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The extracellular loops of *Salmonella* Typhimurium outer membrane protein A (OmpA) maintain the stability of *Salmonella* containing vacuole (SCV) in murine macrophages and protect the bacteria from autophagy-dependent lysosomal degradation

5 **Atish Roy Chowdhury[1, 2], Dipasree Hajra[1,2], and Dipshikha Chakravortty[1,**
6 **2, 3*]**

7 [1]Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore,
8 Karnataka, India-560012

9 [2]Division of Biological Sciences, Indian Institute of Science, Bangalore, Karnataka, India-
10 560012

11 [3]Centre for Biosystems Science and Engineering, Indian Institute of Science, Bangalore,
12 Karnataka, India-560012.

13

14 Current address: Department of Microbiology and Cell Biology, Indian Institute of Science,
15 Bangalore, Karnataka, India-560012

16 * *Corresponding author*

17 Dipshikha Chakravortty

18 Email: dipa@ iisc.ac.in

19 Tel: 0091 80 2293 2842

20 Fax: 0091 80 2360 2697

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24 # Abstract

25 After entering the host cells, *Salmonella* Typhimurium (STM) stays inside a modified
26 membrane-bound compartment called *Salmonella* containing vacuole (SCV). The biogenesis
27 and stability of SCV are crucial for the intracellular proliferation of *Salmonella*. Our research
28 has provided a novel mechanistic view on the role of a bacterial porin OmpA in maintaining
29 the stability of SCV. We found that the deletion of OmpA forces the bacteria to escape from
30 the SCV during the immediate early stage of infection. In the absence of OmpA, the bacteria
31 failed to retain the LAMP-1 and came into the host cell's cytosol. Subsequently, the cytosolic
32 population of STM *ΔompA* activated the host autophagy machinery after colocalizing with
33 syntaxin 17 and LC3B. The autophagosomes carrying STM *ΔompA* were targeted to the
34 lysosomes for degradation. Inhibition of autophagy pathway using bafilomycin A1 restored the
35 intracellular proliferation of STM *ΔompA*. We further showed that the four extracellular loops
36 of OmpA played a crucial role in holding the LAMP-1 pool around the SCV. We have altered
37 the extracellular loop sequences of *Salmonella* OmpA by site-directed mutagenesis and
38 observed that the bacteria failed to maintain the LAMP-1 pool around the SCV, which finally
39 resulted in their release into the cytosol of the host macrophages. Surprisingly, the cytosolic
40 population of *Salmonella* having mutations in the extracellular loops of OmpA didn't activate
41 the lysosomal degradation pathway like STM *ΔompA,* which helped them to survive within the
42 murine macrophages. In summary, our study revealed an OmpA dependent novel strategy
43 utilized by *Salmonella* to combat host autophagy by promoting the stability of SCV.

44 **Keywords:** *Salmonella*-containing vacuole, Outer membrane protein A, Syntaxin 17, LC3B,
45 Autophagy, Lysosome, Lysosome associated membrane protein-1, Early endosome antigen-1.

46 # Introduction

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47 *Salmonella* Typhimurium (STM), the non-typhoidal serovar of the enteric pathogen
48 *Salmonella enterica,* causes self-limiting diarrheal disease and gastroenteritis in humans [1]. It
49 causes typhoid fever-like symptoms in mice and is considered an excellent model to study the
50 pathogenesis of the human pathogen *Salmonella* Typhi. The pathogen enters the body of
51 healthy individuals with contaminated food and water. The Global Burden of Diseases,
52 Injuries, and Risk Factors Study (GBD) estimated an occurrence of 5,35,000 cases of invasive
53 non-typhoidal *Salmonella* infection worldwide, with approximately 77500 deaths in 2017 [2].
54 *Salmonella* Typhimurium can invade a wide range of host cells. After entering the host cells,
55 *Salmonella* resides within a modified membrane-bound acidic compartment called *Salmonella*
56 containing vacuole (SCV) [3-5]. The successful intracellular proliferation of the bacteria
57 depends upon the formation and maintenance of intact SCV within the host cells. The
58 disruption of SCV imposes a dramatic outcome on the fate of the intracellular bacteria. The
59 leakage of SCV and subsequent release of the bacteria into the cytosol of phagocytic cells
60 abrogate bacterial proliferation. In contrast, the cytosol of the non-phagocytic epithelial cells
61 promotes bacterial proliferation [5-9]. *Salmonella* majorly uses SPI-1 and SPI-2 encoded
62 virulent factors to control the biogenesis of SCV. These SPI-encoded virulent effectors work
63 in concert with host proteins to maintain the stability of SCV [4, 10-12]. However, the
64 contributions of non-SPI virulent genes in the biogenesis and stability of SCV have been poorly
65 understood.
66 Outer membrane protein A (OmpA) is a β barrel porin protein found on the outer membrane
67 of *Salmonella* Typhimurium. It consists of eight anti-parallel β sheets, connected by four
68 externally exposed extracellular loops and four periplasmic turns. With the help of its
69 periplasmic domain, it protects *Salmonella* from oxidative stress by changing the outer
70 membrane permeability [13]. The deficiency of OmpA compromises the biofilm-forming
71 ability of the bacteria in response to bile salt stress [14]. In our previous study, we have proved

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72 that deletion of OmpA significantly hampers the stability of the outer membrane of *Salmonella*,
73 which makes the bacteria susceptible to *in vivo* nitrosative stress[15]. We have also found that
74 *ompA* knockout *Salmonella* quits the SCV during the late phase of infection in murine
75 macrophages, suggesting a previously unknown role of OmpA in regulating the stability of
76 SCV.

77 In this current study, we addressed a novel role of *Salmonella* Typhimurium OmpA in
78 maintaining SCV stability. We have shown that the cytosolic population of *Salmonella* lacking
79 OmpA activates the host autophagy machinery and is cleared by the lysosomal degradative
80 pathway. Our study further revealed a strong interaction between the OmpA of intracellular
81 *Salmonella* with host LAMP-1 in macrophages. By introducing mutations in the externally
82 exposed extracellular loops of OmpA, we dissected the role of *Salmonella* OmpA in
83 modulating the intracellular vacuolar life of the pathogen. To the best of our knowledge, this
84 is the first study illustrating the precise role of the extracellular loops of *Salmonella*
85 Typhimurium OmpA in the intracellular virulence of bacteria.

86 Results

87 OmpA deficient strain of *Salmonella* quits SCV during the late phase of infection in
88 murine macrophages and activates host autophagy machinery.

89 Intracellular *Salmonella* Typhimurium residing within the SCV can inhibit phagolysosome
90 maturation [4]. The successful systemic colonization of *Salmonella* Typhimurium depends
91 upon forming a replicative niche within the host cells [16]. However, the biogenesis of SCV is
92 a complicated phenomenon. *Salmonella* employs a plethora of proteins and virulent factors that
93 work in conjunction with host factors for the construction of SCV. Most of the studies that
94 have addressed the intracellular pathogenesis of *Salmonella* have discovered the role of SPI-1
95 and SPI-2 encoded type 3 secretion systems (T3SS1 and T3SS2) and virulent factors in SCV

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96 biogenesis [17]. Earlier, our group has reported the role of outer membrane protein A (OmpA)
97 in maintaining the stability of SCV within murine macrophages[15]. To validate our previous
98 observation, the vacuolar niche of the wild type, *ompA* deficient, and complemented strains of
99 *S*. Typhimurium was checked in murine macrophages. STM *ΔompA* was found to be residing
100 with in the cytoplasm of RAW264.7 cells during the late phase of infection (**Figure 1A**). The
101 poor colocalization of STM *ΔompA* with LAMP-1 proved their release from the SCV (**Figure**
102 **4.1B**). When the *ompA* gene was complemented in the knockout bacteria, there was a reversal
103 in the vacuolar escaping phenotype (**Figure 1A and 1B**). Wild type *Salmonella* can recruit a
104 non-receptor tyrosine kinase named focal adhesion kinase (FAK) on the surface of the SCV in
105 an SPI-2 encoded T3SS2 dependent manner. FAK can suppress the host autophagy machinery
106 by activating the Akt-mTOR signaling pathway [18, 19]. Our previous study proved that
107 intracellular STM *ΔompA* is unable to produce and secrete the SPI-2 encoded translocon
108 proteins into the host cell's cytosol [15], suggesting the formation of a malfunctioning T3SS2.
109 Hence, we have hypothesized that the infection of macrophages with STM *ΔompA* may activate
110 host autophagy machinery. RAW264.7 cells were infected with STM (WT) and *ΔompA* to
111 evaluate the recruitment of autophagy markers (syntaxin 17 and LC3B) around the bacteria
112 during the late phase of infection. Syntaxin 17 is an autophagosomal SNARE protein with a
113 unique C-terminal hairpin structure of two tandem trans-membrane domains which are
114 constructed with glycine zipper motifs and interacts with the autophagosomal membrane [20].
115 Syntaxin 17 can further recruit SNAP 29 and lysosomal SNARE protein VAMP8 [21]. It helps
116 in the fusion of the autophagosome with lysosome and degradation of enclosed contents [22].
117 Microtubule-associated protein 1A/ 1B-light chain 3 (MAP-LC3/ LC3/ Atg8), which loads the
118 cargo in the autophagosome, is considered one of the important autophagy markers. Usually,
119 LC3B is diffused throughout the cytosol. Upon autophagy initiation, LC3B is cleaved by
120 cysteine protease Atg4 to form LC3B-I and further modified by the association of

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121 phosphatidylethanolamine to create LC3B-II. This lipidated form of LC3B (LC3B-II) forms
122 distinct small puncta in the cytosol and helps to seal the membrane of autophagosome carrying
123 cargo [23]. In line with our expectation, an increased recruitment of syntaxin 17 (**Figure 1C**
124 **and 1D**) and LC3B (**Figure 1E and 1F**) was observed around STM *ΔompA* compared to the
125 wild type bacteria during the late phase of infection. This suggests that STM *ΔompA* can
126 damage SCV and eventually activates host autophagy machinery in macrophages. To firmly
127 support this conclusion, the co-staining of LAMP-1 and syntaxin 17 was performed in
128 RAW264.7 cells infected with STM (WT) and *ΔompA* (**Figure S1A and S1B**). It was found
129 that the wild type *Salmonella* staying within intact SCV (**Figure S1A.1**) can restrict the
130 recruitment of syntaxin 17 (**Figure S1A.3 and S1A.5**). In the contrary, the majority of
131 intracellular STM *ΔompA* that hardly colocalize with LAMP-1 (**Figure S1A.2**) profoundly
132 sequester syntaxin 17 (**Figure S1A.4 and S1A.6**). The better colocalization of autophagy
133 markers with STM *ΔompA* (**Figure 1D and 1F**) suggested the formation of syntaxin 17^{+} $LC3B^{+}$
134 autophagosome around the mutant bacteria and subsequent activation of host autophagy
135 machinery (xenophagy). Autophagy can target the pathogen trapped inside the autophagosome
136 to the lysosomes for degradation. We verified this hypothesis by measuring the activation and
137 subsequent fusion of the lysosomes with wild type and *ompA* deficient *Salmonella.* Before
138 infecting the cells, the lysosomes were loaded with Texas red ovalbumin, and their
139 colocalization with the intracellular pathogens was evaluated. Compared to STM (WT), the
140 enhanced colocalization of STM *ΔompA* with Texas red (trapped inside lysosomes) suggested
141 a sharp rise in lysosomal activity (**Figure 1G and 1H**). As a control, STM (WT): *LLO*, a wild
142 type bacterial strain that leaves SCV because of the expression of pore-forming toxin
143 listeriolysin O from *Listeria monocytogenes,* was used. The reduced colocalization of STM
144 (WT): *LLO* with lysosomes robustly proves the role of OmpA to prevent lysosomal fusion with
145 the cytosolic pool of wild type bacteria (**Figure 1G and 1H**). To prove the subsequent

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146 activation of autophagy and lysosomal degradation upon infection of macrophages with STM
147 *ΔompA*, the colocalization between host syntaxin 17 and Texas red ovalbumin was studied. It
148 was found that unlike the wild type *Salmonella* (**Figure S2A.1, S2A.3, and S2A.5**), STM
149 *ΔompA* simultaneously colocalizes with both syntaxin 17 (**Figure S2A.2**) and lysosomes
150 (**Figure S2A.4 and S2A.6**). This suggests that the syntaxin 17^{+} autophagosome carrying STM
151 *ΔompA* is finally targeted to the lysosome for degradation. The enhanced activity of lysosomes
152 upon ingestion of any cargo can be estimated by measuring the activity of lysosomal enzymes
153 such as acid phosphatases [24, 25]. The intense activity of lysosomal enzymes upon infection
154 of macrophages with STM *ΔompA* was measured by acid phosphatase assay. The lysosomal
155 acid phosphatase activity was found impaired when the cells were infected with the wild type
156 and complemented strains of *Salmonella* (**Figure 1I**). The improved activity of acid
157 phosphatases from the cells infected with STM *ΔompA* (**Figure 1I**) suggested an active
158 function of lysosomes in killing the pathogen. Taken together, our data proves that during the
159 late phase of infection in murine macrophages, STM *ΔompA* reaches the cytosol of the host
160 cell and activates host autophagy machinery. As a result, the mutant bacteria trapped inside
161 syntaxin 17^{+}, $LC3B^{+}$ autophagosome is targeted to the lysosomal degradation pathway, which
162 might be a reason behind the clearance of the bacteria from macrophages.

163 **Inhibition of host autophagy using bafilomycin A restored the intracellular proliferation**
164 **of *ompA* deficient strain of *Salmonella* Typhimurium.**

165 Earlier, it was found that when the macrophages were infected with STM *ΔompA,* there was an
166 activation of the host autophagy machinery. This observation was strengthened by inhibiting
167 the autophagy pathway using bafilomycin A1, a macrolide antibiotic isolated from
168 *Streptomyces gresius*. Bafilomycin A1 inhibits the acidification of lysosomes by abrogating
169 the vacuolar H^{+} ATPase pump [26]. In the absence of bafilomycin A1, we have seen that a
170 significant population of STM *ΔompA* colocalizes with syntaxin17 (**Figure 2A and 2B**) and

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171 LC3B (**Figure 2C and 2D**) compared to the wild type bacteria, which is consistent with our
172 previous findings. This suggests the formation of an active autophagosome which traps the
173 mutant bacteria and restricts the infection within macrophages. When the cells were treated
174 with bafilomycin A1 (50 nM), an impeded recruitment of syntaxin 17 and LC3B around STM
175 *ΔompA* (**Figure 2A and 2C**) was found. Bafilomycin A1 treatment significantly reduced the
176 percent colocalization between syntaxin 17 with STM *ΔompA* compared to the untreated
177 macrophages infected with STM *ΔompA* (**Figure 2A and 2B**). On the other hand, a drastic
178 abrogation in the formation of LC3B puncta was also noted around STM *ΔompA* under
179 bafilomycin A1 treatment (**Figure 2C and 2D**). Normally, STM *ΔompA* has been found to be
180 deficient in replication in murine macrophages compared to wild type bacteria (**Figure 2E**).
181 When bafilomycin A1 was used, a significant recovery in the proliferation of STM *ΔompA* was
182 observed. The acidification of SCV helps in the intracellular proliferation of wild type
183 *Salmonella* by inducing SPI-2 gene expression. Bafilomycin A1 non-specifically inhibits the
184 acidification of the cells' acidic compartments, including both SCV and lysosomes. Hence it
185 can slow down the growth of wild type bacteria residing within SCV, which can be considered
186 as the reason behind the unaltered proliferation of STM (WT) upon bafilomycin A1 treatment
187 (**Figure 2E**). Taken together, our data illustrate that *Salmonella* Typhimurium OmpA protects
188 the bacteria against host autophagy machinery by improving the intracellular vacuolar life of
189 the pathogen.

190 **STM *ΔompA* quits the SCV before the early stage of infection and remains in the cytosol**
191 **of murine and human monocyte-derived macrophages.**

192 We further wanted to find out the time at which bacteria escape the SCV. To answer this
193 question, both RAW264.7 and PMA activated U937 cells were infected with wild type, *ompA*
194 mutant, and complemented strains of *Salmonella* and the vacuolar niche of the pathogen was
195 investigated during early (2h post-infection) and middle (6h post-infection) stages of infection.

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196 It was found that compared to the STM (WT), the colocalization of STM *ΔompA* with LAMP-
197 1 is significantly less at 2 hours post-infection in RAW 264.7 cells (**Figure 3A and 3B**), stating
198 that the knockout bacteria leave the vacuole even before the early stage of infection in
199 macrophages. The reduced colocalization of the mutant bacteria with LAMP-1 was restored in
200 the complemented strain (**Figure 3A and 3B**). Similarly, the poor colocalization of STM
201 *ΔompA* with LAMP-1 at 6 hours post-infection suggests that majority of the bacteria that quit
202 the vacuole before 2 hours remain in the cytosol during the middle stage of infection as well
203 (**Figure 3C and 3D**). To inspect whether this phenotype is cell type-specific or not, the same
204 experiment was carried out in PMA activated U937 cells. In accordance with our previous
205 observation, it was found that STM *ΔompA* has a higher propensity towards abandoning the
206 SCV before the early stage of infection than STM (WT) in U937 cells (**Figure S3A – S3F**).
207 Hence it was decided to study the vacuolar niche of the pathogen during the immediate early
208 phase of infection.

