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

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Abstract

After entering the host cells, *Salmonella* Typhimurium (STM) stays inside a modified acidic compartment called *Salmonella* containing vacuole (SCV). The biogenesis and stability maintenance of SCV are critical but inadequately understood areas. Our research has provided a novel mechanistic view on the role of a bacterial porin OmpA in maintaining the stability of SCV. We found that the deletion of OmpA forces the bacteria to escape from the SCV during the immediate early stage of infection. Within murine and human monocyte-derived macrophages, in the absence of OmpA, the bacteria were inefficient in retaining the LAMP-1, a standard SCV marker around itself. The cytosolic population of the mutant bacteria already left the SCV can activate the host autophagy machinery. On the contrary to the wild type bacteria confined within the SCV, STM *ΔompA* colocalizes more with autophagy markers syntaxin 17 and LC3B. Inhibition of autophagy pathway using bafilomycin A1 restored the intracellular proliferation of STM *ΔompA*. The activation of the autophagy pathway further targeted the autophagosomes carrying STM *ΔompA* to the lysosomes for degradation. We further showed that the four extracellular loops of OmpA exposed outside played a crucial role in retaining the LAMP-1 pool around the SCV. We have altered the extracellular loop sequences of OmpA by site-directed mutagenesis and observed that the bacteria could not hold the LAMP-1 pool around the SCV and slowly started losing the SCV membrane, which finally led to the release of the loop mutants into the cytosol of the host macrophages. We ultimately proved that the cytosolic population of STM having mutations in the OmpA extracellular loops doesn't activate the lysosomal degradation pathway like STM *ΔompA,* which helps them to survive within the murine macrophages.

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

Introduction

Salmonella Typhimurium (STM), the non-typhoidal serovar of the enteric pathogen *Salmonella enterica*, causes self-limiting diarrheal disease and gastroenteritis in humans [1]. It causes typhoid fever-like symptoms in mice and is considered an excellent model to study the pathogenesis of the human pathogen *Salmonella* Typhi. The pathogen enters the body of healthy individuals with contaminated food and water. The Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) estimated an occurrence of 5,35,000 cases of invasive non-typhoidal *Salmonella* infection worldwide, with approximately 77500 deaths in 2017 [2]. *Salmonella* Typhimurium can invade a wide range of host cells. After entering the host cells, *Salmonella* resides within a modified acidic compartment called *Salmonella* containing vacuole (SCV) [3-5]. The successful intracellular proliferation of the bacteria depends upon the formation and maintenance of intact SCV within the host cells. The disruption of SCV imposes a dramatic outcome on the fate of the intracellular bacteria. The leakage of SCV and subsequent pathogen release into the cytosol of phagocytic cells abrogate bacterial proliferation. In contrast, the cytosol of the non-phagocytic epithelial cells promotes bacterial proliferation [5-9]. *Salmonella* uses SPI-1 and SPI-2 encoded virulent factors to control the biogenesis of SCV. These SPI-encoded virulent effectors work in concert with host proteins to maintain the stability of SCV [4, 10-12]. However, the contributions of non-SPI virulent genes in the biogenesis and stability maintenance of SCV have been poorly understood.

Outer membrane protein A (OmpA) is a β barrel porin protein found on the outer membrane of *Salmonella* Typhimurium. It consists of eight anti-parallel β sheets, connected by four externally exposed extracellular loops and four periplasmic turns. Apart from its structural role, it protects *Salmonella* from oxidative stress by changing the outer membrane permeability [13]. The deficiency of OmpA compromises the biofilm-forming ability of the bacteria in response to bile salt stress [14]. In our previous study, we have proved that deletion of OmpA

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

In this current study, we addressed a novel role of *Salmonella* Typhimurium OmpA in maintaining SCV stability. We have shown that the cytosolic population of *Salmonella* lacking OmpA activates the host autophagy machinery and is cleared by the lysosomal degradative pathway. Our study further revealed a strong interaction between the OmpA of intracellular *Salmonella* with the host SCV marker LAMP-1. By introducing mutations in the externally exposed extracellular loops of OmpA, we dissected the role of *Salmonella* OmpA in modulating the intracellular vacuolar life of the pathogen. To the best of our knowledge, this is the first study illustrating the precise role of extracellular loops of an outer membrane porin of *Salmonella* Typhimurium (OmpA) in controlling the biogenesis and stability of SCV in macrophages.

