least 300
1110 infected cells belonging to three independent experiments were counted per time point. (E)
1111 Quantification of intracellular bacteria in transfected MH-S cells with plasmid pcDNA3 or with
1112 Rab14 dominant-negative construct (DN-Rab14) at 3.5 h post infection. Data, shown as CFU/well,
1113 are the average of three independent experiments. *, $P <$ 0.05 (results are significantly different
1114 from the results for cells transfected with control plasmid pcDNA3; Mann-Whitney U test). (F)
1115 Immunofluorescence showing the lack of colocalization of the KCV and EGFP-Rab14 (images
1116 were taken 3.5 h post infection) in AKT X treated cells. Bacteria were stained using rabbit anti-
1117 *Klebsiella* and goat anti-rabbit conjugated to Cascade blue antibodies. Images are representative of
1118 triplicate coverslips in two independent experiments.

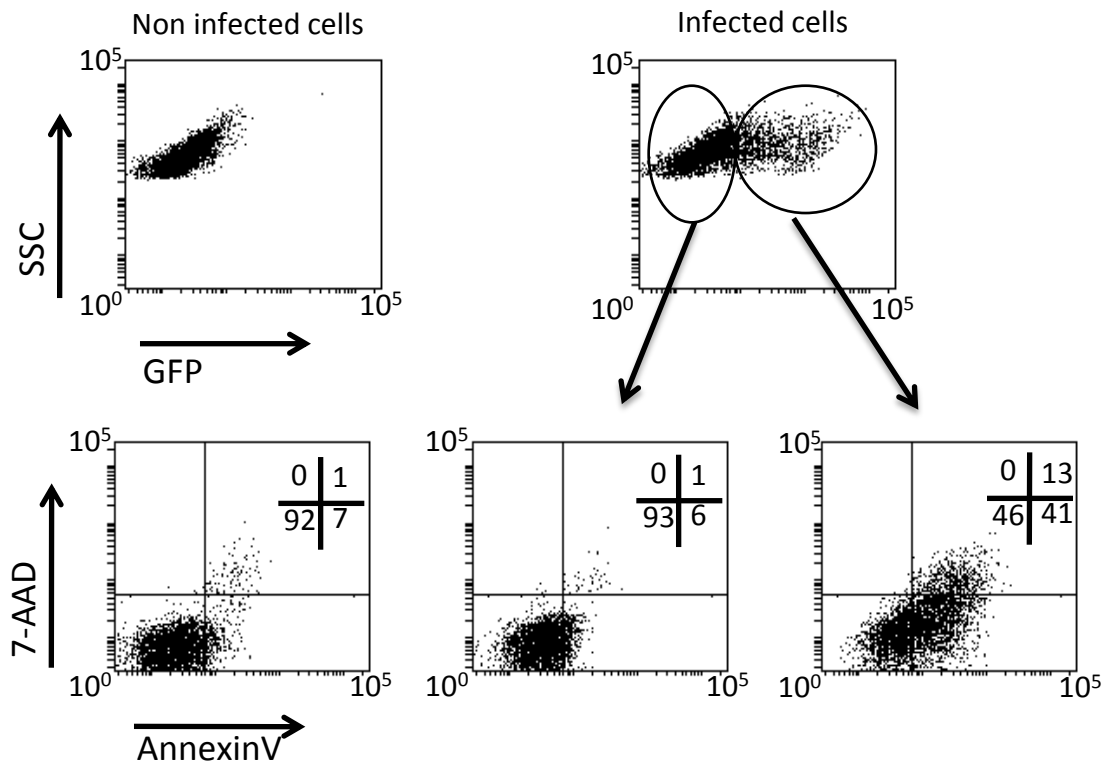
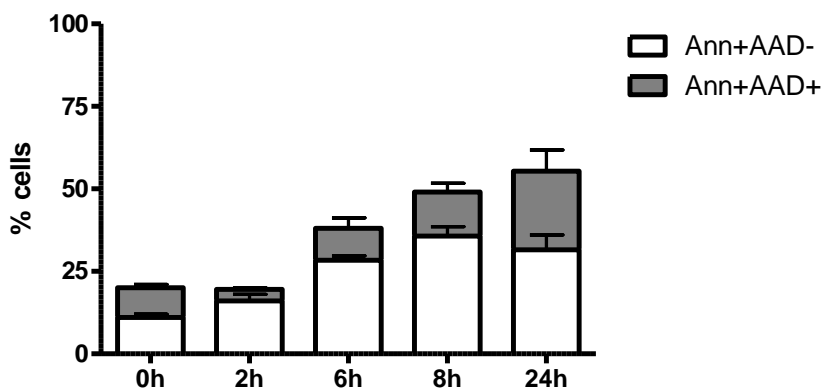
1119 **FIGURE 8. Role of CPS in *K. pneumoniae* intracellular survival.**

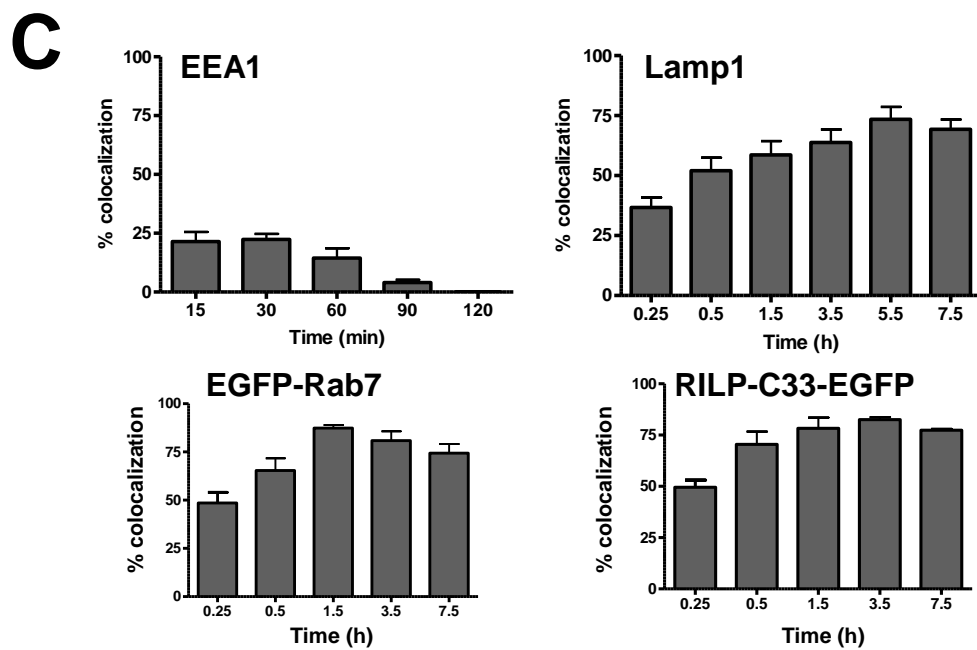