209 **STM *ΔompA* quits the SCV during the immediate early stage of infection in macrophages.**

210 The formation of SCV inside the host cell is a dynamic process. Immediately after entering the
211 cells, the wild type *Salmonella* residing within early SCV attracts early endosome membrane
212 markers such as early endosome antigen-1 (EEA-1), Rab5, and transferrin receptors, which are
213 replaced within 20 to 40 minutes post-infection by late endosome membrane markers like-
214 LAMP-1/2/3, Rab7, and V-ATPase [5, 27]. The lack of significant difference between the
215 recruitment of EEA1 around STM (WT) and STM *ΔompA* at 15 minutes post-infection in
216 activated U937 cells (**Figure 4A and 4B**) suggested that both the bacteria reside within the
217 early SCV decorated with EEA1 during the immediate early stage of infection. In this
218 experiment, PFA-fixed dead bacteria were used as a control. Taken together, this data also
219 suggests that deletion of *ompA* from *Salmonella* does not hamper the SCV biogenesis during
220 infection. Simultaneously, the colocalization of wild type and mutant bacteria with LAMP-1

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221 was tested at 15-, 30- and 120 minutes post-infection in activated U937 (**Figure 4C- 4F**) and
222 RAW264.7 cells (**Figure 4G- 4J**). At 15 minutes post-infection, STM *ΔompA* was found to be
223 recruiting more LAMP-1 compared to the wild type bacteria in U937 (**Figure 4C and 4F**) and
224 RAW264.7 (**Figure. 4G and 4J**) cells. With an increase in time (at 30- and 120 minutes post-
225 infection), the wild type *Salmonella* was found to be acquiring more LAMP-1 in both the cells
226 (**Figure 4D- 4F and Figure 4H- 4J**). In contrast, STM *ΔompA* was unable to retain the
227 acquired LAMP-1 and started losing the SCV membrane. Taken together, our data
228 demonstrated an essential role of outer membrane protein A of *Salmonella* to maintain a stable
229 interaction with LAMP-1. Earlier, it was found that wild type *Salmonella* uses SPI-1 encoded
230 virulent factor SipC (*Salmonella* invasion protein C) to acquire LAMP-1 from Golgi in a host
231 syntaxin 6 dependent manners [28]. Our study revealed that STM *ΔompA* is unable to obtain
232 LAMP-1 and escape the SCV. Hence, we hypothesized that STM *ΔompA* is deficient in
233 producing SipC. To test this hypothesis, RAW264.7 cells were infected with wild type and
234 *ompA* knockout strains of *Salmonella,* and the bacterial colocalization with SipC was studied.
235 Surprisingly, no significant difference in the colocalization of SipC with intracellular STM
236 (WT) and STM *ΔompA* (**Figure S4A and S4B**) was observed. Simultaneously, the expression
237 of *sipC* transcripts from intracellular wild type and mutant bacteria was quantified (**Figure**
238 **S4C**). There was no significant difference between the expression of *sipC* in wild type and
239 *ompA* knockout strains of *Salmonella* proliferating in macrophages (**Figure S4C**), which is
240 consistent with our previous findings. These data led us to conclude that OmpA plays a SipC
241 independent role in maintaining a stable interaction of *Salmonella* with LAMP-1.

242 ***Salmonella* Typhimurium OmpA plays a critical role in maintaining host LAMP-1 (SCV)**
243 **interaction during infection in macrophages.**

244 Earlier, we have shown an enhanced expression of *ompA* transcript in the wild type *Salmonella*
245 Typhimurium growing intracellularly in murine macrophages at 9th and 12th hours post-

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246 infection, which suggested the requirement of OmpA for the intracellular survival of bacteria
247 [15]. Our current study revealed that in the absence of OmpA, *Salmonella* could not stay inside
248 the SCV. Hence, we hypothesized that outer membrane protein A plays a direct role in
249 stabilizing the SCV membrane by retaining the LAMP-1 pool. To prove the interaction
250 between *Salmonella* OmpA and host LAMP-1, the macrophages were infected with wild type
251 bacteria, and the percent colocalization between OmpA and LAMP-1 during different time
252 points was investigated. In RAW264.7 cells, the wild type bacteria use OmpA in maintaining
253 a stable interaction with LAMP-1 at 2- and 6 hours post-infection (**Figure 5B and 5C**). This
254 interaction was further increased during the late phase of infection (at 16 hours post-infection)
255 (**Figure 5B and 5C**). In activated U937 cells, the interaction between OmpA with the LAMP-
256 1 was found to be increasing significantly with time (**Figure 5D and 5E**). Taken together, our
257 data suggested that indeed wild type *Salmonella* Typhimurium uses OmpA to retain the LAMP-
258 1 around the SCV firmly. When OmpA is deleted, the bacteria could not hold the LAMP-1
259 pool and were gradually released into the cytosol of macrophages from the SCV. OmpA is
260 embedded into the outer membrane of *Salmonella* Typhimurium with the help of its cylindrical
261 structure consisting of eight anti-parallel β sheets. The β sheets are connected to each other by
262 four periplasmic turns and four extracellular loops, which are exposed outside (**Figure 6A**),
263 and likely to be interacting with LAMP-1. Hence, we decided to find out the role of these
264 extracellular loops in establishing the interaction between the bacteria with LAMP-1. The
265 *Salmonella* OmpA extracellular loop sequences were compared with *Escherichia coli* K1
266 (**Figure 6A**), another Gram-negative bacterial pathogen causing meningitis in neonates [29,
267 30]. Without hampering the membrane-embedded cylindrical structure of OmpA, the
268 conserved and unique domains of the loops were altered by site-directed mutagenesis (**Figure**
269 **6A**), and six different variants, namely L1-1, L1-2, L2-1, L2-2, L3-1, L4-1, were generated.
270 The mutated versions of the gene were expressed in the *ompA* knockout background of

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271 *Salmonella* and used for infection. The latest research from our group demonstrated that the
272 depletion of OmpA increases the permeability of the bacterial outer membrane [15]. To
273 validate the proper folding and localization of OmpA on the bacterial outer membrane despite
274 receiving mutations in the extracellular loop regions, the outer membrane permeability of STM
275 (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-
276 2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and
277 *ΔompA*: pQE60-*ompA*-L4-1 was checked by $DiBAC_4$ staining (**Figure 6B and 6C**). In line
278 with our expectations, an enhanced uptake of $DiBAC_4$ was observed in STM *ΔompA* compared
279 to the wild type and complemented strains of *Salmonella*. The complementation of the *ompA*
280 knockout strain with mutated variants of *ompA* significantly reduced the entry of $DiBAC_4$,
281 suggesting a restoration of the outer membrane stability of the bacteria due to proper folding
282 and localization of mutated OmpA (**Figure 6B and 6C**).

283 **Mutation in the extracellular loops of *Salmonella* Typhimurium OmpA hampers the**
284 **stability of SCV but is not sufficient to target the bacteria to the lysosome.**

285 To find out the role of the extracellular loops of OmpA in maintaining the interaction of the
286 bacteria with LAMP-1, the loop mutants of OmpA were used for infecting murine
287 macrophages, and the recruitment of LAMP-1 was studied at 6- and 16 hours post-infection.
288 Altering the aminoacid sequences in any one of the four extracellular loops of OmpA gave
289 outcomes similar to STM *ΔompA*. Unlike the wild type and the complemented strains, all the
290 loop mutants quit the vacuole at the 6th hour post-infection (**Figure S5A**) and stayed in the
291 cytosol of macrophages during the late phase of infection (16th hour post-infection) as well
292 (**Figure 7A**). The cytosolic localization of the loop mutants was confirmed by quantifying their
293 percent colocalization with LAMP-1, which was comparable to the *ompA* knockout bacteria
294 (**Figure S5B and 7B**). This suggests that the extracellular loops of *Salmonella* OmpA execute
295 an important role in maintaining the stability of the SCV membrane by retaining LAMP-1.

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296 Bacteria were unable to hold the LAMP-1 around the SCV either in the absence of the entire
297 OmpA (*ompA* knockout *Salmonella*) or due to the structural and functional ineffectiveness of
298 the extracellular loops of OmpA. We further wanted to check the effect of the mutations in the
299 loop region of OmpA on the intracellular survival of the bacteria. An intracellular survival
300 assay was performed with the loop mutants in macrophages. Despite their cytosolic
301 inhabitation, mutations in the loop region did not cast any impact on the intracellular survival
302 of the bacteria. All the loop mutants were found to be surviving better than the STM *ΔompA*
303 while infecting the macrophage (**Figure 7C**). Despite having mutations in the extracellular
304 loop regions, the presence of intact OmpA on the outer membrane of the mutant bacteria might
305 be the reason behind their better survival in the cytosol. We speculated that the cylindrical
306 structure of OmpA, present in the outer membrane of the loop mutants, might be protecting the
307 cytosolic bacteria from being targeted to the lysosome. To test our hypothesis, the
308 colocalization of the loop mutants with the lysosomes was checked during the late phase of
309 infection in murine macrophages (**Figure 8A and 8B**). Unlike the wild type and the
310 complemented strains of *Salmonella*, STM *ΔompA* was found to be engulfed by the lysosomes
311 during the late phase of infection in murine macrophages (**Figure 8A and 8B**). In accordance
312 with our expectations, it was observed that the *ompA* variants having mutations in the
313 extracellular loops are less prone to be captured by the lysosomes, which further explains their
314 better survival within murine macrophages.

315 ## Discussion

316 While infecting the host cell, *Salmonella* Typhimurium stays inside a modified compartment
317 called *Salmonella* containing vacuole (SCV) [3, 4]. The low pH of the SCV triggers the
318 activation of the PhoP/Q two-component system, which finally upregulates the expression of
319 SPI-2 genes [31-33]. With the help of SPI-2 encoded T3SS2 and other virulent factors,
320 *Salmonella* can inhibit the phagolysosome maturation and suppresses the lysosome biogenesis

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321 in the host cells [25, 34]. The SPI-2 genes of intracellular bacteria further impede the
322 recruitment of iNOS and NADPH phagocytic oxidase around the SCV and ensure the
323 successful proliferation of the bacteria within the host cells [35, 36]. The formation of SCV is
324 a dynamic and complex process that employs a wide array of host and bacterial proteins.
325 Immediately after entering the host cells, the bacteria-containing vacuole recruits the early
326 endosome membrane markers such as EEA1, transferrin receptors, and Rab5. With time, the
327 early endosomal proteins are replaced with late endosome membrane markers such as LAMP-
328 1, LAMP-2, Rab7, etc. [4]. *Salmonella* uses SPI-1 and SPI-2 encoded virulent factors to
329 regulate the biogenesis and stability of the SCV. SPI-1 encoded virulent factor SopB
330 phosphatase can reduce the membrane charge of SCV and prevent the fusion of lysosomes with
331 SCV [11]. *Salmonella* employs SPI-2 encoded protein SpiC to inhibit the fusion of lysosomes
332 with SCV [34]. Another SPI-2 protein, SifA, forms *Salmonella*-induced filaments in epithelial
333 cells to maintain the integrity of SCV by downregulating the recruitment of kinesins [37, 38].
334 SifA interacts with Rab9 to block the retrograde transport of mannose-6- phosphate receptors
335 and MPR hydrolases to the Golgi apparatus, which ultimately inhibits lysosomal fusion with
336 SCV [39]. Tampering (by point mutation or deletion) the host and bacterial effectors that
337 control the stability of SCV create a tremendous impact on the intracellular fate of the pathogen.
338 Introducing point mutation in host Rab7 or deleting *sifA* from *Salmonella* released the bacteria
339 into the host cytosol from SCV, which makes the bacteria hyper-virulent in epithelial cells and
340 replication-deficient in macrophages [7, 8]. On the contrary, deletion of SPI-2 gene *sseJ* in *sifA*
341 null background further restored the vacuolar status of the pathogen [9]. However, very little
342 is known about the contribution of non-SPI virulent genes of *Salmonella* on the biogenesis and
343 integrity of SCV.

344 Our study revealed a novel role of *Salmonella* Typhimurium outer membrane protein A
345 (OmpA) in maintaining the stability of SCV. Outer membrane protein A is an outer membrane-

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346 bound porin of *Salmonella* that protects the pathogen from oxidative and nitrosative stress [13,
347 15]. The deletion of OmpA compromises the intracellular vacuolar status of the bacteria. STM
348 *ΔompA* was unable to recruit LAMP-1 around the SCV and gradually was released into the
349 host cytosol. The SCV quitting phenomenon of the pathogen was reversed when the gene was
350 complemented in the knockout bacteria. Earlier, our group has shown that this cytosolic
351 population of the bacteria becomes hyper-proliferative inside the epithelial cells, and their
352 intracellular proliferation was significantly attenuated in macrophages [15]. Our study further
353 depicted that STM *ΔompA* is unable to recruit T3SS2 translocon proteins (SseC and SseD) on
354 its surface and is deficient in producing SPI-2 effectors such as SsaV and SpiC [15]. Available
355 literature suggests that *Salmonella* deficient in making active T3SS2 and with damaged SCV
356 cannot suppress host autophagy machinery. Hence, we wanted to investigate the autophagy-
357 inducing ability of the cytosolic population of STM *ΔompA*. Compared to the wild type
358 bacteria, which stay within LAMP-1 decorated SCV, the cytosolic population of STM *ΔompA*
359 colocalizes more with syntaxin 17 and LC3B. The inhibition of autophagy using bafilomycin
360 A1 not only reduced the recruitment of autophagy markers (syntaxin 17 and LC3B) on STM
361 *ΔompA* but also improved their intracellular life. It was speculated that the activation of the
362 autophagy pathway would recruit lysosomes around the mutant bacteria. By Texas red
363 ovalbumin pulse-chase experiment, an enhanced colocalization of lysosomes with STM
364 *ΔompA* was observed in macrophages. When STM (WT): *LLO* (which stays in the macrophage
365 cytosol with intact OmpA on its outer membrane) was used for infection, a reduced
366 colocalization between the lysosome and bacteria was observed, suggesting that OmpA
367 protects the cytosolic bacteria from being targeted to lysosomes. This proves that the syntaxin
368 17 and LC3B recruited around the cytosolic bacteria lacking OmpA can target them into the
369 lysosomes. We confirmed this result by measuring the enhanced activity of lysosomal acid
370 phosphatases in the cells infected with STM *ΔompA*. Earlier, it has been reported that

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371 *Salmonella* uses SPI-2 encoded virulent factor SpiC to inhibit phagosome-lysosome fusion
372 [34]. STM *ΔompA* has been found to be deficient in producing SpiC, which might be the reason
373 behind the enhanced colocalization between the bacteria with host lysosomes.

374 We further wanted to check at what time the bacteria quit the vacuole in the absence of OmpA.
375 A significant population of STM *ΔompA* escapes the SCV during the early stage of infection
376 in macrophages. Once this bacterial population left the SCV, it remained in the cytosol during
377 the rest of the infection. This result helped us speculate that the departure of STM *ΔompA* from
378 SCV is happening even before the early stage of infection. Hence the recruitment of LAMP-1
379 around the bacteria was examined at 15-, 30- and 120 minutes post-infection. It was found that,
380 immediately after entering the macrophages, both the wild type and the mutant bacteria stay
381 within $EEA1^{+}$ early SCV, suggesting uninterrupted biogenesis of SCV. Moreover, at this stage
382 of infection (15 minutes p.i.), STM *ΔompA* recruits more LAMP-1 than wild type bacteria.
383 With an increase in time, STM *ΔompA* was unable to retain the LAMP-1 pool and gradually
384 lost the SCV membrane, unlike the wild type bacteria. Taken together, it was concluded that
385 without changing the biogenesis of SCV, the absence of OmpA in *Salmonella* only hampers
386 the integrity of SCV by restricting the recruitment of LAMP-1. Wild type *Salmonella* uses SPI-
387 1 effector protein SipC to recruit LAMP-1 from Golgi in host syntaxin 6 dependent manners
388 [28]. As it was observed that STM *ΔompA* is unable to hold the LAMP-1 pool, we speculated
389 that STM *ΔompA* is deficient in producing SipC. The comparable colocalization of SipC
390 between the intracellular wild type and mutant bacteria proved that the OmpA deletion mutant
391 works in a SipC independent manner. This conclusion was supported by measuring the
392 unaltered *sipC* transcript level from intracellular wild type and mutant bacteria. This result
393 motivated us to hypothesize that OmpA plays an important structural role in maintaining the
394 interaction of LAMP-1 with the bacteria confined inside the SCV. The direct interaction
395 between the LAMP-1 and the OmpA of wild type bacteria was measured to verify the

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396 hypothesis. The intracellular wild type *Salmonella* was stained with anti-*Salmonella* OmpA
397 antibody, and the recruitment of LAMP-1 around the bacteria was estimated. We have seen
398 that *Salmonella* Typhimurium OmpA maintains a stable interaction with the host LAMP-1
399 during the early and middle stages of infection in macrophages, which further increases during
400 the late phase of infection.

401 Outer membrane protein A is embedded into the bacterial outer membrane with the help of its
402 cylindrical β barrel structure. The anti-parallel β sheets that constitute the wall of this barrel
403 structure are connected by four extracellular loops (L1, L2, L3, and L4), which are exposed
404 outside. The externally exposed extracellular loops were thought to maintain the integrity of
405 SCV by directly interacting with host LAMP-1. Our speculation was validated by introducing
406 mutations in the loop regions of *Salmonella* OmpA by site-directed mutagenesis. We have
407 performed a comparative study on the amino acid sequences of the extracellular loops of OmpA
408 between *Salmonella* Typhimurium and *Escherichia coli* K1 [40]. The role of the extracellular
409 loops of *E. coli* K1 OmpA has already been deciphered. The introduction of mutations in the
410 loop regions of *E. coli* K1 OmpA made the bacteria proliferation deficient in immune cells and
411 reduced their ability to cause meningitis in a neonatal mouse model [29, 41]. The unique and
412 the conserved sequences of the extracellular loops of *Salmonella* OmpA were targeted to make
413 them deformed structurally and functionally. The amino acid sequences of the loop region were
414 changed to nonpolar amino acids like- alanine, leucine, and valine. It was found that each of
415 these individual loop mutants plays a critical role in maintaining the interaction with LAMP-1
416 during the middle and late stages of infection in murine macrophages. All the OmpA loop
417 mutants came into the cytosol of macrophages after abandoning the SCV. Surprisingly, it was
418 observed that the mutations in the loop regions do not hamper the intracellular proliferation of
419 the bacteria within the cytosol of macrophages. To find out the reason behind the better survival
420 of the *Salmonella* loop mutants in murine macrophages, their colocalization with the lysosomal

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421 compartments was examined. The reduced percent colocalization of the loop mutants with
422 Texas red ovalbumin suggested poor recruitment of lysosomes on the cytosolic niche of
423 *Salmonella* Typhimurium *ompA* variants. In the previous study, we have proved that the
424 presence of intact OmpA maintains the integrity of the bacterial outer membrane and protects
425 it from nitrosative stress. The final observation from this study led us to conclude that despite
426 mutations in the extracellular loops, the intact OmpA present on the bacterial outer membrane
427 could protect the cytosolic bacteria from lysosomal degradation in murine macrophages.

428 Altogether, our study provides an OmpA dependent novel mechanism used by *Salmonella*
429 Typhimurium to maintain the stability and integrity of the SCV. *Salmonella* uses the
430 extracellular loops of OmpA to retain the LAMP-1 pool around the SCV. In the absence of
431 OmpA, the bacteria fail to hold the LAMP-1 and come into the cytosol after quitting the SCV.
432 The cytosolic bacteria lacking OmpA further activate the host autophagy machinery. They are
433 unable to prevent the maturation of the phagolysosome, which further leads to the clearance of
434 the bacteria by the lysosomal degradation pathway.

435 Materials and methods

436 Bacterial strains, media, and culture conditions

437 The wild type (WT) bacteria *Salmonella enterica* serovar Typhimurium strain 14028S used in
438 this study was a generous gift from Professor Michael Hensel, Max Von Pettenkofer-Institute
439 for Hygiene and Medizinische Mikrobiologie, Germany. The bacterial strains were revived
440 from glycerol stock (stored in -80^{0}C) and plated either only on LB agar (purchased from
441 HiMedia) (for the wild type *Salmonella*) or LB agar along with appropriate antibiotics like-
442 kanamycin (50 µg/mL) (for the *ompA* knockout strains), ampicillin (50 µg/mL) (for the wild
443 type *Salmonella* expressing mCherry/ RFP, GFP, and LLO), and kanamycin and ampicillin
444 together (both 50 µg/mL), (for the complemented, loop mutants, mCherry and GFP expressing

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445 *ompA* knockout strains). *Salmonella- Shigella* agar was used for plate cell lysates/ cell
446 suspensions to calculate the bacterial burden in infected cell lines. The complete list of strains
447 and plasmids has been listed below. (Description in Table- 4.1) Dead bacteria used in several
448 experiments were produced from viable wild type bacteria by treating the bacteria with 3.5%
449 paraformaldehyde for 30 minutes.

450 **Eukaryotic cell lines and growth conditions**

451 The murine macrophage-like cell lines RAW 264.7 used in this study were maintained in
452 Dulbecco's Modified Eagle's Media (Sigma-Aldrich) supplemented with 10% FCS (Fetal calf
453 serum, Gibco) at 37^0C temperature in the presence of 5% CO_2. Human monocyte cell line U937
454 cells were maintained in Roswell Park Memorial Institute 1640 media (Sigma-Aldrich)
455 supplemented with 10% FCS (Fetal calf serum, Gibco). Phorbol Myristate Acetate (Sigma-
456 Aldrich) (concentration- 20 ng/ mL) was used for the activation of U937 cells for 24 hours at
457 37^0C temperature in the presence of 5% CO_2, followed by the replacement of the media
458 carrying PMA with normal RPMI supplemented with 10% FCS and further incubating the cells
459 for 24 hours before starting the experiments.

460 **RNA isolation from intracellular bacteria and RT PCR**

461 The RAW264.7 cells were infected with STM (WT) and *ΔompA* at MOI of 50. 12hours post-
462 infection, the infected macrophages were lysed with TRIzol reagent (RNAiso Plus, Takara)
463 and stored at -80^0C overnight. The lysed supernatants were further subjected to chloroform
464 extraction followed by precipitation of total RNA by adding an equal volume of isopropanol.
465 The pellet was washed with 70% RNA-grade ethanol, air-dried, and suspended in 20 µL of
466 DEPC water. The RNA concentration was estimated in nano-drop and run on 1.5% agarose gel
467 to assess RNA quality. To make cDNA, 3 µg of RNA sample was subjected to DNase treatment
468 (Thermo Fischer Scientific) at 37^0C for two hours. The reaction was stopped by adding 5mM

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469 Na_2EDTA (Thermo Fischer Scientific), followed by heating the sample at 65^0C for 10 min.
470 The samples were incubated with random hexamers at 65^0C for 10 minutes and then
471 supplemented with 5X RT buffer, RT enzyme, dNTPs, and DEPC treated water at 42^0C for an
472 hour. Quantitative real-time PCR was done using SYBR/ TB Green RT PCR kit (Takara Bio)
473 in Bio-Rad real-time PCR detection system, and the expression level of target genes was
474 measured using *Salmonella* Typhimurium *sipC* specific RT primers (Table- 4.2). 16S rRNA
475 transcript level was used to normalize the expression levels of the target genes.

476 **Intracellular proliferation assay**

477 RAW264.7 (1.5 to 2 X 10^5 cells seeded per well) were infected with STM (WT), *ΔompA,*
478 *ΔompA*: pQE60-*ompA*, *ΔompA*:pQE60-*ompA* L1-1, *ΔompA*:pQE60-*ompA* L1-2,
479 *ΔompA*:pQE60-*ompA* L2-1, *ΔompA*:pQE60-*ompA* L2-2, *ΔompA*:pQE60-*ompA* L3-1, and
480 *ΔompA*:pQE60-*ompA* L4-1 at MOI of 10. After centrifuging the cells at 800 rpm for 5 minutes,
481 the infected cells were incubated at 37^0C temperature in the presence of 5% CO_2 for 25 minutes.
482 Next, the cells were washed thrice with PBS to remove all the unattached extracellular bacteria
483 and subjected to 100 μg/ mL concentration of gentamicin treatment for 1 hour. This was
484 followed by washing the cells with sterile PBS and subjecting them to a 25 μg/ mL
485 concentration of gentamicin treatment till the lysis. The cells were lysed with 0.1% triton-X-
486 100 at 2 hours and 16 hours post-infection. The lysates were plated on *Salmonella- Shigella*
487 Agar, and the corresponding CFU at 2 hours and 16 hours were determined. The intracellular
488 proliferation of bacteria (Fold proliferation) was determined using a simple formula-

489 **Fold proliferation= [CFU at 16 hours]/ [CFU at 2 hours]**

490 In some sets of experiments, the fold proliferation of STM (WT) and *ΔompA* in the
491 macrophages (RAW 264.7) was measured in the presence of autophagy inhibitor bafilomycin
492 A (50 nM). Bafilomycin A was added to the cells infected with STM (WT) and *ΔompA* along

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493 with 25 µg/ mL of gentamycin solution and incubated till the lysis. As usual, the cells were
494 lysed with 0.1% triton-X-100 at 2 hours and 16 hours post-infection. The lysates were plated
495 on *Salmonella- Shigella* Agar, and the corresponding CFU at 2 hours and 16 hours were
496 calculated to determine Fold proliferation.

497 **Confocal microscopy**

498 RAW 264.7 or U937 cells were seeded at a 1.5 to 2 X 10^5 cells density per sterile glass
499 coverslips. U937 cells were activated using PMA (as mentioned earlier in 4.2.2). The cells
500 were infected with appropriate bacterial strains at MOI 20. The cells were washed thrice with
501 PBS and fixed with 3.5% paraformaldehyde for 15 minutes at indicated time points post-
502 infection. The cells were first incubated with specific primary antibody raised against- wild
503 type *Salmonella* Typhimurium (rabbit raised anti- *Salmonella* O antigen), mouse lysosome-
504 associated membrane protein-1 (LAMP-1) (rat raised anti-mouse LAMP-1), human LAMP-1
505 (mouse raised anti-human LAMP-1), mouse LC3B (rabbit raised anti-mouse LC3B), mouse
506 syntaxin 17 (rabbit raised anti-mouse syntaxin 17), *Salmonella* Typhimurium SipC (mouse
507 raised anti-*Salmonella* SipC), *S.* Typhimurium OmpA (rabbit raised anti-*Salmonella* OmpA
508 antibody) and human EEA1 (mouse raised anti-human EEA1) as per the requirements of
509 experiments. The primary antibodies were diluted in 2.5% BSA and 0.01% saponin (dilution
510 1: 100, duration 6 to 8 hours at 4^0C temperature). This was followed by incubating the cells
511 with appropriate secondary antibodies conjugated with fluorophores (dylight 488, alexa fluor
512 647, and cy3) (dilution 1: 200, duration 1 hours at room temperature). The coverslips were
513 mounted with anti-fade reagent and fixed on a glass slide with transparent nail paint. Samples
514 were imaged by confocal laser scanning microscopy (Zeiss LSM 710) using a 63X oil
515 immersion objective lens. The images were analyzed with ZEN Black 2009 software provided
516 by Zeiss.

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517 **Texas red ovalbumin pulse-chase experiment**

518 1.5 to 2 X 10^5 RAW 264.7 cells were seeded on the top of sterile glass coverslips in the wells
519 of a 24 well plate. The cells were fed with a 50 µg/ mL concentration of Texas red ovalbumin
520 (resuspended in DMEM media) for 30 minutes at 37^0C in the presence of 5% CO_2. After this,
521 the labeling media was removed, and the cells were washed with sterile PBS. The cells were
522 further incubated for 30 minutes with fresh DMEM media and infected with overnight grown
523 10- 12 hours old stationary phase culture of STM (WT), STM (WT): *GFP*, *ΔompA*: *GFP,* and
524 STM (WT): *LLO* at MOI of 20. PFA-fixed dead bacteria were used for infection as control at
525 MOI of 25. The cells were washed thrice with PBS and fixed with 3.5% paraformaldehyde for
526 15 minutes at indicated time points post-infection. Rabbit-raised anti-*Salmonella* primary
527 antibody stained the STM (WT): *LLO* and PFA fixed dead bacteria. The coverslips were
528 mounted with anti-fade reagent and fixed on a glass slide with transparent nail paint to visualize
529 the lysosomal arrangements in the infected macrophage cells. Samples were imaged by
530 confocal laser scanning microscopy (Zeiss LSM 710) using a 63X oil immersion objective lens.
531 The images were analyzed with ZEN Black 2009 software provided by Zeiss.

532 **Acid phosphatase assay**

533 The protocol of acid phosphatase assay has been followed as mentioned earlier [25, 42, 43].
534 1.5 to 2 X 10^5 RAW 264.7 cells were seeded into the wells of a 24 well plate and infected with
535 overnight grown stationary phase culture of STM (WT), *ΔompA, ΔompA*: pQE60-*ompA*,
536 *ΔompA*: pQE60, and STM (WT): *LLO* at MOI of 10. PFA-fixed dead bacteria were used as a
537 control for the infection (MOI-20). The infected cells were incubated for 12 hours under
538 gentamycin treatment (as mentioned earlier). At the end of the incubation period, the cells were
539 washed with PBS and incubated for 4 hours at 37^0C with a buffer containing a 0.1 (M) sodium
540 acetate of pH= 5, 0.1% triton-X-100, 5mM of *p*-nitrophenyl phosphate (pNPP). The absorbance

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541 of the supernatant was measured at 405 nm using a microplate reader. The non-enzymatic
542 hydrolysis of pNPP (negligible) was measured media control without macrophage cells.

543 **Generation of extracellular loop mutants of *ompA* by site-directed mutagenesis**

544 The protocol for site-directed mutagenesis was followed, as mentioned earlier [44]. Primer
545 pairs (from Table- 4.2) carrying mutations in the desired locations of the four extracellular
546 loops of *Salmonella* Typhimurium OmpA (Figure 4.12.B) were used to amplify the pQE60-
547 *ompA* recombinant plasmid (Size- 4.5 kb) using Phusion high fidelity DNA polymerase (NEB).
548 The reaction mixture was heated at 95^{0}C for 10 minutes for the plasmid's denaturation,
549 followed by 35 amplification cycles at 95^{0}C for 30 seconds, 54^{0}C for 60 seconds, and 72^{0}C for
550 2 minutes 30 seconds with a final extension time of 10 minutes. The amplified PCR product
551 was then digested with DpnI and transformed into *E. coli* TG1. The transformants were then
552 selected on ampicillin plates. The plasmids were isolated from the transformant colonies, and
553 the mutations were verified by sequencing. Once the confirmation was done, the recombinant
554 plasmids carrying desired mutations were transformed into STM *ΔompA* by electroporation to
555 create specific loop mutant *Salmonella*. These loop mutants were used for infecting RAW264.7
556 cells.

557 **Measurement of outer membrane porosity of *Salmonella* Typhimurium OmpA loop**
558 **mutants**

559 Outer membrane porosity of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-
560 *ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-
561 L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 grown in low magnesium
562 acidic F medium (pH= 5.4) for 12 hours was measured using a dye called bis-(1,3-dibutyl
563 barbituric acid)-trimethylene oxonol (Invitrogen) ($DiBAC_4$). Briefly, 4.5 X 10^7 CFU of each
564 bacterial strain was incubated with 1 μg/ml of DiBAC4 for 30 minutes in a 37^{0}C shaker

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565 incubator. The $DiBAC_4$ treated bacterial cells were analyzed by flow cytometry (BD
566 FACSVerse by BD Biosciences-US) to evaluate the porosity of the bacterial outer membrane.

567 **Statistical analysis**

568 Each experiment has been independently repeated 2 to 3 times [as mentioned in the figure
569 legends. The *in vitro* data and the results obtained from cell line experiments were analyzed by
570 unpaired student's *t*-test by GraphPad Prism 8.4.3 (686) software, and *p* values below 0.05
571 were considered significant. The results are expressed as mean ± SEM. Differences between
572 experimental groups were deemed to be significant for $p < 0.05$.

573 Abbreviations

574 STM: *Salmonella* Typhimurium

575 OmpA: Outer membrane protein A

576 LC3B: Microtubule-associated protein 1A/ 1B-light chain 3

577 Stx17: Syntaxin 17

578 LLO: Listeriolysin O

579 SCV: *Salmonella* containing vacuole

580 LAMP-1: Lysosome associated membrane protein-1

581 EEA1: Early endosome antigen 1

582 RFP: Red fluorescent protein

583 GFP: Green fluorescent protein

584 SipC: *Salmonella* invasion protein C

585

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586 **Table 4.1. Strains and plasmids used in this study**

Strains/ plasmids	Characteristics	Source/ references
Salmonella enterica serovar Typhimurium ATCC strain14028S	Wild type (WT)	Gifted by Prof. M. Hensel
S. Typhimurium *ΔompA*	Kan^R	Laboratory stock
S. Typhimurium *ΔompA*: pQE60-*ompA*	Kan^R, Amp^R	Laboratory stock
S. Typhimurium *ΔompA*: pQE60	Kan^R, Amp^R	Laboratory stock
pQE60 vector	Low copy number plasmid, Amp^R	Laboratory stock
pFV- mCherry (RFP)	Amp^R	Laboratory stock
pFV: GFP	Amp^R	Laboratory stock
STM (WT): *LLO*	Amp^R	Laboratory stock
S. Typhimurium *ΔompA*: pQE60-*ompA* L1-1	Kan^R, Amp^R	This study
S. Typhimurium *ΔompA*: pQE60-*ompA* L1-2	Kan^R, Amp^R	This study

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S. Typhimurium *ΔompA*: pQE60-*ompA* L2-1	Kan^R, Amp^R	This study
S. Typhimurium *ΔompA*: pQE60-*ompA* L2-2	Kan^R, Amp^R	This study
S. Typhimurium *ΔompA*: pQE60-*ompA* L3-1	Kan^R, Amp^R	This study
S. Typhimurium *ΔompA*: pQE60-*ompA* L4-1	Kan^R, Amp^R	This study

587

588 **Table 4.2. Primers used in this study (5’-3’)**

589 *sipC* forward- GACCTGGGGTTGAGTCCTAC

590 *sipC* reverse- ACGGCACTGGAAGACATTC

591 *16S rRNA* forward-CGGACGGGTGAGTAATGT

592 *16S rRNA* reverse-TGCTTCTTCTGCGGGTAA

593

594 ***ompA* site directed mutagenesis primers (5’-3’)**

595 *ompA* Loop 1-1 forward- GCTTCATTCACAATGTGGTGGTGACTCATGAAAACC

596 *ompA* Loop 1-1 reverse- TGGTTTTCATGAGTCACCACCACATTGTGAATGAAGCCG

597 *ompA* Loop 1-2 forward- ACAATGATGGCCCGGCGGCGGCGAACCAACTGGGCG

598 *ompA* Loop 1-2 reverse-TGCGCCCAGTTGGTTCGCCGCCGCCGGGCCATCATTGTG

599 *ompA* Loop 2-1 forward- GTTAGGCCGTATGGCGGCGGCGGGCGACAACATC

600 *OmpA* Loop 2-1 reverse- TGATGTTGTCGCCCGCCGCCGCCATACGGCCTAACC

601 *OmpA* Loop 2-2 forward- ATGCCGTACAAAGTGGTGAACATCAATGGCGC

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602 *OmpA* Loop 2-2 reverse- AGCGCCATTGATGTTCACCACTTTGTACGGC

603 *OmpA* Loop 3-1 forward- AGACACCAAGTCTCTGGCGCTGGGCGGCCCGTCTAC

604 *OmpA* Loop 3-1 reverse- TAGACGGGCCGCCCAGCGCCAGAGACTTGGTGTC

605 *OmpA* Loop 4-1 forward- ACTAACAACATCGTGGTGGCCAACACCATC

606 *OmpA* Loop 4-1 reverse- CCGATGGTGTTGGCCACCACGATGTTGTTAG

607 *ompA* SDM confirmatory primer- ATGAAAAAGACAGCTATCGC

608 Author Contributions

609 ARC and DC conceived the study and designed the experiments. ARC performed all the
610 experiments, analyzed the data, and wrote the original draft of the manuscript. DH performed
611 the experiments, participated in proofreading and editing of the manuscript with ARC. DC
612 supervised the study and reviewed the manuscript. All the authors have read and approved the
613 manuscript.

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632 # Availability of data and materials

633 All data generated and analyzed during this study, including the supplementary information
634 files, have been incorporated in this article. The data is available from the corresponding author
635 on reasonable request.