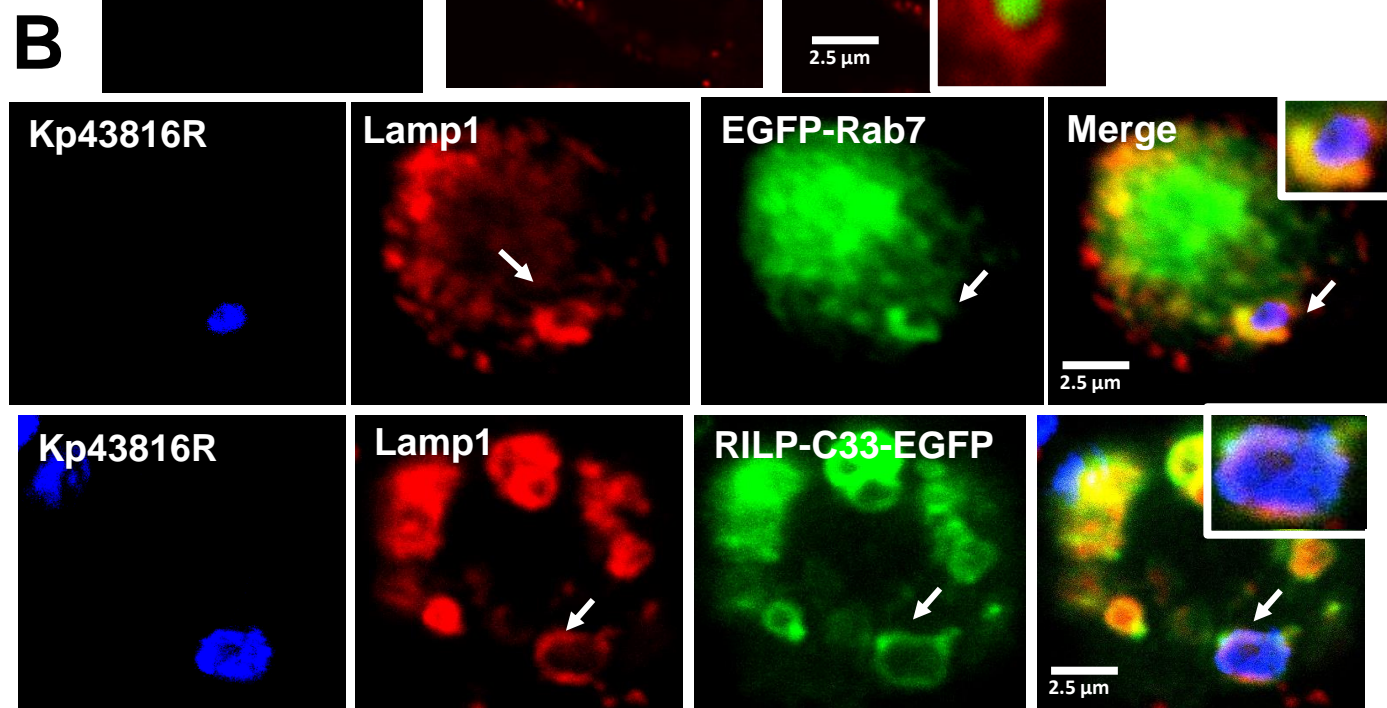
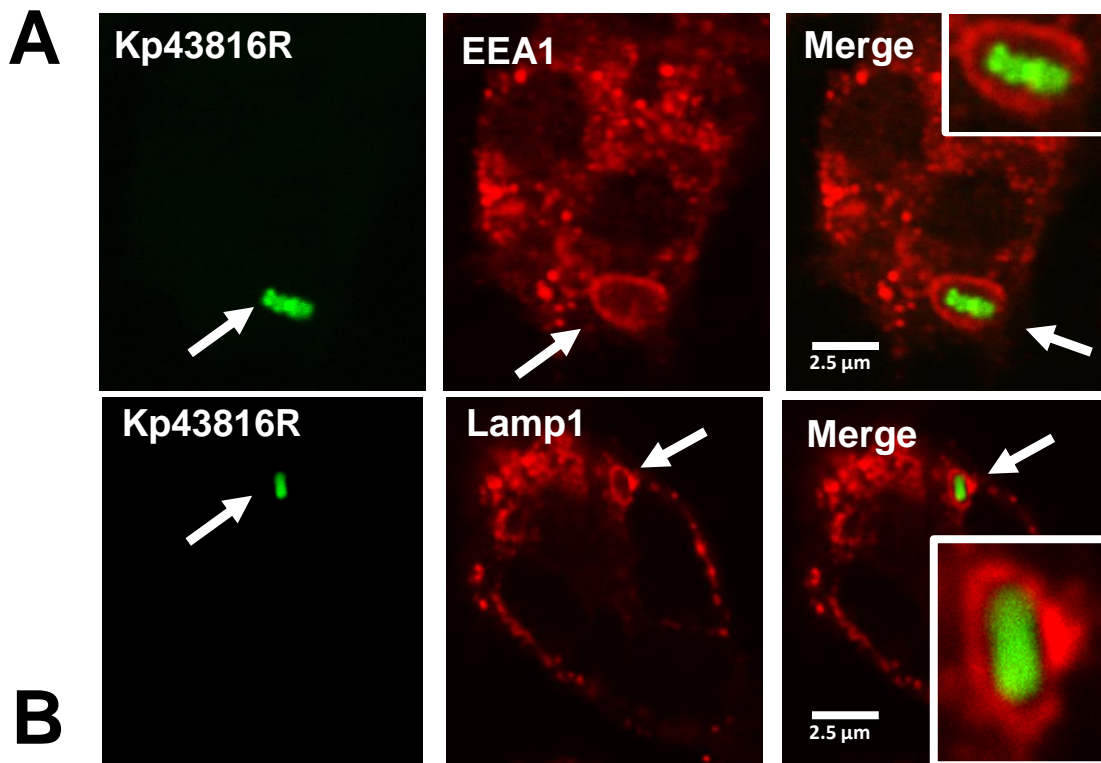
1120 (A) MH-S or mTHP-1 cells were infected with Kp43816R (black symbols) or the capsule mutant
1121 ($43\Delta manCKm$; white symbols). Intracellular bacteria were quantified by lysis, serial dilution and

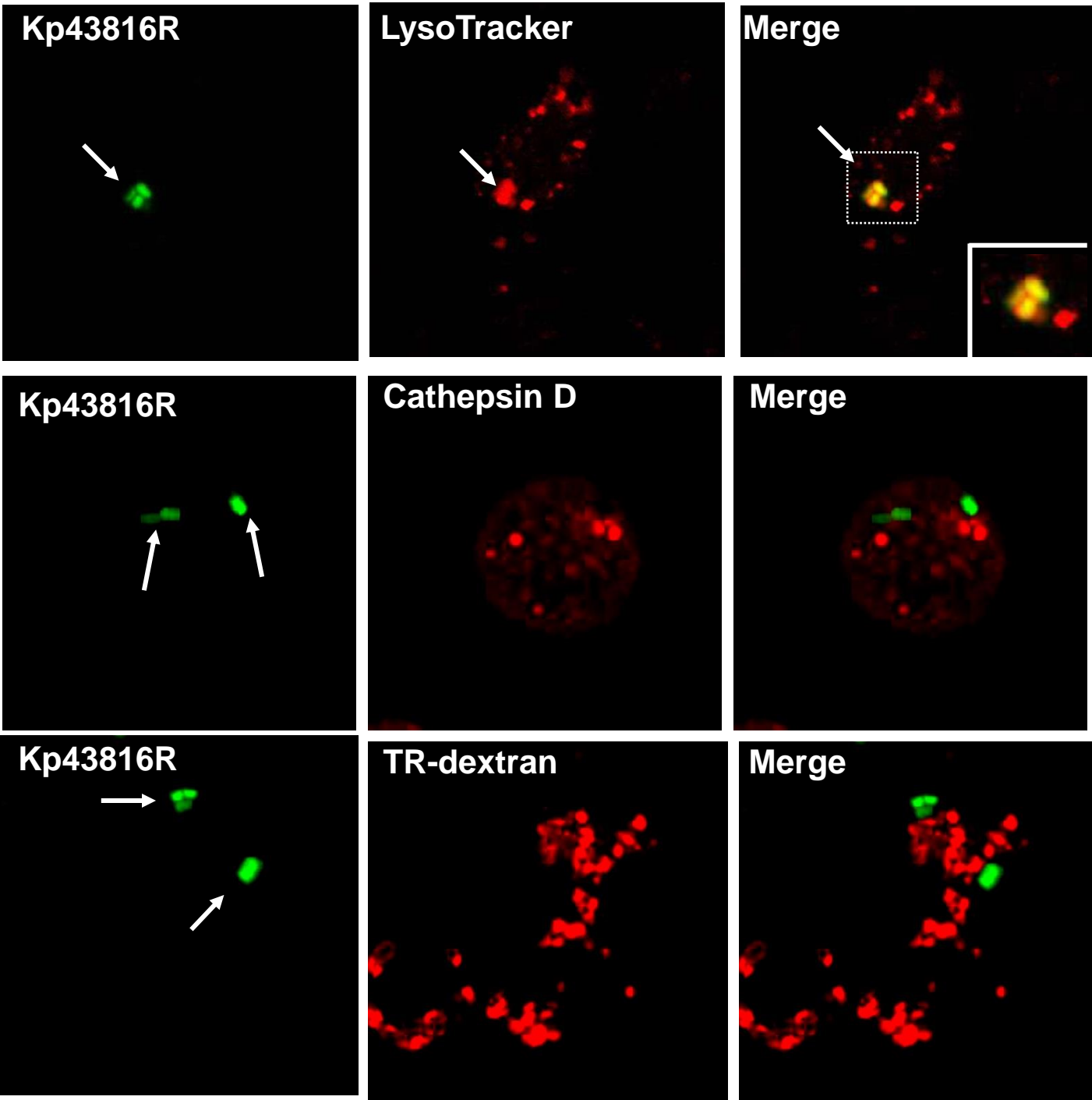
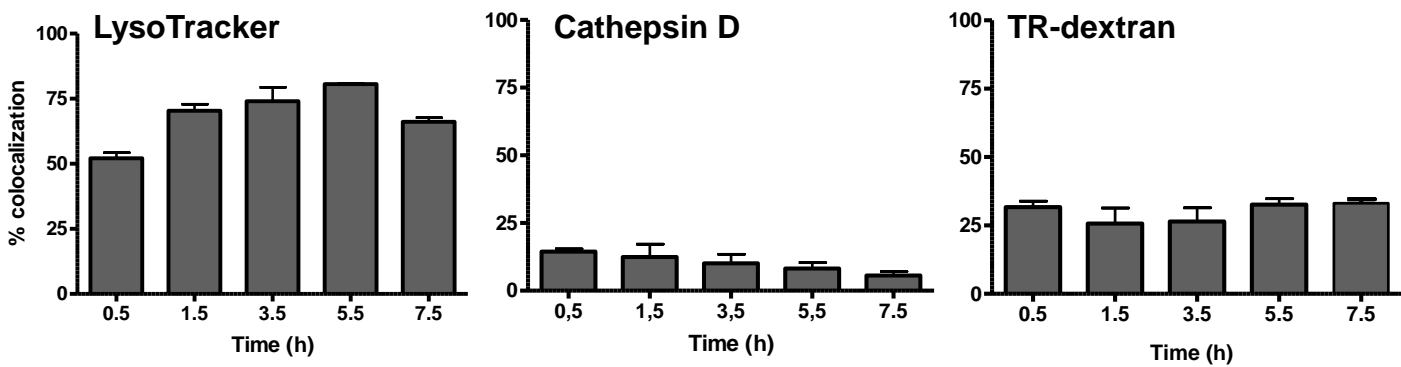
1122 viable counting on LB agar plates. Data, shown as $\text{Log}_{10}\text{CFU/well}$, are the average of three
1123 independent experiments. (B) Opsonization with 1% normal human sera (NHS) increased the
1124 phagocytosis of the capsule mutant (Kp43816Rdes) by mTHP-1 cells. Data, shown as CFU/well,
1125 are the average of three independent experiments. *, $P < 0.05$ (results are significantly different
1126 from the results for cells infected with the non-opsonized capsule mutant; Mann-Whitney U
1127 test); n.s., no significant difference. (C) mTHP-1 cells were infected for 30 min with Kp43816R
1128 or the capsule mutant ($43\Delta\text{manCKM}$; ΔmanCKM) which were either opsonized or not.