636 # Declarations

637 **Ethics statement**

638 Not applicable.

639 **Consent for publication**

640 Not applicable.

641 # Competing interests

642 The authors declare to have no conflict of interest.

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749 # Figure Legends

750 **Figure 1.**

751 **OmpA deficient strain of *Salmonella* quits SCV during the late phase of infection in**
752 **murine macrophages and activates host autophagy machinery**

753 (A) RAW264.7 cells were infected with STM (WT): RFP, *ΔompA*: RFP, and *ΔompA*: pQE60-
754 *ompA* at MOI of 20. Cells were fixed at 16 hours post-infection & LAMP-1 was labeled with
755 anti-mouse LAMP-1 antibody. To stain the complemented strain anti-*Salmonella* antibody was
756 used. (B) The quantification of LAMP-1 recruitment on bacteria in RAW 264.7 cells has been
757 represented in a graph. Percent colocalization was determined after analyzing more than 60
758 different microscopic stacks from two independent experiments. Scale bar = 5μm, [n≥60, N=2].
759 (C-F) RAW264.7 cells were infected with STM (WT): *RFP* and *ΔompA*: *RFP* at MOI of 20.
760 Cells were fixed at 16 hours post-infection. Two autophagy markers, (C) syntaxin 17 and (E)
761 LC3B, were stained with rabbit-raised anti-mouse syntaxin 17 and LC3B-II primary
762 antibodies, respectively. The quantification of (D) syntaxin 17 and (F) LC3B recruitment on
763 STM (WT): *RFP* and *ΔompA*: *RFP* has been represented in the form of two graphs. (D) The
764 percent colocalization of syntaxin 17 with bacteria was determined after analyzing 100
765 microscopic stacks from three independent experiments (n=100. N=3). (F) The percent
766 colocalization between the bacteria and LC3B was determined after analyzing more than 60
767 different microscopic stacks from two independent experiments. Scale bar = 5μm, [n≥60, N=2].
768 To stain the lysosomes, RAW 264.7 cells were pre-treated with Texas red ovalbumin for 30
769 minutes. (G) The cells are washed thrice with PBS after that and infected with STM (WT):
770 *GFP*, *ΔompA*: *GFP*, and STM (WT): *LLO*, respectively, at MOI of 20. PFA-fixed dead bacteria

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771 were used for infection at MOI of 25. To stain STM (WT): *LLO* and PFA fixed dead bacteria
772 rabbit-anti *Salmonella* O primary and anti-rabbit dylight 488 secondary antibodies were used.
773 (H) The colocalization of lysosomes with bacteria has been represented in the form of a graph.
774 The percent colocalization between Texas red and the bacteria was determined after counting
775 50 microscopic stacks from two independent experiments (n=50, N=2). Scale bar = 5µm. (I)
776 To measure the acid phosphatase activity of lysosomes, RAW 264.7 cells were infected with
777 STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, STM (WT): *LLO*, and PFA fixed
778 dead bacteria at MOI of 10. Twelve hours post-infection, the cells were washed with PBS and
779 further incubated for 4 hours at 37^0C with a buffer containing sodium acetate, triton-X-100,
780 and *p*-nitrophenyl phosphate (pNPP). The absorbance of the supernatant was measured at 405
781 nm using a microplate reader (n=6, N=2).

782 ***(p) **< 0.005*, *(p) ***< 0.0005*, *(p) ****< 0.0001* (Student's *t*-test).**

783 **Figure 2.**

784 **Inhibition of host autophagy using bafilomycin A restored the intracellular proliferation**
785 **of *ompA* deficient strain of *Salmonella* Typhimurium**

786 RAW264.7 cells were infected with STM (WT): *RFP* and *ΔompA*: *RFP* at MOI of 20. One set
787 of cells infected with STM *ΔompA*: *RFP* were treated with bafilomycin A (50 nM). Cells were
788 fixed at 16 hours post-infection. Two autophagy markers, (A) syntaxin 17 and (C) LC3B, were
789 stained with rabbit-raised anti-mouse syntaxin 17 and LC3B-II primary antibodies,
790 respectively. The quantification of (B) syntaxin 17 and (D) LC3B recruitment on STM (WT):
791 *RFP*, *ΔompA*: *RFP*, and *ΔompA*: *RFP* under bafilomycin A treatment have been represented in
792 the form of two graphs. (B) The percent colocalization of syntaxin 17 with bacteria was
793 determined after analyzing 100 microscopic stacks from three independent experiments
794 (n=100. N=3). (D) The percent colocalization between the bacteria and LC3B was determined

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795 after analyzing more than 60 different microscopic stacks from two independent experiments.
796 Scale bar = 5µm, [n≥60, N=2]. (E) Intracellular survival of STM (WT) and *ΔompA* (MOI-10)
797 in RAW264.7 cells (16 hours post-infection) in presence and absence of autophagy inhibitor
798 bafilomycin A (50 nM). The bacteria's fold proliferation was calculated by normalizing the
799 CFU at 16 hours to CFU at 2 hours (n=3, N=2).

800 ***(p)* ***< *0.0005*, *(p)* ****< *0.0001*, ns= non-significant (Student's *t*-test).**

801 **Figure 3.**

802 **STM *ΔompA* quits the SCV in murine macrophages before the early stage of infection**

803 (A-D) RAW264.7 cells were infected with STM (WT): RFP, *ΔompA*: RFP, and *ΔompA*:
804 pQE60-*ompA* at MOI of 20. Cells were fixed at (A) 2 hours (early phase) and (C) 6 hours
805 (middle phase) post-infection & LAMP-1 was labeled with anti-mouse LAMP-1 antibody. To
806 stain the complemented strain and the PFA fixed dead bacteria anti-*Salmonella* antibody was
807 used. The quantification of LAMP-1 recruitment on bacteria in RAW 264.7 cells at (B) 2 hours
808 and (D) 6 hours post-infection has been represented in the form of two graphs. (B) During the
809 early stage of infection (2 hours post-infection), the percent colocalization of bacteria with
810 LAMP-1 was determined after analyzing more than 50 different microscopic stacks from two
811 independent experiments [n≥50, N=2]. (D) During the middle stage of infection (6 hours post-
812 infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing
813 more than 40 different microscopic fields from two independent experiments [n≥40, N=2].
814 Scale bar = 5µm.

815 ***(P)* *< 0.05, *(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

816 **Figure 4.**

817 **STM *ΔompA* quits the SCV during the immediate early stage of infection in macrophages**

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818 PMA activated U937 cells were infected with STM (WT), *ΔompA*, and PFA fixed dead bacteria
819 at MOI of 25. (A) Cells were fixed at 15 minutes post-infection (immediate early stage of
820 infection). The bacteria have been stained with rabbit-raised anti-*Salmonella* O primary
821 antibody. Early endosome antigen (EEA1) was labeled with an anti-human EEA1 antibody
822 raised in the mouse. (B) The quantification of EEA1 recruitment on bacteria in U937 cells has
823 been represented in the form of three graphs. During the immediate early stage of infection (15
824 minutes post-infection), the percent colocalization of bacteria with EEA1 was determined after
825 analyzing more than 30 different microscopic stacks from two independent experiments [n≥30,
826 N=2]. PMA activated U937 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at
827 MOI of 20. Cells were fixed at (C) 15 minutes (immediate early phase), (D) 30 minutes, and
828 (E) 2 hours post-infection & LAMP-1 was labeled with anti-human LAMP-1 antibody. (F) The
829 quantification of LAMP-1 recruitment on bacteria in U937 cells at 15 minutes, 30 minutes, and
830 2 hours post-infection has been represented in the form of a graph. (F) The percent
831 colocalization of bacteria with LAMP-1 was determined after analyzing more than 50 different
832 microscopic stacks from two independent experiments [n≥50, N=2]. Scale bar = 5μm.
833 RAW264.7 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at MOI of 20. Cells
834 were fixed at (G) 15 minutes (immediate early phase), (H) 30 minutes, and (I) 2 hours post-
835 infection & LAMP-1 was labeled with anti-mouse LAMP-1 antibody. (J) The quantification of
836 LAMP-1 recruitment on bacteria in RAW264.7 cells at 15 minutes, 30 minutes, and 2 hours
837 post-infection has been represented in the form of a graph. (J) The percent colocalization of
838 bacteria with LAMP-1 was determined after analyzing more than 60 different microscopic
839 stacks from two independent experiments [n≥60, N=2]. Scale bar = 5μm.

840 ***(P)* **< 0.005, *(P)* ***< 0.0005, *(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

841 **Figure 5.**

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842 ***Salmonella* Typhimurium OmpA plays a critical role in maintaining the interaction with**
843 **host LAMP-1 (SCV) during infection in macrophages.**

844 (A) The structure of *Salmonella* Typhimurium OmpA obtained with the help of SWISS-
845 MODEL software (B) RAW 264.7 cells were infected with STM (WT) at MOI of 20. Cells
846 were fixed at 2 hours (early phase), 6 hours (middle phase), and 16 hours (late phase) post-
847 infection. Intracellular *Salmonella* was stained with rabbit-raised anti-*Salmonella* OmpA
848 primary antibody. In RAW264.7 cells, LAMP-1 was labeled with rat-raised anti-mouse
849 LAMP-1 antibody. (C) The quantification of LAMP-1 recruitment on bacteria in RAW264.7
850 cells at 2 hours, 6 hours, and 16 hours post-infection has been represented in the form of a
851 graph. (C) The percent colocalization of bacteria with LAMP-1 was determined after analyzing
852 50 different microscopic stacks from two independent experiments [n=50, N=2]. (D) PMA
853 activated U937 cells were infected with STM (WT) at an MOI of 20. Cells were fixed at 2
854 hours (early phase), 6 hours (middle phase), and 16 hours (late phase) post-infection.
855 Intracellular *Salmonella* was stained with rabbit-raised anti-*Salmonella* OmpA primary
856 antibody. In U937 cells, LAMP-1 was labeled with an anti-human LAMP-1 antibody. (E) The
857 quantification of LAMP-1 recruitment on bacteria in U937 cells at 2 hours, 6 hours, and 16
858 hours post-infection has been represented in the form of a graph. (E) The percent colocalization
859 of bacteria with LAMP-1 was determined after analyzing 50 different microscopic stacks from
860 three independent experiments [n=50, N=3]. Scale bar = 5µm.

861 ***(P)* *< 0.05, *(P)* **< 0.005, *(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

862 **Figure 6.**

863 **Introducing mutation in the extracellular loops of *Salmonella* Typhimurium OmpA by**
864 **site-directed mutagenesis.**

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865 (A) The outer membrane-embedded β barrel structure of *Salmonella* Typhimurium OmpA with
866 extracellular loops (Loop1, Loop2, Loop3, and Loop4). Comparison between the extracellular
867 loop sequences of *Escherichia coli* K1 and *Salmonella* Typhimurium. Two different mutations
868 were introduced in loop1 (L1- L1-1 and L1-2) and loop2 (L2- L2-1 and L2-2) separately. Two
869 distinct single mutations (L3-1 and L4-1) were introduced in loop3 (L3) and loop4 (L4). (B-C)
870 Measurement of membrane porosity of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*:
871 pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*:
872 pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1, and *ΔompA*: pQE60-*ompA*-L4-1 in acidic F
873 media (12 hours post-inoculation) using DiBAC4 (final concentration- 1 μg/ mL) by flow
874 cytometry. Unstained bacterial cells were used as control (Autofluorescence). Both dot plots
875 (SSC-A vs. DiBAC4) and histograms (Count vs. DiBAC4) have been represented. The percent
876 population of DiBAC4 positive bacterial cells has been represented here. Data are represented
877 as mean ± SEM (n=5, N=2).

878 ***(P) *< 0.05*, *(P) **< 0.005*, *(P) ***< 0.0005*, *(P) ****< 0.0001*, ns= non-significant**

879 **Figure 7.**

880 **Mutation in the extracellular loops of *Salmonella* Typhimurium OmpA reduces the**
881 **retention of LAMP-1 around the bacteria in murine macrophages.**

882 RAW264.7 cells were infected with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*:
883 pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*:
884 pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 at MOI of
885 20. Cells were fixed at (A) 16 hours (late phase) post-infection. Intracellular *Salmonella* was
886 stained with rabbit-raised anti-*Salmonella* O primary antibody. LAMP-1 was labeled with rat-
887 raised anti-mouse LAMP-1 primary antibody. The quantification of LAMP-1 recruitment on
888 bacteria in RAW 264.7 cells at 16 hours post-infection has been represented in a graph. (B)

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889 During the late stage of infection (16 hours post-infection), the percent colocalization of
890 bacteria with LAMP-1 was determined after analyzing 100 different microscopic fields from
891 three independent experiments [n=100, N=3]. Scale bar = 5μm. (C) Intracellular survival of
892 STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-
893 *ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-
894 L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 (MOI-10) in RAW264.7 cells (16 hours post-infection).
895 The bacteria's fold proliferation was calculated by normalizing the CFU at 16 hours to CFU at
896 2 hours (n=3, N=3).

897 ***(P)* *< 0.05, *(P)* **< 0.005, *(P)* ***< 0.0005, *(P)* ****< 0.0001, ns= non-significant,**
898 **(Student's *t*-test).**

899 **Figure 8.**

900 **The mutation in the extracellular loops of OmpA is not sufficient to send the cytosolic**
901 **population of *Salmonella* to the lysosomal degradation pathway.**

902 (A) RAW 264.7 cells were pre-treated with Texas red ovalbumin for 30 minutes and infected
903 with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*:
904 pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*:
905 pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 respectively, at MOI of 20. Rabbit-raised
906 anti-*Salmonella* O primary and anti-rabbit dylight 488 secondary antibodies were used to stain
907 the intracellular bacteria. (B) The percent colocalization of bacteria with lysosome have been
908 represented in the form of a graph. The percent colocalization between bacteria and texas red
909 was determined after analyzing 100 microscopic stacks from two independent experiments
910 (n=100, N=2). Scale bar = 5μm.

911 ***(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

912 **Figure 9.**

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913 **The hypothetical working model of activation of autophagy upon SCV damage by STM**
914 ***ΔompA*.**

915 (A) After entering the host cell, STM (WT) stays inside the early endosomes. As time passes,
916 EEA1 (early endosomal marker) is replaced with LAMP-1 (SCV marker). STM (WT) staying
917 inside SCV can down-regulate lysosome biogenesis and suppress host autophagy machinery in
918 SPI-2 encoded T3SS dependent manner. The down-regulation of lysosome biogenesis
919 facilitates the successful proliferation of the bacteria inside macrophages. (B) Intracellular
920 STM *ΔompA* is unable to retain LAMP-1 and comes into the cytosol after damaging SCV
921 during the immediate early stage of infection. The extracellular loops of *Salmonella* OmpA
922 play an essential role in maintaining the interaction between SCV and LAMP-1. The cytosolic
923 population of STM *ΔompA* activates host autophagy machinery. After being colocalized with
924 syntaxin 17 and LC3B, a significant fraction of cytosolic STM *ΔompA* is targeted to lysosomal
925 degradation.

926 ## Supplementary Figures

927 **Figure S1.**

928 **The damage of SCV by STM *ΔompA* activates host autophagy machinery.**

929 (A) RAW264.7 cells were infected with STM (WT): RFP and *ΔompA*: RFP at MOI of 20. Cells
930 were fixed at 16 hours post-infection. LAMP-1 and syntaxin 17 were labeled with rat-raised
931 anti-mouse LAMP-1 and rabbit-raised anti-mouse syntaxin 17 primary antibodies,
932 respectively. (B) The quantification of LAMP-1 and syntaxin 17 recruitment on bacteria in
933 RAW 264.7 cells have been represented in the form of a graph. Percent colocalization was
934 determined after analyzing more than 150 different microscopic stacks from two independent
935 experiments. Scale bar = 5μm, [n≥150, N=2].

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936 ***(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

937 **Figure S2.**

938 **Activation of host autophagy machinery targets the *ompA* deficient *Salmonella* to the**
939 **lysosome.**

940 To stain the lysosomes, RAW 264.7 cells were pre-treated with Texas red ovalbumin for 30
941 minutes and infected with STM (WT): *GFP* and *ΔompA*: *GFP,* respectively, at MOI of 20.
942 Host syntaxin 17 was labeled with rabbit-raised anti-mouse syntaxin 17 primary and anti-rabbit
943 alexa fluor 647 secondary antibodies, respectively. (H) The colocalization of bacteria with
944 lysosomes and syntaxin 17 has been represented in the form of a graph. The percent
945 colocalization of the bacteria with texas red and syntaxin 17 were determined after counting
946 more than 50 microscopic stacks from two independent experiments (n≥ 50, N=2). Scale bar =
947 5μm.

948 ***(P)* **< 0.005, *(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

949 **Figure S3.**

950 **STM *ΔompA* quits the SCV in human monocyte-derived macrophages before the early**
951 **stage of infection.**

952 PMA activates U937 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at MOI of
953 20. Cells were fixed at (A) 2 hours (early phase), (C) 6 hours (middle phase), and (E) 16 hours
954 (late phase) post-infection & LAMP-1 were labeled with anti-human LAMP-1 antibody. The
955 quantification of LAMP-1 recruitment on bacteria in U937 cells at (B) 2 hours, (D) 6 hours,
956 and (F) 16 hours post-infection has been represented in the form of three graphs. (B) During
957 the early stage of infection (2 hours post-infection), the percent colocalization of bacteria with
958 LAMP-1 was determined after analyzing more than 40 different microscopic stacks from two

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959 independent experiments [n≥40, N=2]. (D) During the middle stage of infection (6 hours post-
960 infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing
961 more than 30 different microscopic stacks from two independent experiments [n≥30, N=2]. (F)
962 During the late stage of infection (16 hours post-infection), the percent colocalization of
963 bacteria with LAMP-1 was determined after analyzing more than 50 different microscopic
964 stacks from two independent experiments [n≥50, N=2]. Scale bar = 5µm.

965 ***(P)* *< 0.05, *(P)* **< 0.005, *(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

966 **Figure S4.**

967 **The inability of STM *ΔompA* to retain LAMP-1 does not depend upon the production of**
968 **SipC.**

969 RAW264.7 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at MOI of 20. (A)
970 Cells were fixed at 2 hours post-infection & SPI-1 effector protein SipC produced by
971 intracellular *Salmonella* was labeled with anti-mouse SipC antibody. (B) The quantification of
972 SipC arrangement around the bacteria in RAW264.7 cells at 2 hours post-infection has been
973 represented in the form of a graph. (B) The percent colocalization of bacteria with LAMP-1
974 was determined after analyzing 100 different microscopic stacks from two independent
975 experiments [n=100, N=2]. Scale bar = 5µm. (C) The transcript level expression of *sipC* in
976 STM (WT) and *ΔompA* growing intracellularly in RAW264.7 cells 12 hours post-infection
977 (n=5, N=3).

978 **ns= non-significant, (Student's *t*-test).**

979 **Figure S5.**

980 **Mutation in the extracellular loops of *Salmonella* Typhimurium OmpA reduces the**
981 **retention of LAMP-1 around the bacteria in murine macrophages.**

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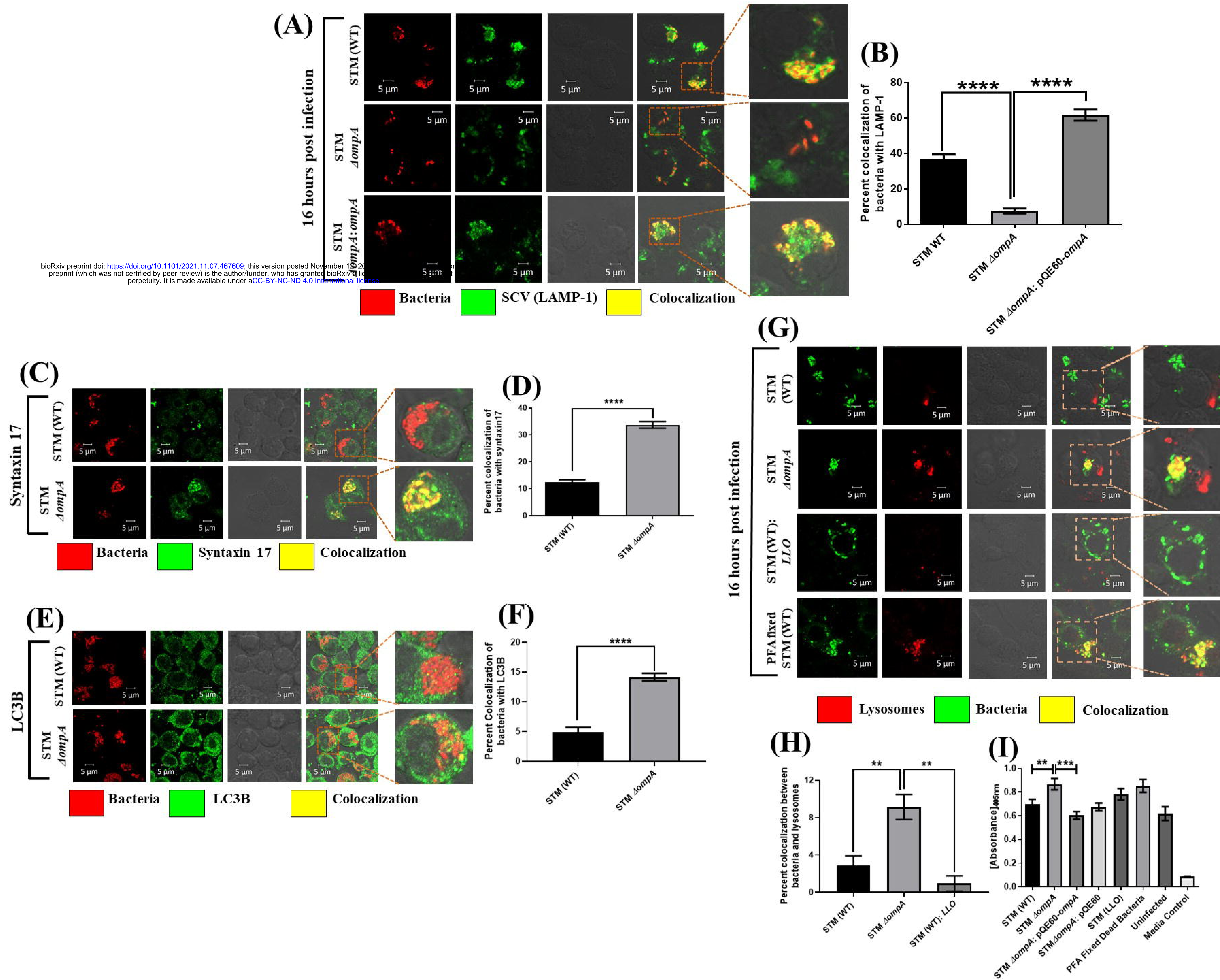
982 RAW264.7 cells were infected with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*:
983 pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*:
984 pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 at MOI of
985 20. Cells were fixed at (A) 6 hours (late phase) post-infection. Intracellular *Salmonella* was
986 stained with rabbit-raised anti-*Salmonella* O primary antibody. LAMP-1 was labeled with rat-
987 raised anti-mouse LAMP-1 primary antibody. The quantification of LAMP-1 recruitment on
988 bacteria in RAW 264.7 cells at 6 hours post-infection has been represented in the form of a
989 graph. (B) During the late stage of infection (6 hours post-infection), the percent colocalization
990 of bacteria with LAMP-1 was determined after analyzing 100 different microscopic fields from
991 two independent experiments [n=100, N=2]. Scale bar = 5μm.

992 ***(P)* **< 0.005, *(P)* ****< 0.0001, ns= non-significant, (Student's *t*-test).**

993

994



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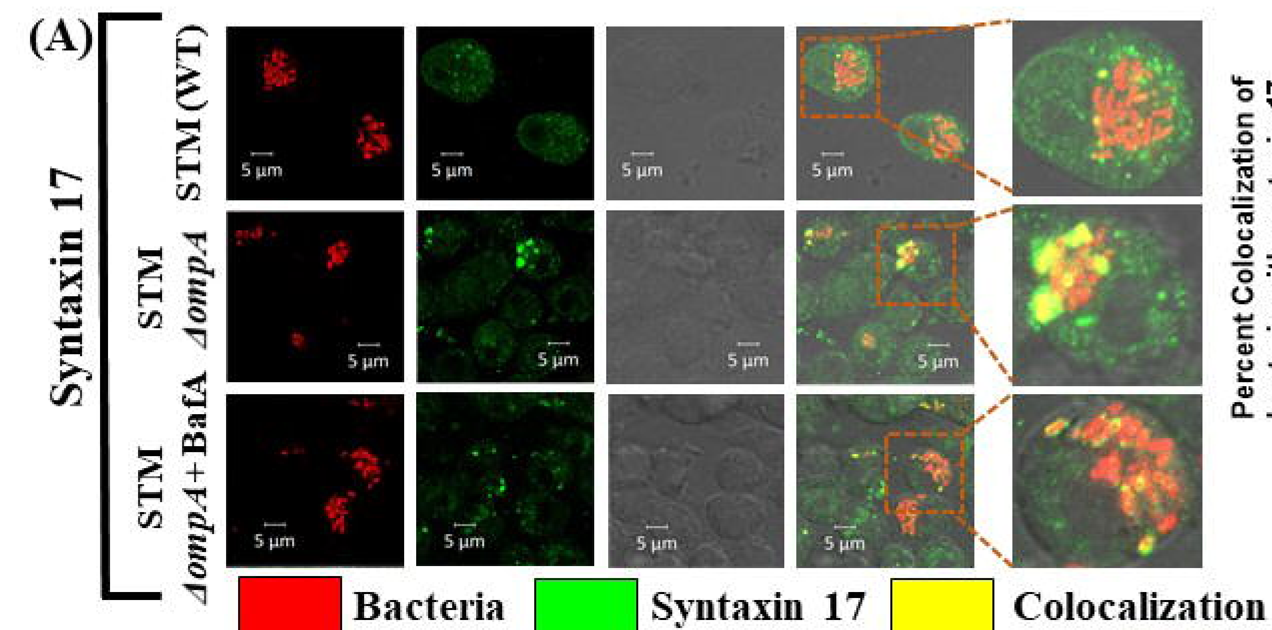
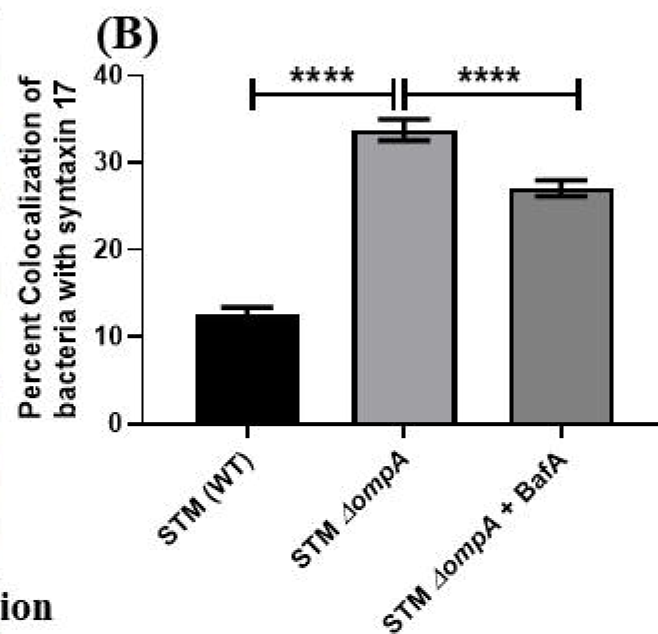
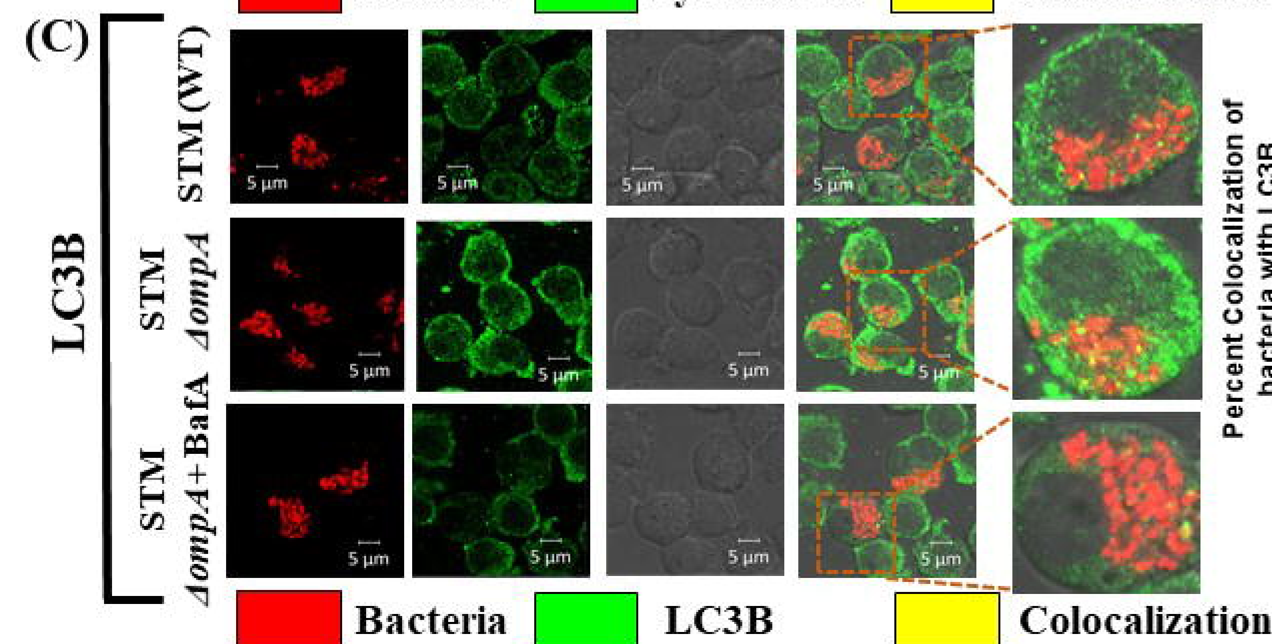


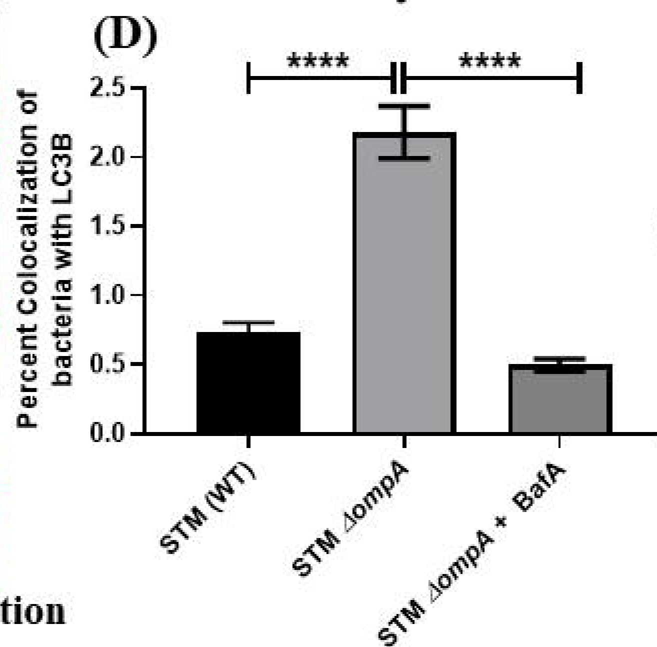
(A)
Syntaxin 17
STM (WT)
STM ΔompA
STM ΔompA+ BafA
5 µm
Bacteria
Syntaxin 17
Colocalization



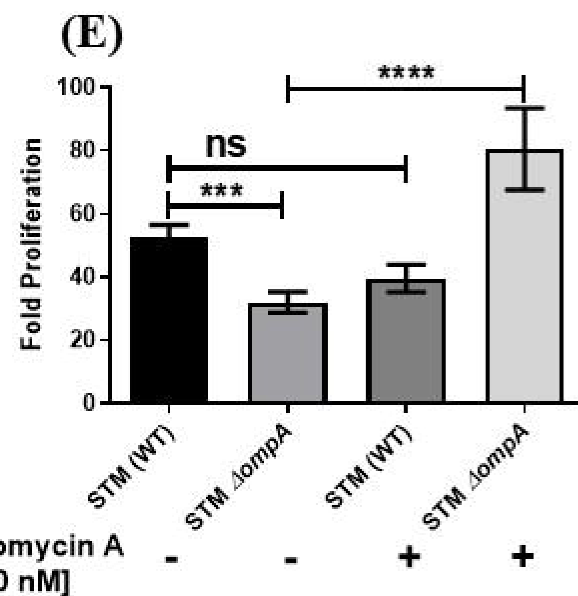
(B)
Percent Colocalization of bacteria with syntaxin 17
****
****
0
10
20
30
40
STM (WT)
STM ΔompA
STM ΔompA + BafA



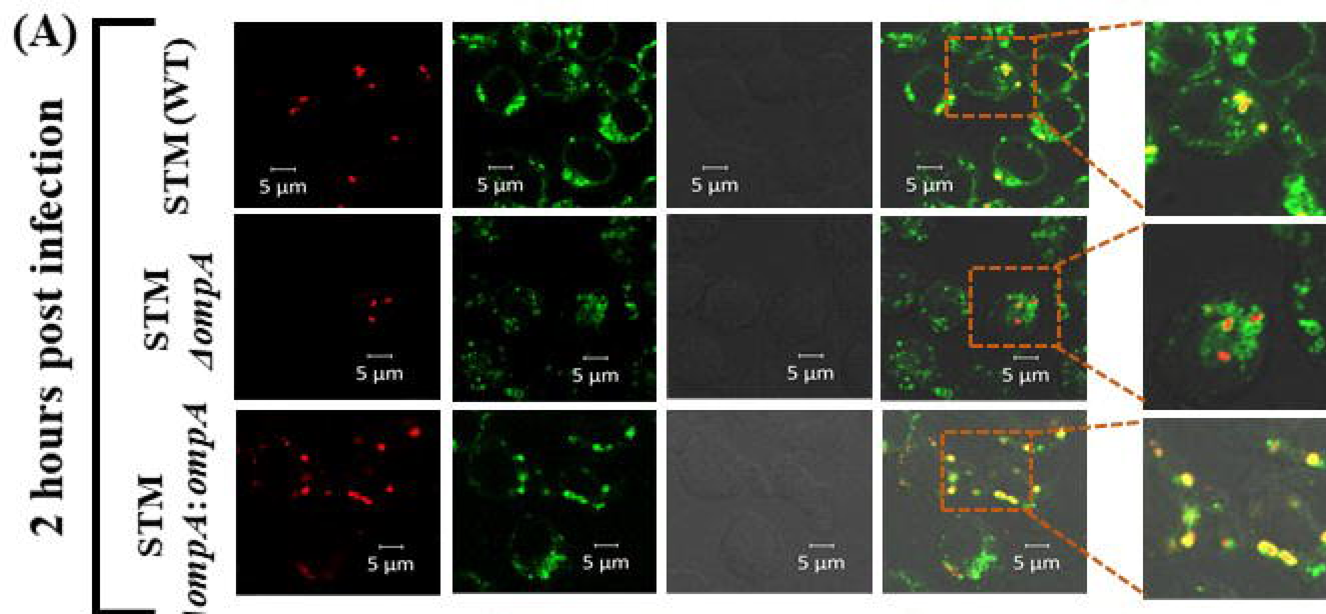
(C)
LC3B
STM (WT)
STM ΔompA
STM ΔompA+ BafA
5 µm
Bacteria
LC3B
Colocalization