1129 Intracellular bacteria were quantified by lysis, serial dilution and viable counting on LB agar
1130 plates. Data, shown as $\text{Log}_{10}\text{CFU/well}$, are the average of three independent experiments.
1131 Significance testing performed by Log Rank test. *, $P < 0.05$. (D) Analysis of *cps::gfp*
1132 expression over time by flow cytometry. Analysis was performed staining the bacteria using
1133 rabbit anti-*Klebsiella* and donkey anti-rabbit conjugated to Rhodamine antibodies (red
1134 histogram). GFP fluorescence (green histogram) was analyzed in the gated Rhodamine labelled
1135 (antibody stained) population. Grey histogram represents GFP fluorescence for the negative-
1136 control sample, and the area of the histogram is considered negative for GFP fluorescence.
1137 Panels show the overlay of the different histograms. Results are representative of three
1138 independent experiments. (E) Fluorescence levels of Kp43816R containing pPROBE'43Procps.
1139 Data, shown as relative fluorescence units (RFUs), are the average of three independent
1140 experiments. *, $P < 0.05$ (results are significantly different from the results for cells grown in
1141 medium buffered to pH 7.5; Mann-Whitney U test). (F) *wzi*, *orf7* and *gnd* mRNA levels assessed
1142 by RT-qPCR. Data are presented as mean \pm SD (n = 3).*, $P < 0.05$ (results are significantly
1143 different from the results for cells grown in medium buffered to pH 7.5; Mann-Whitney U test).

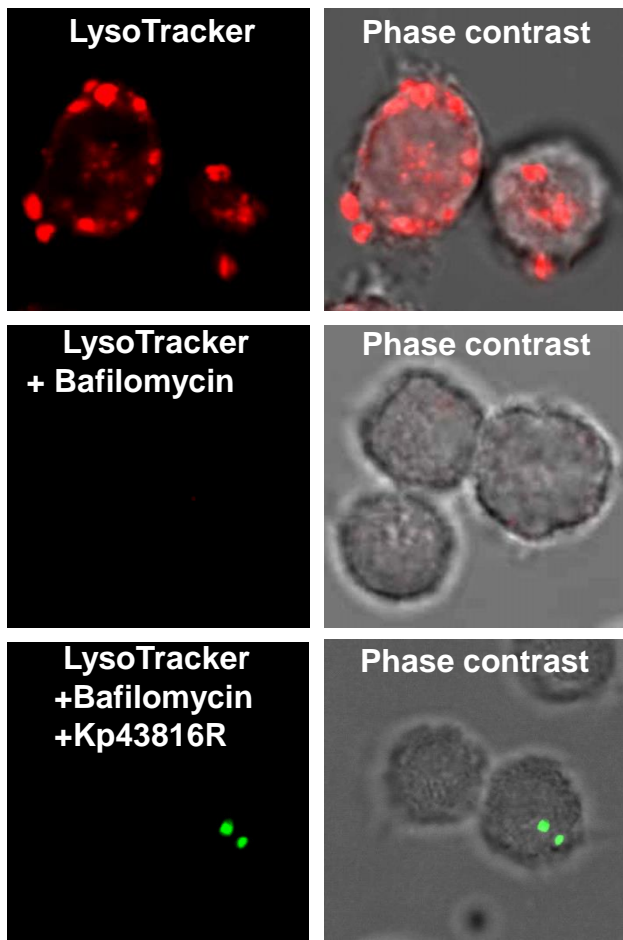
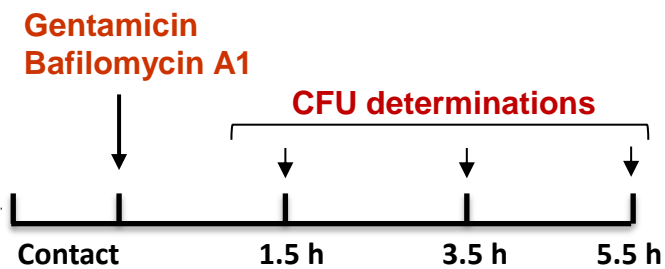
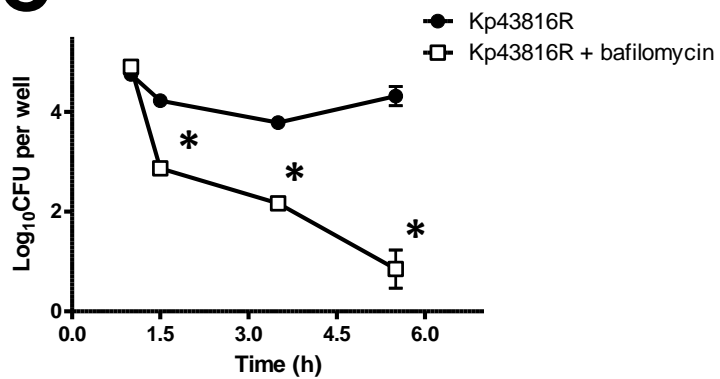