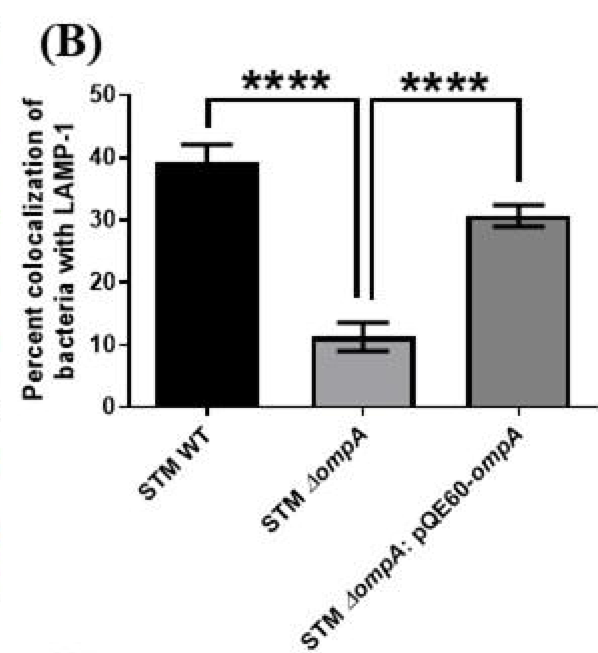


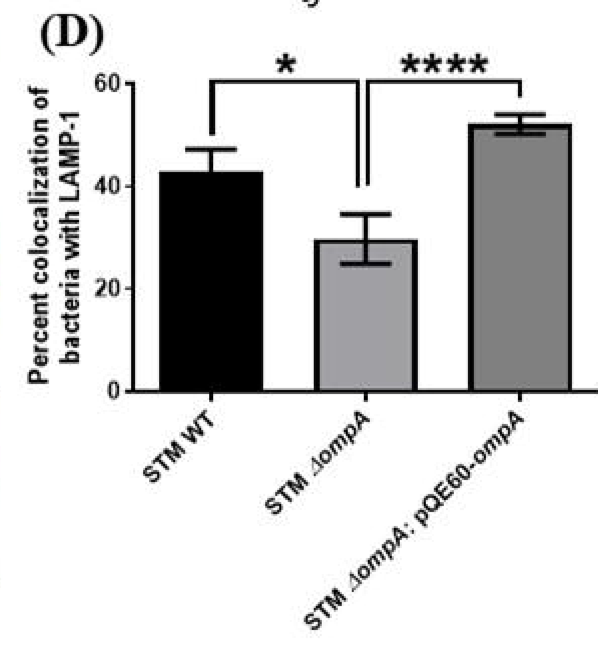
(D)
Percent Colocalization of bacteria with LC3B
****
****
0.0
0.5
1.0
1.5
2.0
2.5
STM (WT)
STM ΔompA
STM ΔompA + BafA

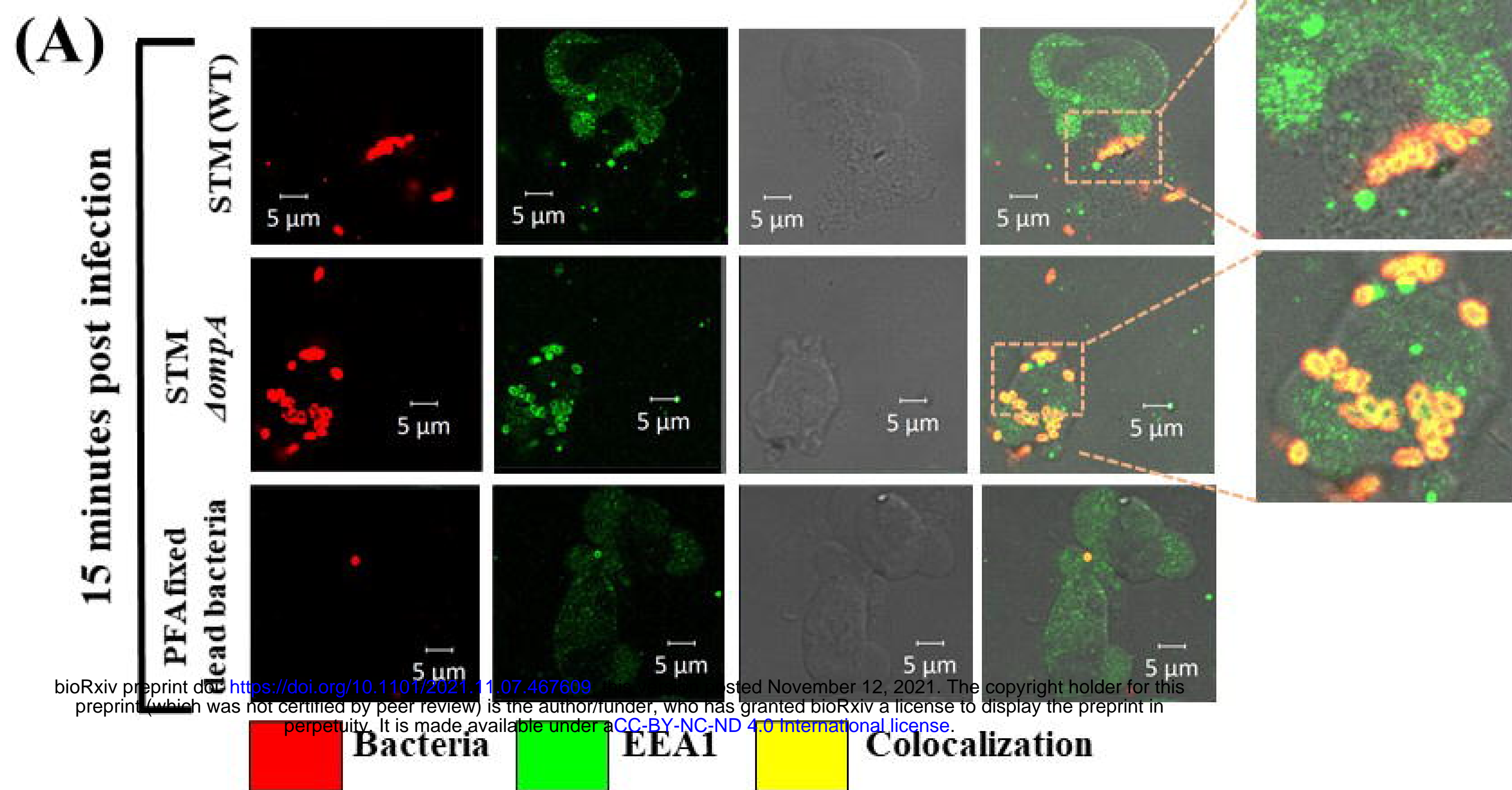


(E)
Fold Proliferation
****
ns
***
0
20
40
60
80
100
STM (WT)
STM ΔompA
STM (WT)
STM ΔompA
Bafilomycin A [50 nM]
-
-
+
+


(A)
2 hours post infection
STM (WT)
STM ΔompA
STM ΔompA:ompA
5 μm
(C)
6 hours post infection


(B)
Percent colocalization of bacteria with LAMP-1
****
****
0
10
20
30
40
50
STM WT
STM ΔompA
STM ΔompA: pQE60-ompA


(D)
Percent colocalization of bacteria with LAMP-1
*
****
0
20
40
60
STM WT
STM ΔompA
STM ΔompA: pQE60-ompA



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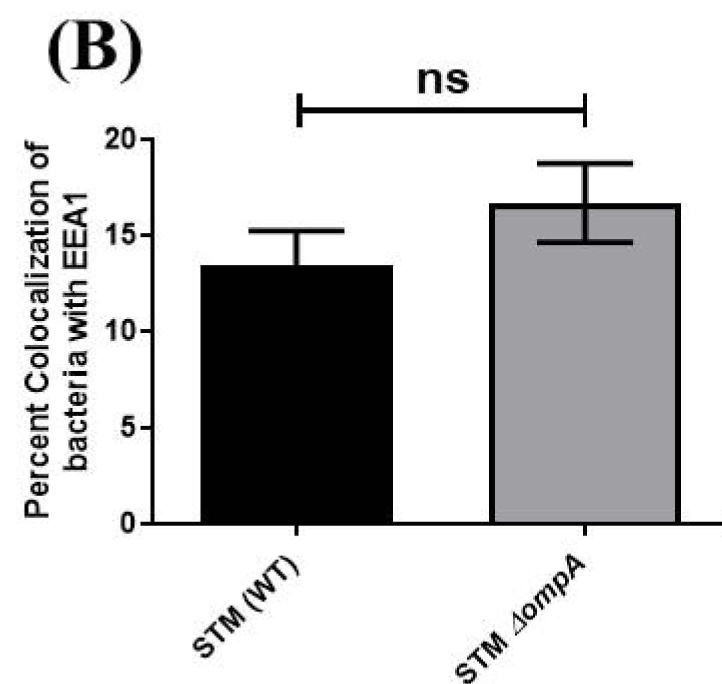
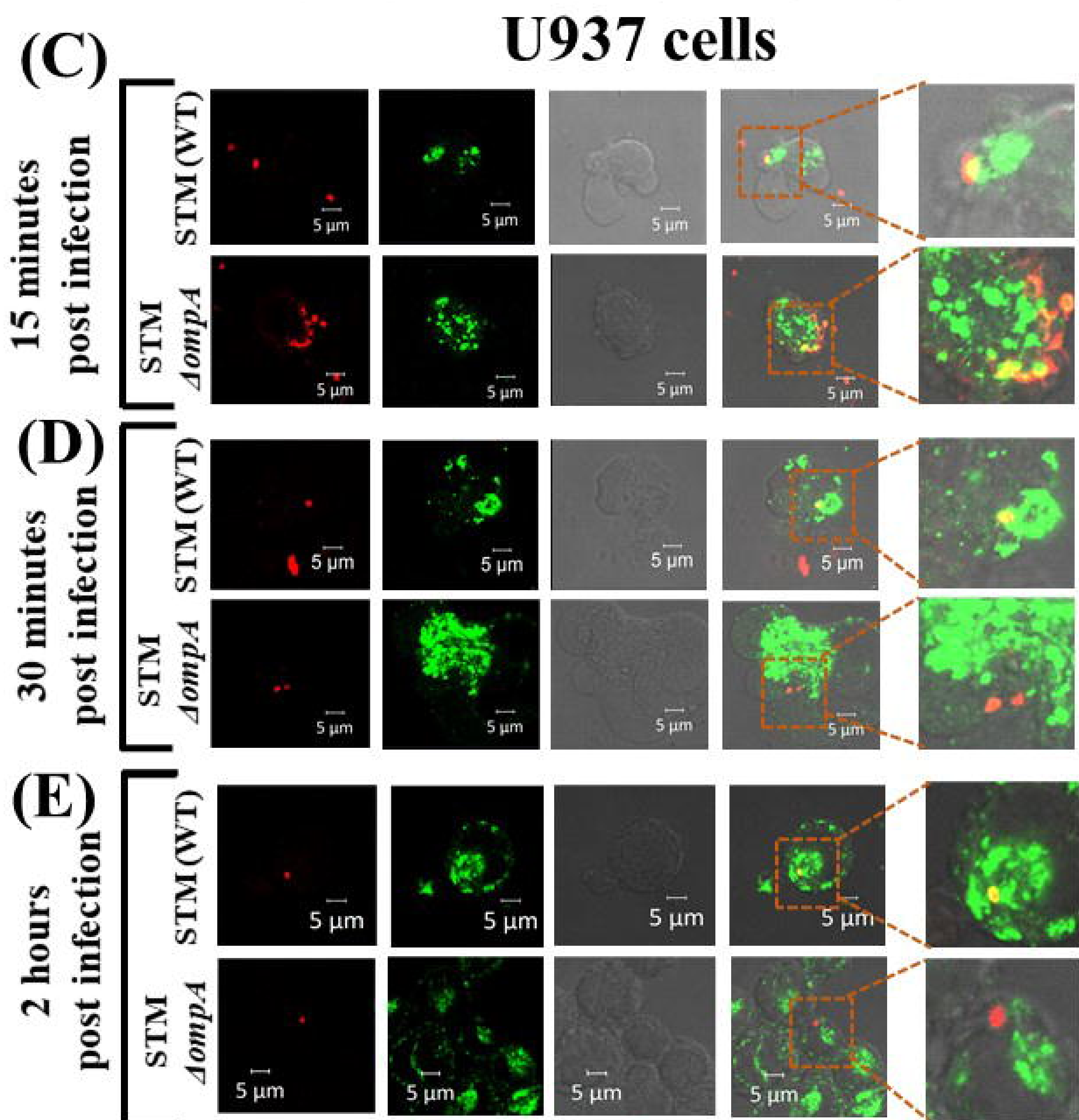
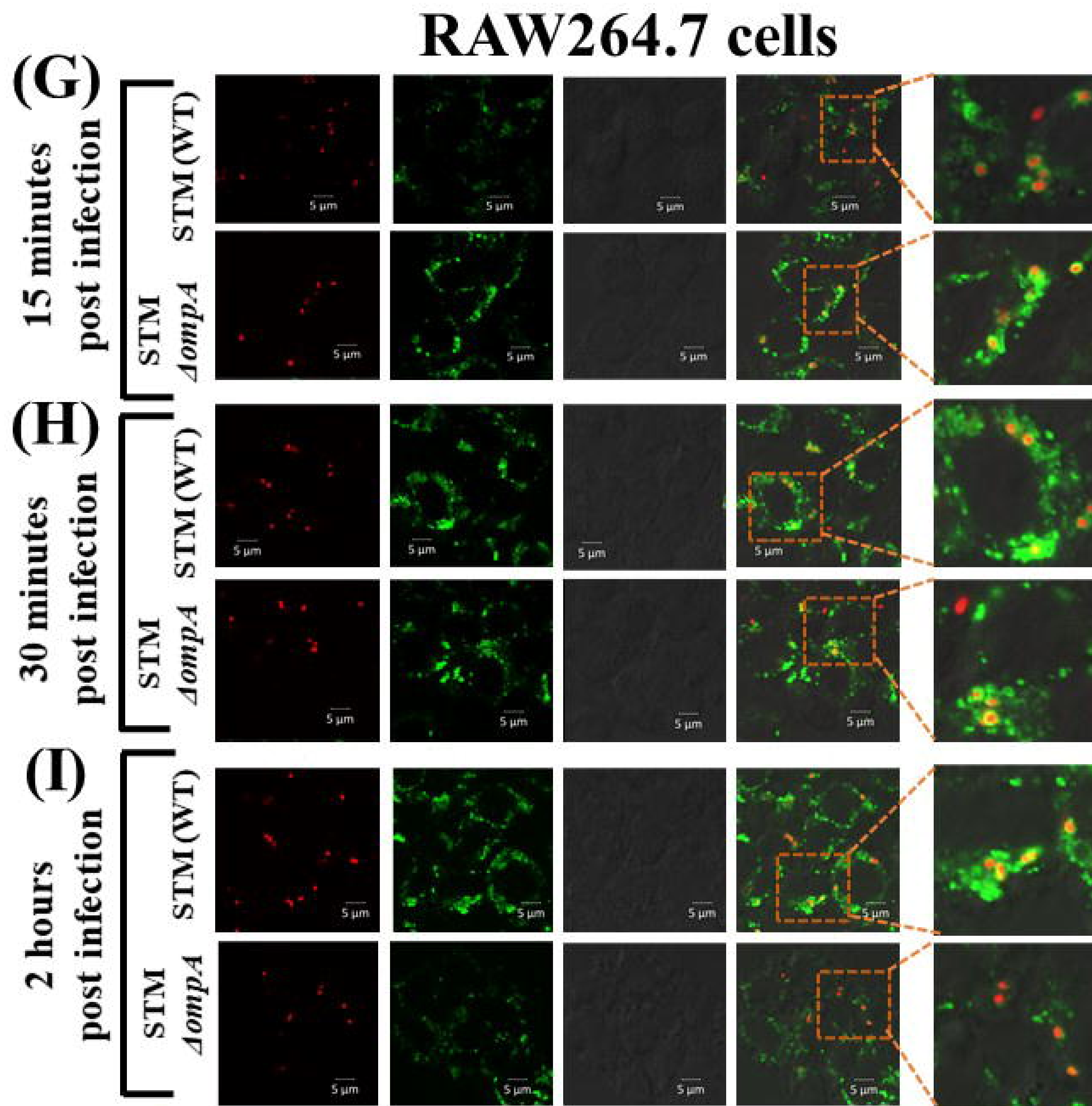
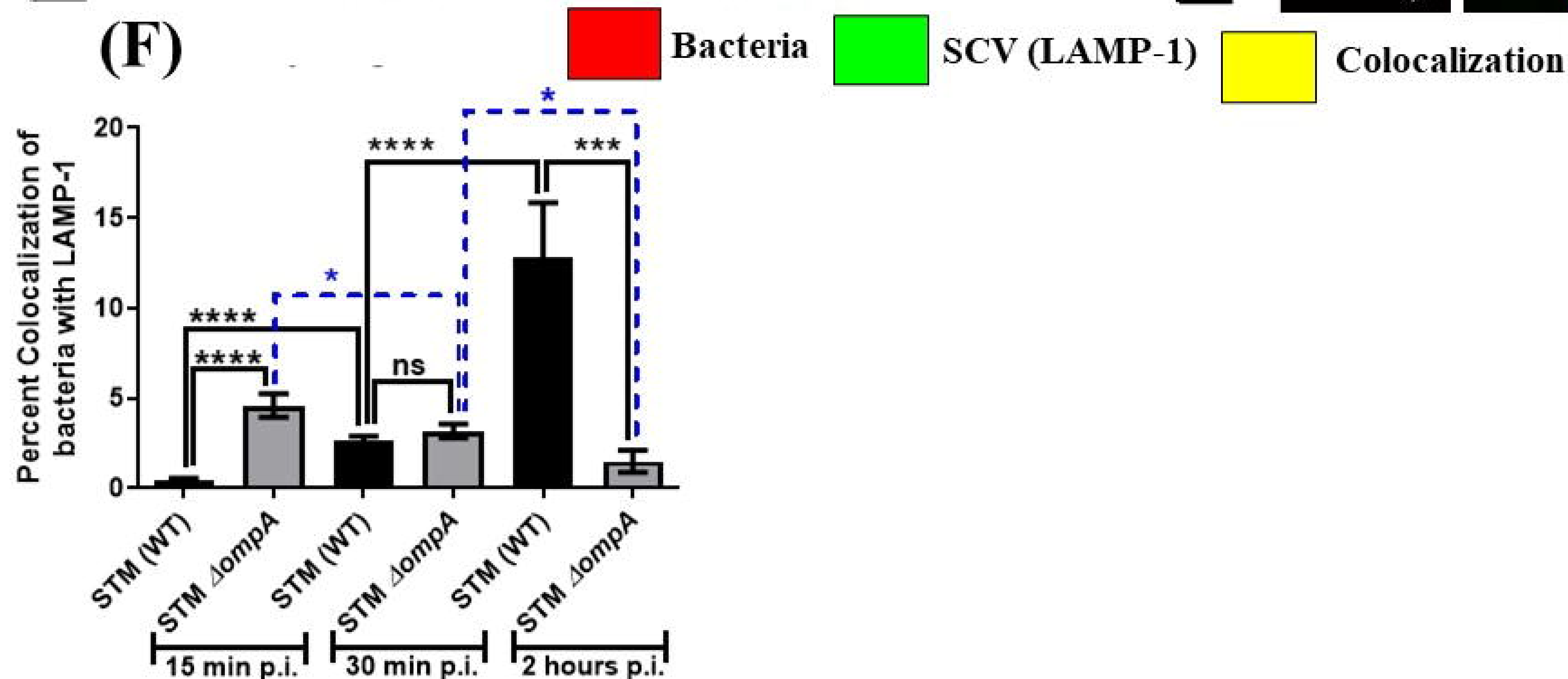
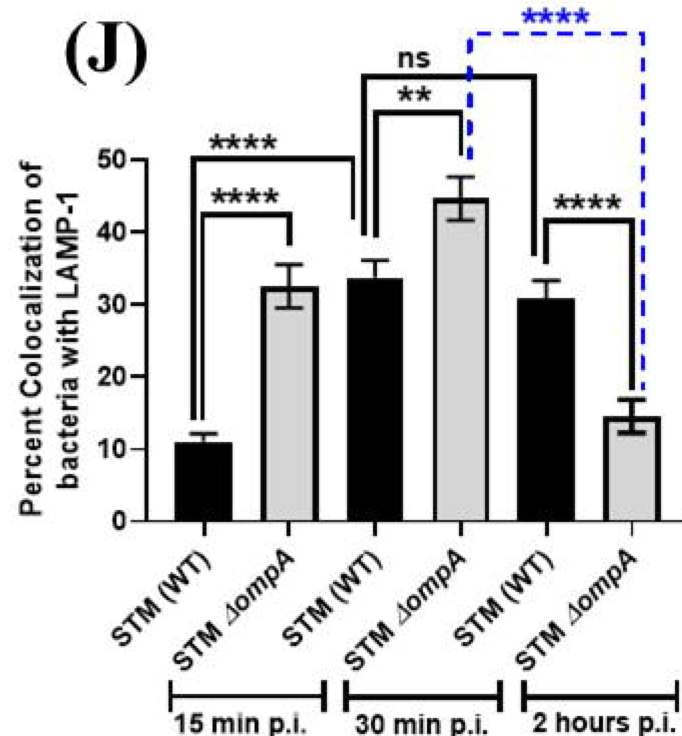


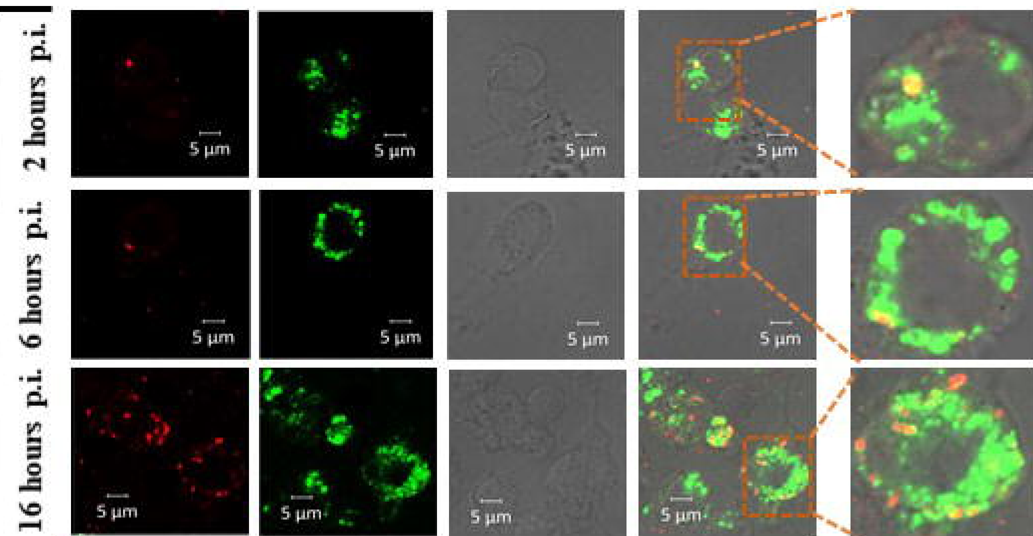


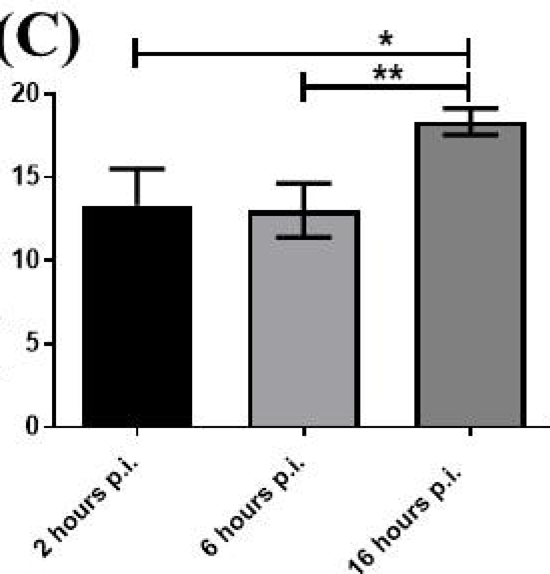


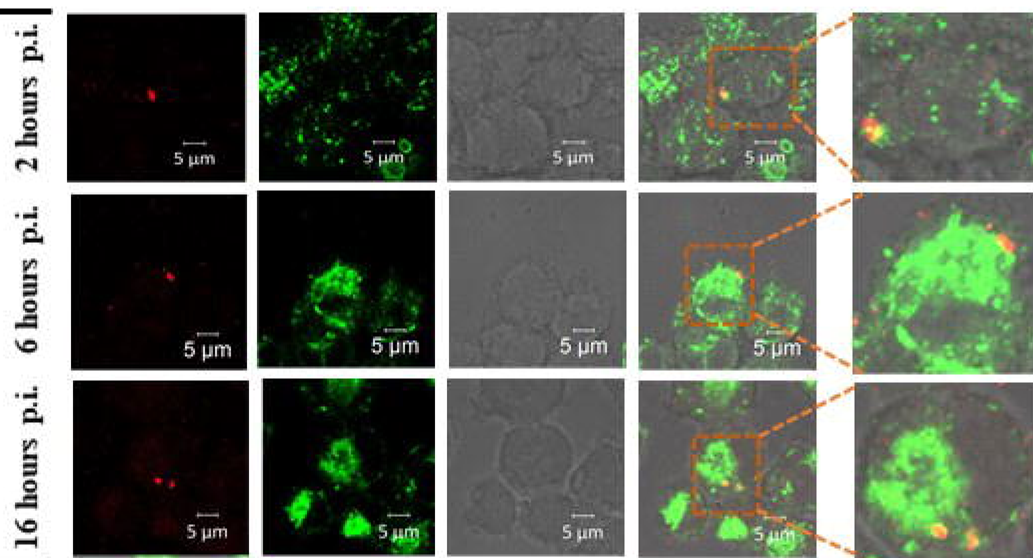


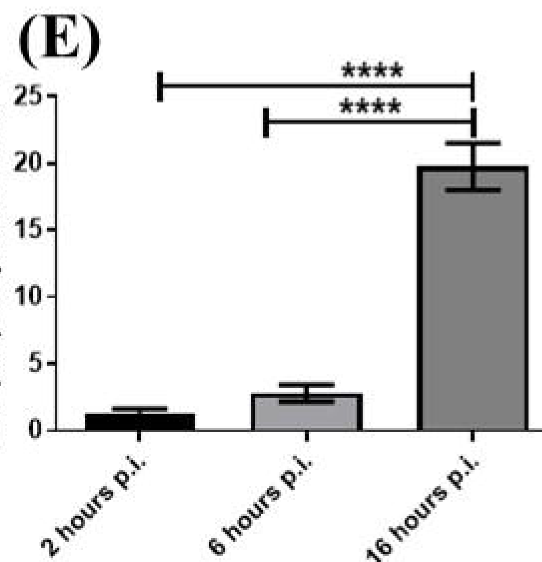


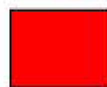
(A)
OmpA
[350 amino acids]

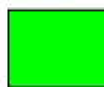

(B)
RAW264.7 cells
2 hours p.i.
6 hours p.i.
16 hours p.i.
5 µm

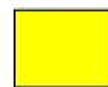

(C)
Percent Colocalization between STM (WT) OmpA and LAMP
0
5
10
15
20
2 hours p.i.
6 hours p.i.
16 hours p.i.
*
**

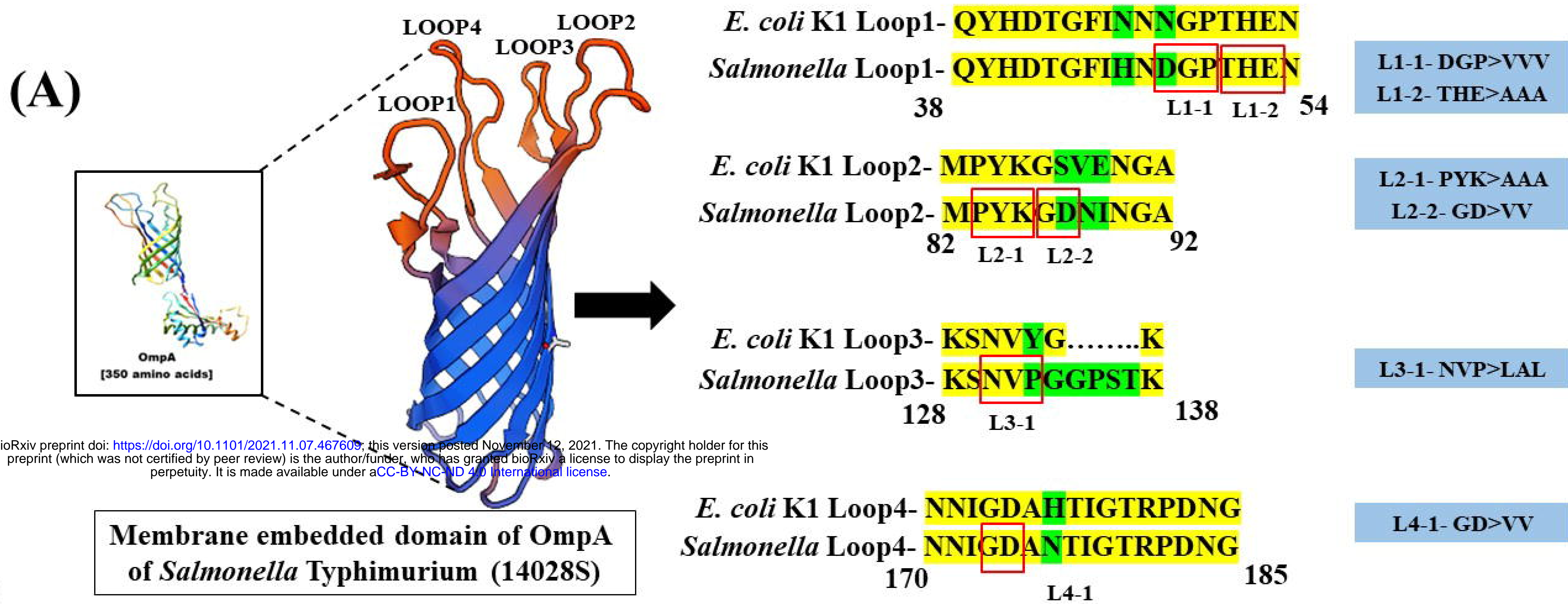

(D)
U937 cells
2 hours p.i.
6 hours p.i.
16 hours p.i.
5 µm


(E)
Percent Colocalization between STM (WT) OmpA and LAMP-1
0
5
10
15
20
25
2 hours p.i.
6 hours p.i.
16 hours p.i.
****
****


OmpA


SCV (LAMP-1)


Colocalization



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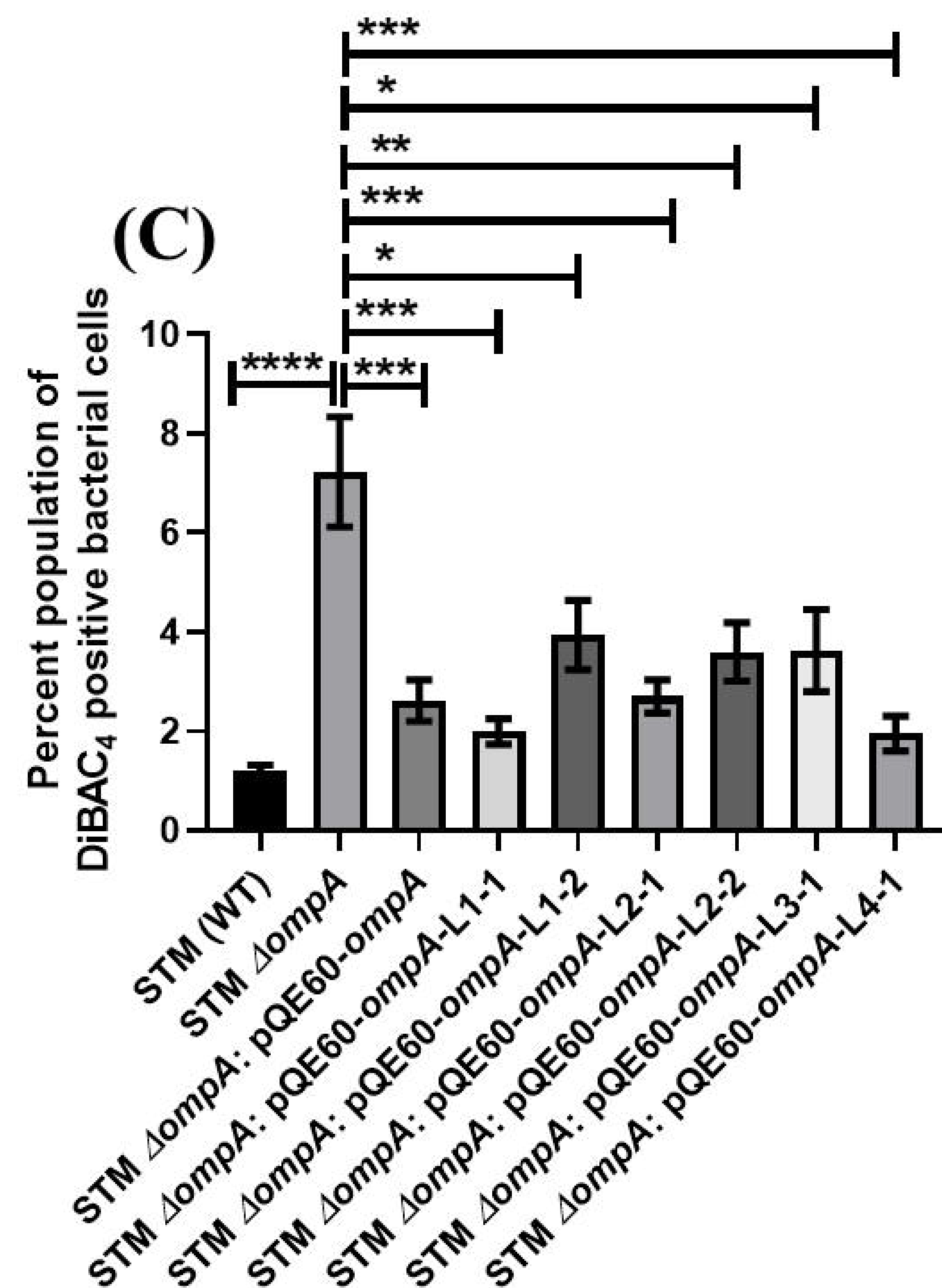
(B)

Autofluorescence 0.1%
STM (WT) 0.98%
STM ΔompA 11.01%
STM ΔompA: ompA 3.44%
STM ΔompA: ompA-L1-1 2.34%
STM ΔompA: ompA-L1-2 4.6%
STM ΔompA: ompA-L2-1 3.36%
STM ΔompA: ompA-L2-2 4.6%
STM ΔompA: ompA-L3-1 5.42%
STM ΔompA: ompA-L4-1 2.51%



(A)

16 hours post infection

STM (WT)

STM ΔompA

STM ΔompA: ompA

STM ΔompA: L1-1

STM ΔompA: L1-2

STM ΔompA: L2-1

STM ΔompA: L2-2

STM ΔompA: L3-1

STM ΔompA: L4-1

5 μm

Bacteria

SCV (LAMP-1)

Colocalization

(B)

Percent colocalization of bacteria with LAMP-1

0
10
20
30

****

STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60-ompA-L1-1
STM ΔompA: pQE60-ompA-L1-2
STM ΔompA: pQE60-ompA-L2-1
STM ΔompA: pQE60-ompA-L2-2
STM ΔompA: pQE60-ompA-L3-1
STM ΔompA: pQE60-ompA-L4-1

(C)

Fold Proliferation

0
50
100
150

**
ns
*

STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60-ompA- L1-1
STM ΔompA: pQE60-ompA- L1-2
STM ΔompA: pQE60-ompA- L2-1
STM ΔompA: pQE60-ompA- L2-2
STM ΔompA: pQE60-ompA- L3-1
STM ΔompA: pQE60-ompA- L4-1

(A)

16 hours post infection

STM (WT)