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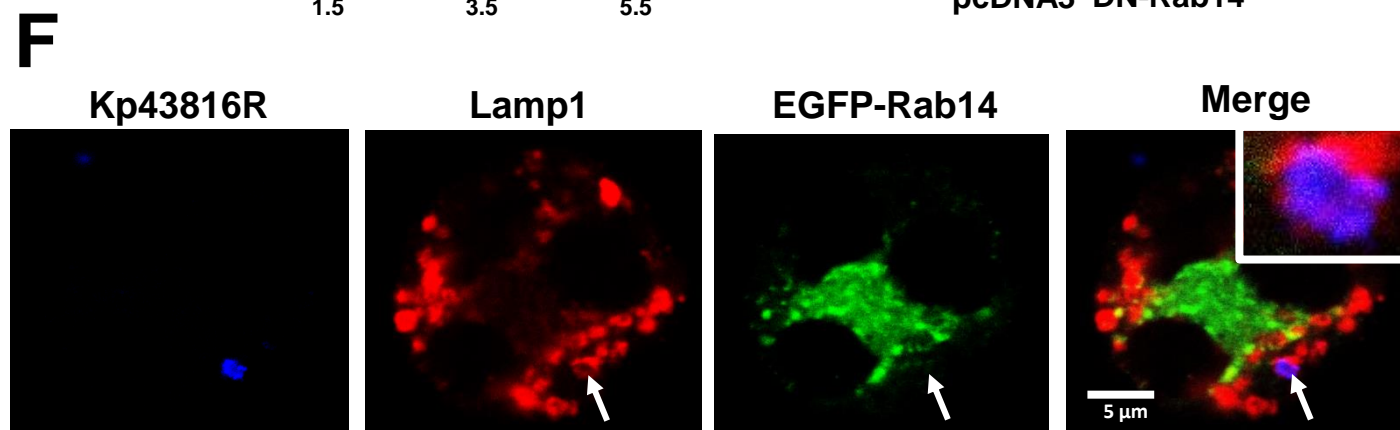
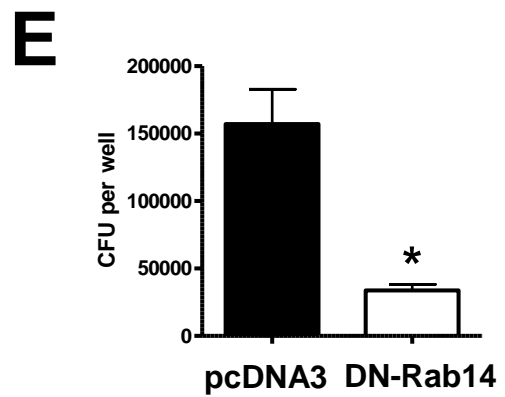
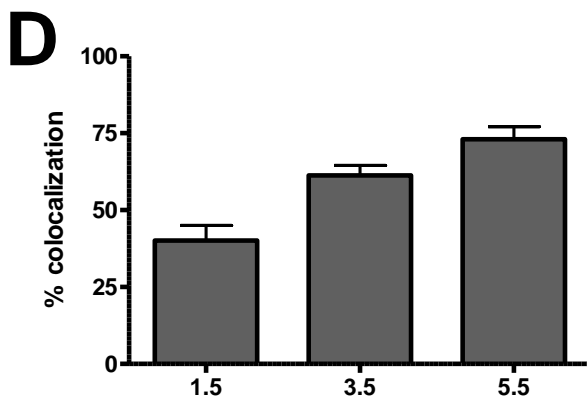
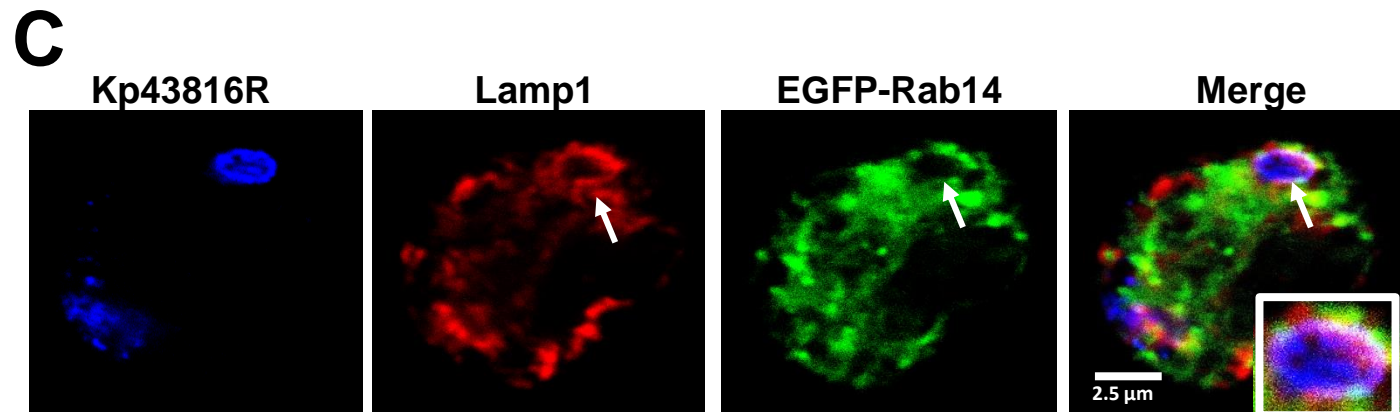
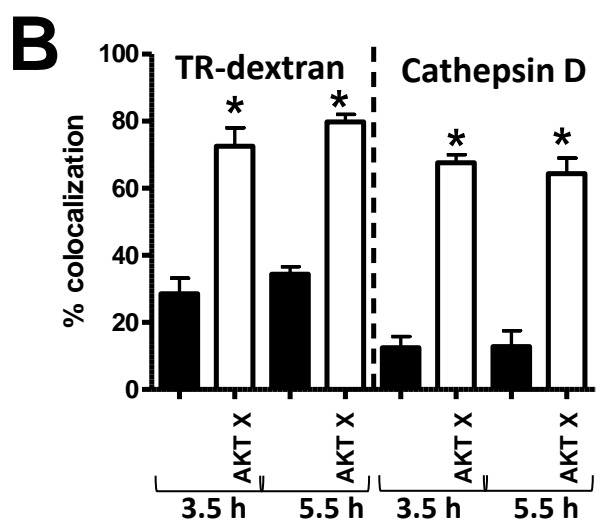
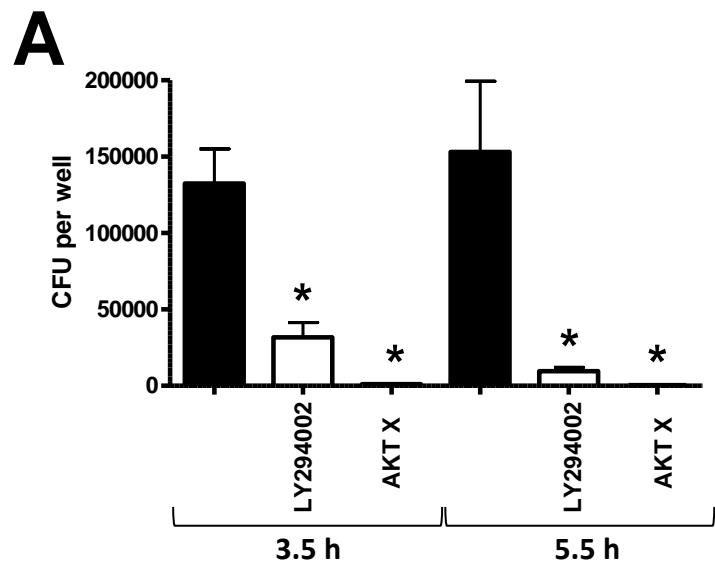


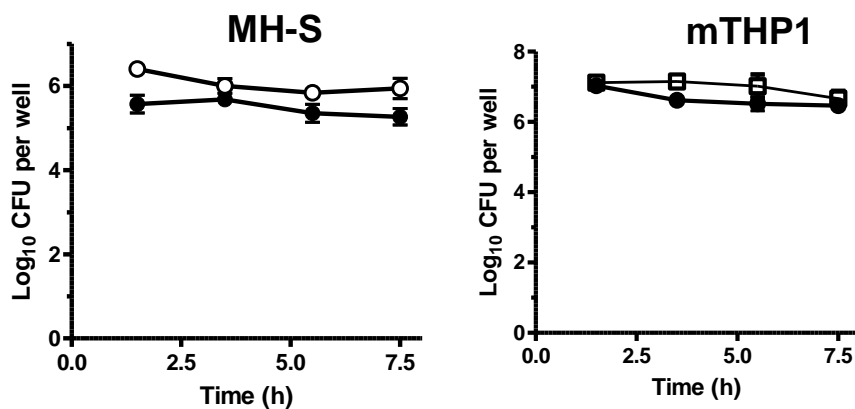
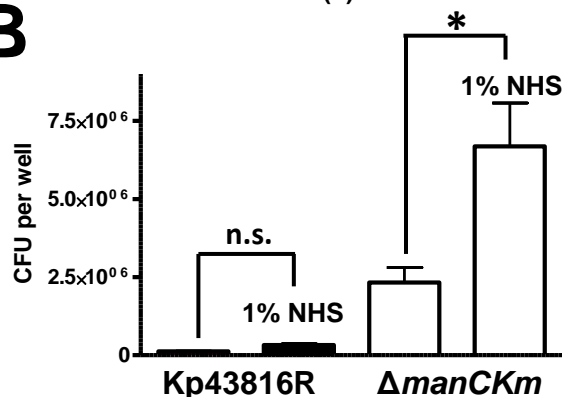
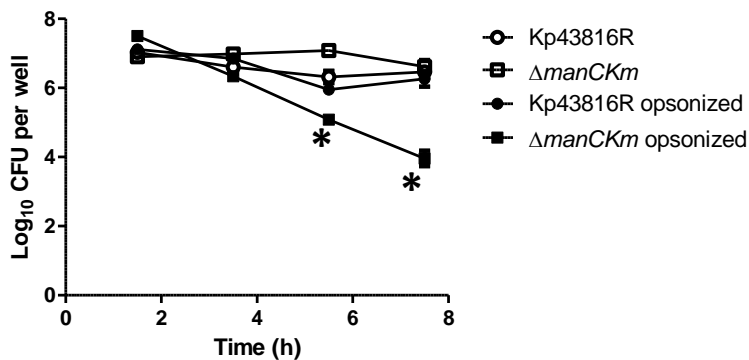
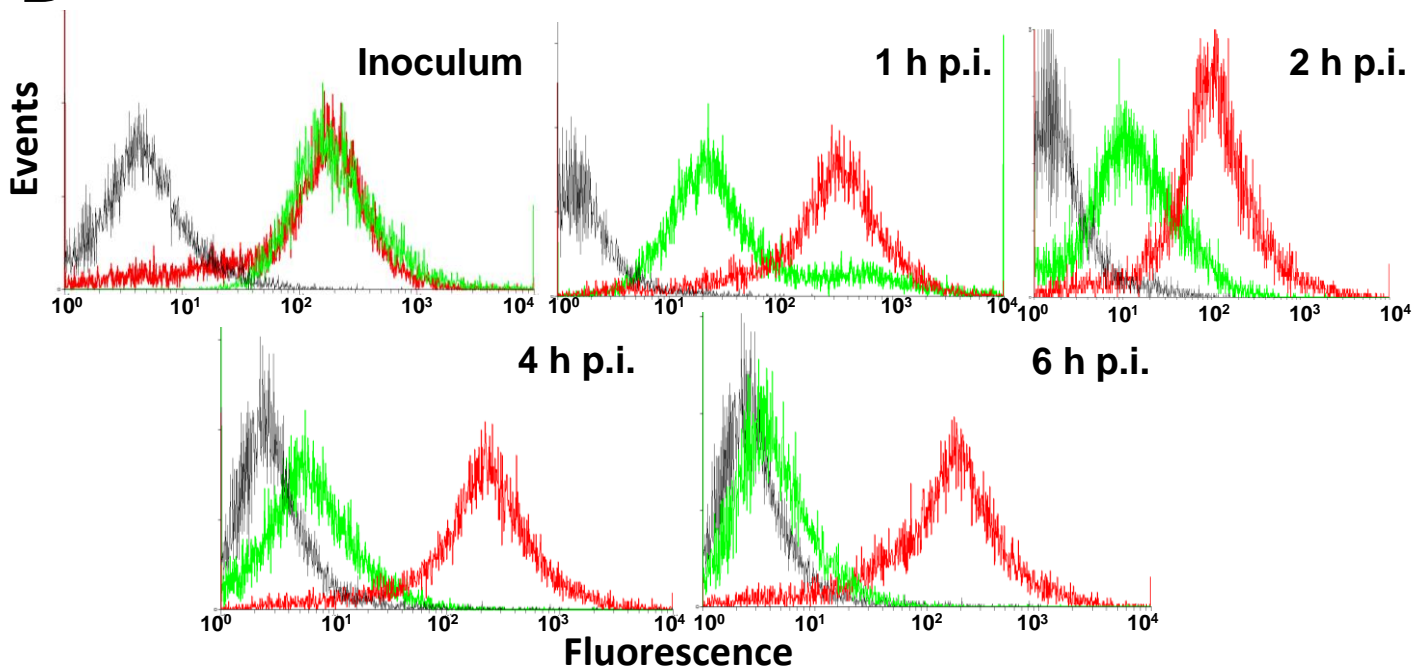
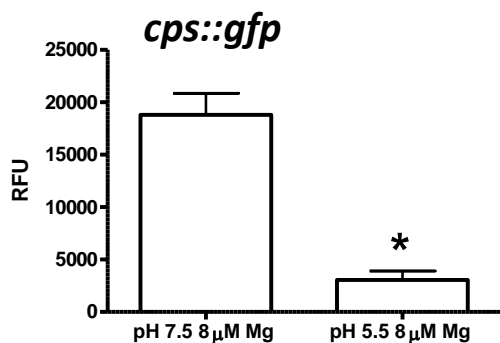
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