STM ΔompA

STM ΔompA: ompA

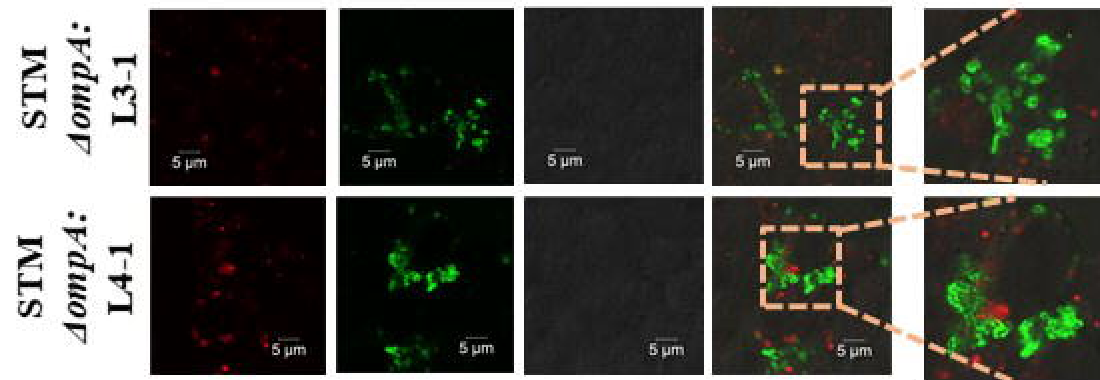
STM ΔompA: L1-1

STM ΔompA: L1-2

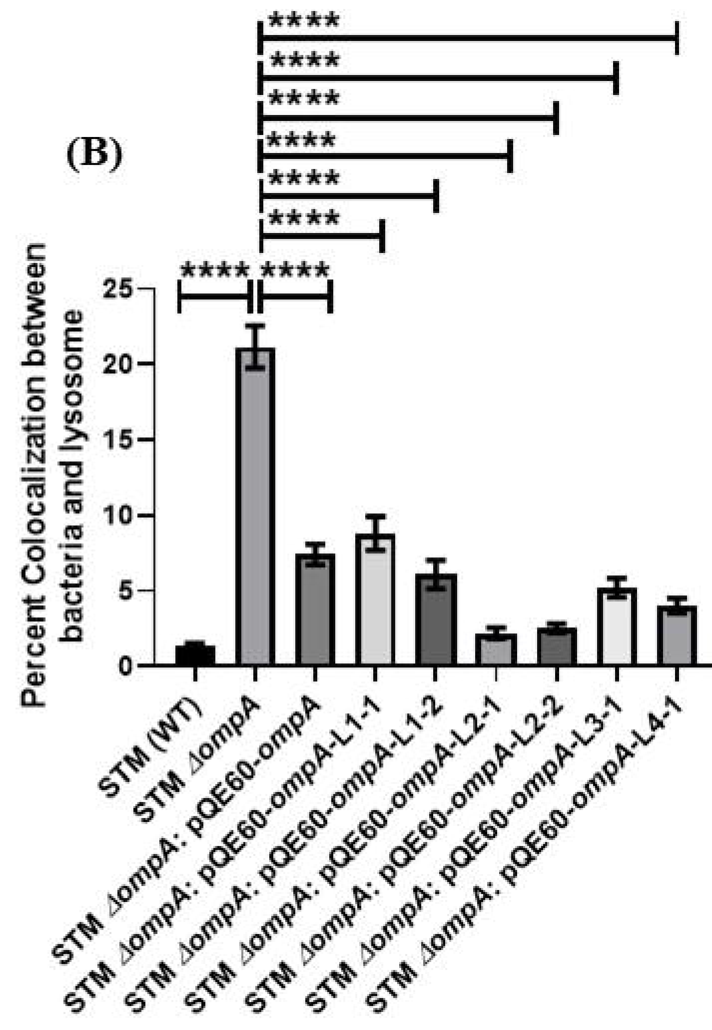
STM ΔompA: L2-1

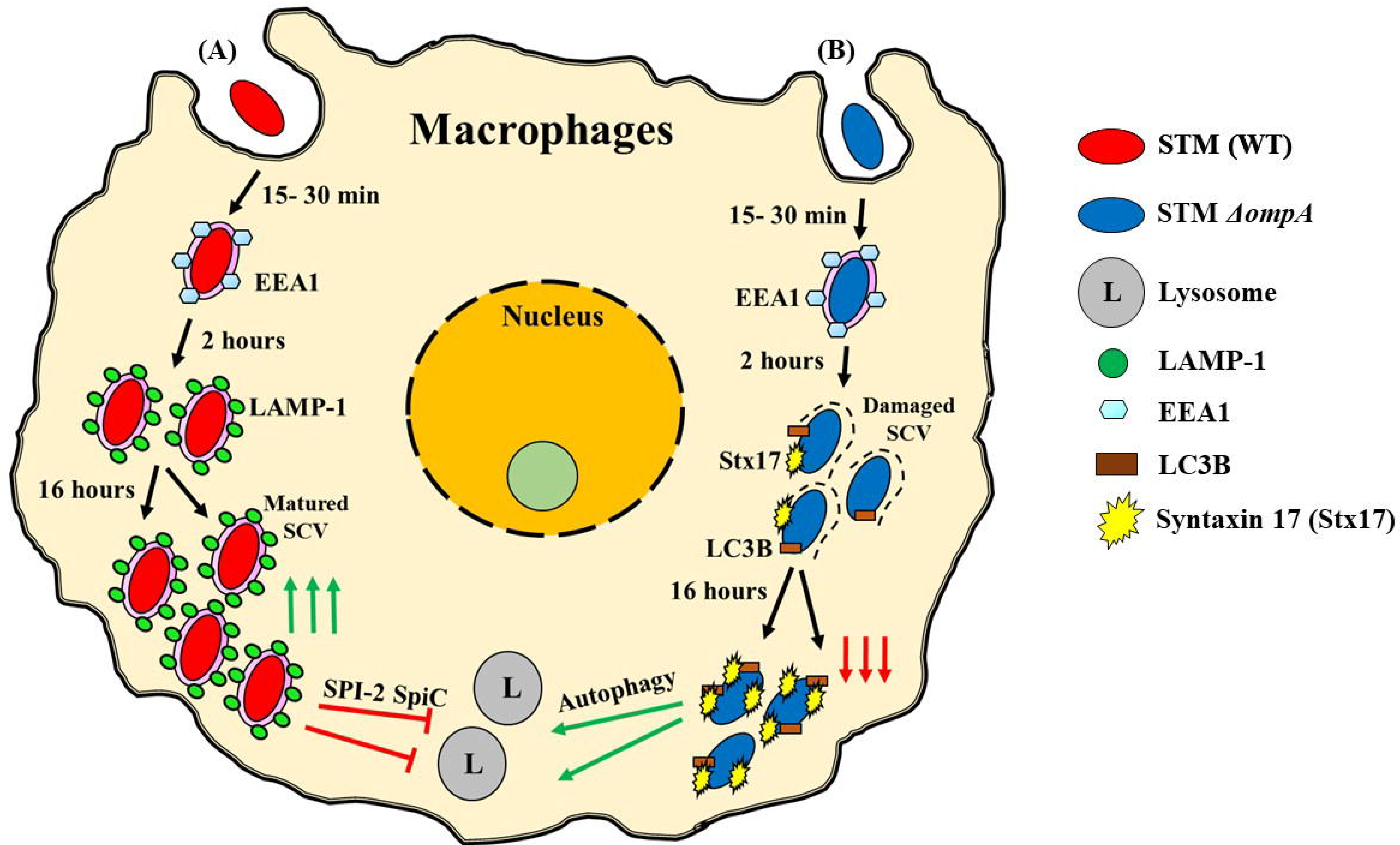
STM ΔompA: L2-2

5 µm



Lysosome Bacteria Colocalization




(A)
(B)
Macrophages
15- 30 min
EEA1
2 hours
LAMP-1
16 hours
Matured SCV
Nucleus
15- 30 min
EEA1
2 hours
Damaged SCV
Stx17
LC3B
16 hours
SPI-2 SpiC
L
L
Autophagy
STM (WT)
STM ΔompA
Lysosome
LAMP-1
EEA1
LC3B
Syntaxin 17 (Stx17)

(A)

A.1 A.3 A.5

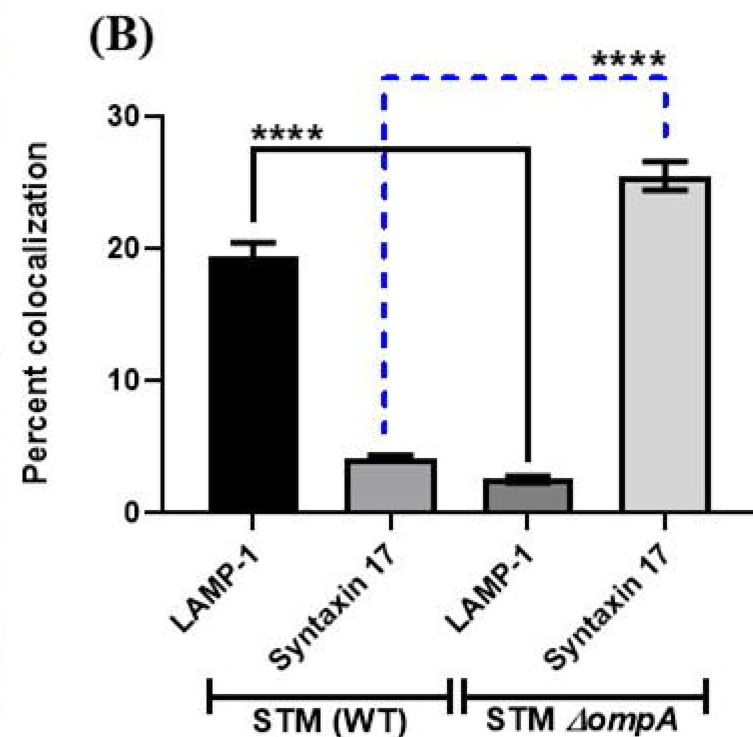
STM (WT)

5 µm 5 µm 5 µm 5 µm

STM *ΔompA*

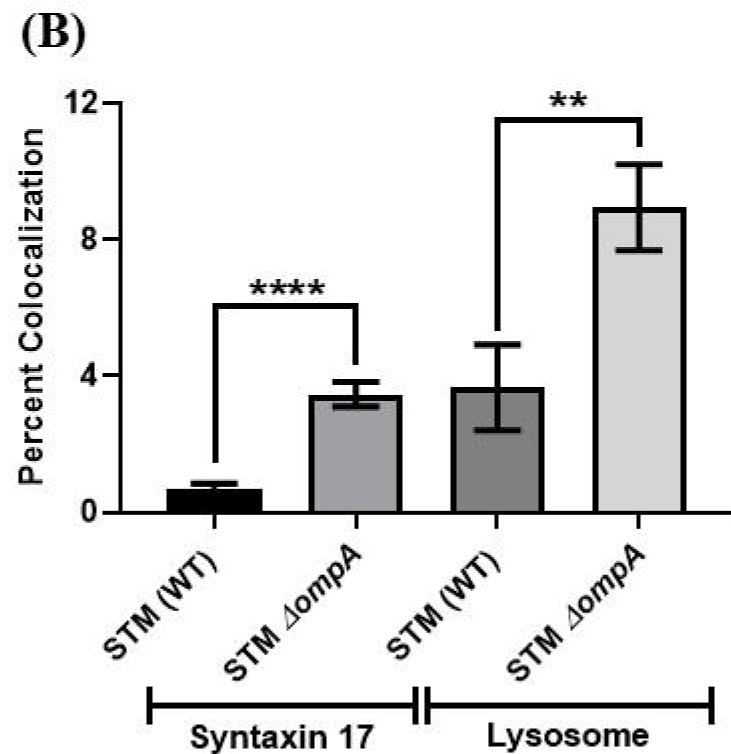
5 µm 5 µm 5 µm 5 µm

A.2 A.4 A.6

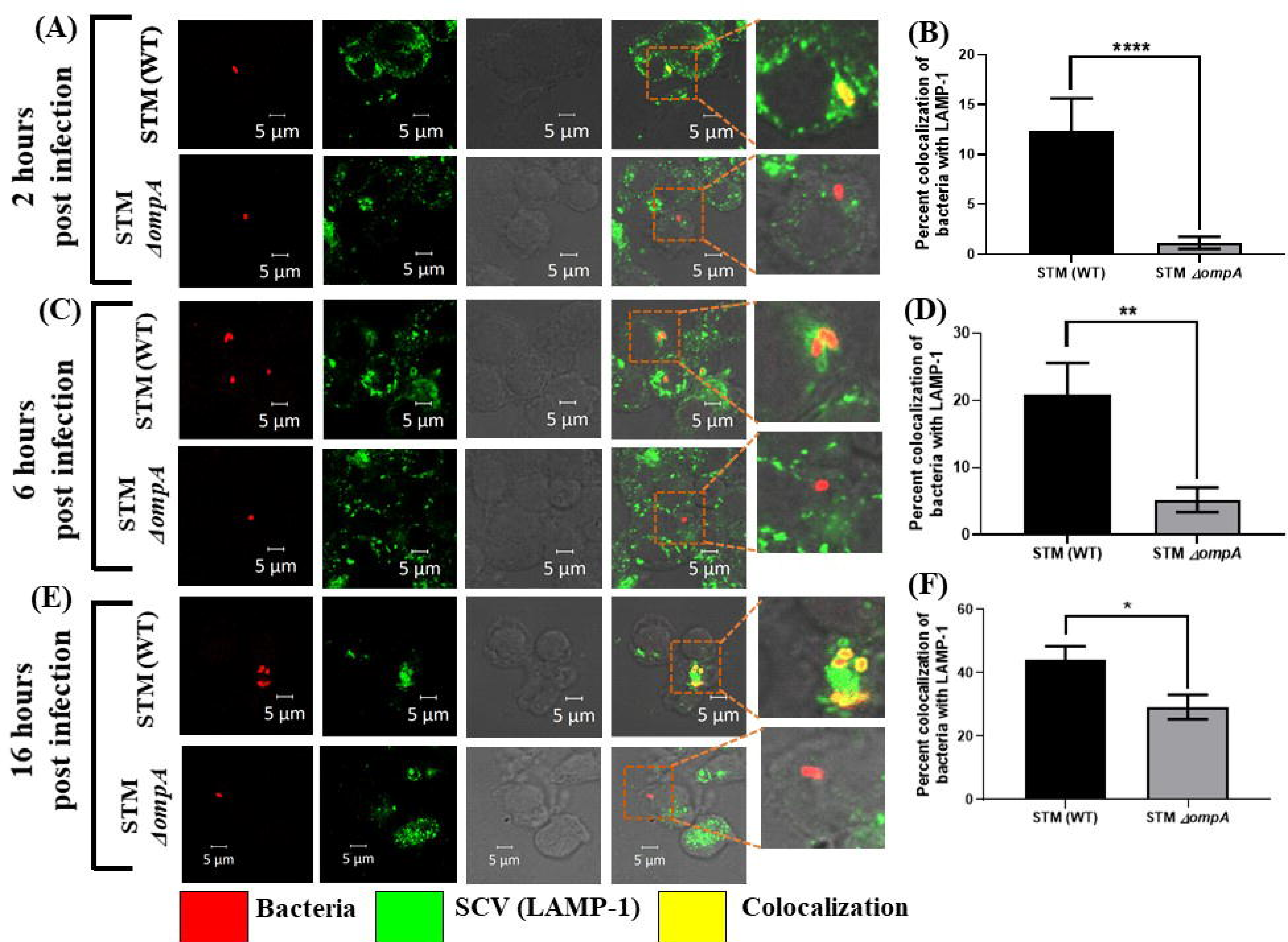


Salmonella LAMP-1 Syntaxin 17 Colocalization between bacteria and LAMP-1

Colocalization between bacteria and Syntaxin 17



Lysosome Bacteria Syntaxin 17 Colocalization between bacteria and lysosome



(A)
2 hours post infection
STM (WT)
STM ΔompA
5 µm
(B)
Percent colocalization of bacteria with LAMP-1
****
STM (WT)
STM ΔompA
(C)
6 hours post infection
(D)
**
(E)
16 hours post infection
(F)
*
Bacteria
SCV (LAMP-1)
Colocalization

(A)

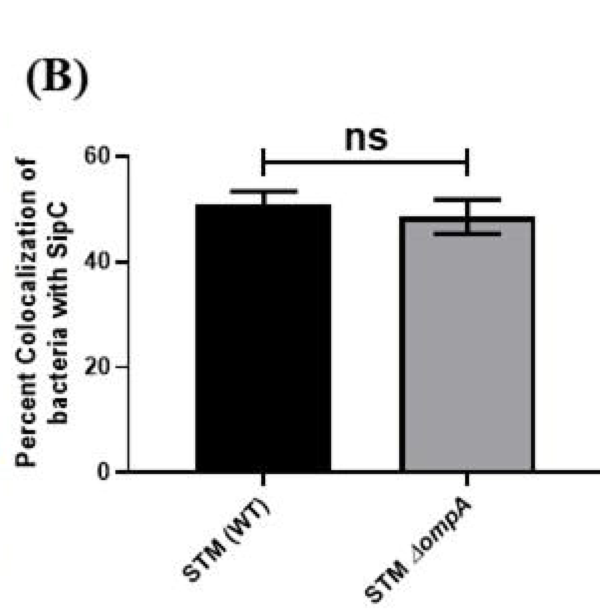
2 hours post infection

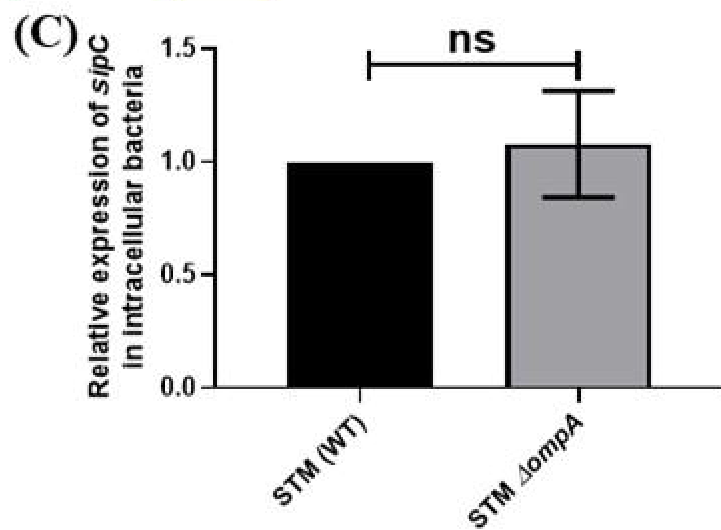
STM (WT)

STM *ΔompA*

5 μm

Bacteria SipC Colocalization





(A)

6 hours post infection

STM (WT)

STM ΔompA

STM ΔompA: ompA

STM ΔompA: L1-1

STM ΔompA: L1-2

STM ΔompA: L2-1

STM ΔompA: L2-2

Bacteria

SCV (LAMP-1)

Colocalization