Results

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

Salmonella Typhimurium is an intracellular vacuolar pathogen, which inhibits phagolysosome maturation after entering the host cells and resides within a modified compartment called SCV [4]. The successful systemic colonization by *Salmonella* Typhimurium depends upon forming a replicative vacuolar niche within the host cells [16]. However, the biogenesis of SCV within the host cells is a complicated phenomenon. *Salmonella* employs a plethora of proteins and virulent factors that work in conjunction with host factors for the biogenesis of SCV. Most of the studies that have addressed the intracellular behavior of *Salmonella* so far have discovered

the role of SPI-1 and SPI-2 encoded type 3 secretion systems (T3SS1 and T3SS2) and virulent factors in SCV biogenesis [17]. Earlier, our group has reported the role of outer membrane protein A in maintaining the stability of SCV within murine macrophages[15]. To validate our previous observation, the vacuolar niche of the wild type, *ompA* deficient, and complemented strains of *S*. Typhimurium in murine macrophages was checked. During the late phase of infection in macrophages, it was found that, unlike the wild type bacteria, STM *ΔompA* ceases to be within SCV and comes into the cytoplasm (**Figure 1A**). The poor colocalization of STM *ΔompA* with LAMP-1 (SCV marker) concomitantly supported the fact (Figure 4.1B). When the *ompA* gene was complemented in the knockout bacteria, there was a reversal in the vacuolar escaping phenotype (**Figure 1A and 1B**). Wild type *Salmonella* can recruit non-receptor tyrosine kinase or focal adhesion kinase on the surface of the SCV in an SPI-2 encoded T3SS2 dependent manner to suppress the host autophagy machinery by activating the Akt-mTOR signaling pathway [18, 19]. A previous study from our group proved that intracellular STM *ΔompA* is unable to produce and secrete the SPI-2 encoded translocon proteins into the host cell's cytosol [15], suggesting the formation of a malfunctioning T3SS2. Hence, we have hypothesized that the infection of murine macrophages with STM *ΔompA* may activate host autophagy machinery. Murine macrophages were infected with STM (WT) and *ΔompA* to check the recruitment of autophagy markers (syntaxin 17 and LC3B) around the bacteria during the late phase of infection. Syntaxin 17 is an autophagosomal SNARE protein with a unique C-terminal hairpin structure of two tandem trans-membrane domains mainly constructed with glycine zipper motifs. It interacts with the autophagosomal membrane [20]. Syntaxin 17 can interact with SNAP 29 and lysosomal SNARE protein VAMP8 [21]. It helps in the fusion of the autophagosome with lysosome and degradation of enclosed contents [22]. Microtubule-associated protein 1A/ 1B-light chain 3 (MAP-LC3/ LC3/ Atg8), which loads the cargo in the autophagosome, is considered as one of the important markers of autophagy. Usually, LC3B

is diffused throughout the cytosol. Upon autophagy initiation, LC3B is cleaved by cysteine protease Atg4 to form LC3B-I and further modified by the association of phosphatidylethanolamine to create LC3B-II. This lipidated form of LC3B (LC3B-II) forms distinct small puncta in the cytosol and helps to seal the membrane of autophagosome carrying cargo [23]. In line with our expectation, an increased recruitment of syntaxin 17 (**Figure 1C and 1D**) and LC3B (**Figure 1E and 1F**) was observed around STM *ΔompA* compared to the wild type bacteria during the late phase of infection in murine macrophages. Our data provided a considerable amount of evidences in the favour SCV damage by STM *ΔompA* which eventually activates host autophagy machinery in macrophages. To firmly support this conclusion, the co-staining of LAMP-1 and syntaxin 17 in murine macrophages infected with STM (WT) and *ΔompA* (**Figure S1A and S1B**) was performed. It was found that the wild type *Salmonella* staying within intact SCV (**Figure S1A.1**) can restrict the recruitment of syntaxin 17 around itself (**Figure S1A.3 and S1A.5**). In the contrary, the majority of intracellular STM *ΔompA* that hardly colocalize with LAMP-1 (**Figure S1A.2**) profoundly sequester syntaxin 17 (**Figure S1A.4 and S1A.6**). The better colocalization of autophagy markers with STM *ΔompA* (**Figure 1D and 1F**) suggested syntaxin 17^{+} $LC3B^{+}$ autophagosome formation around the mutant bacteria and subsequent activation of host autophagy machinery (xenophagy). Autophagy can target the pathogen trapped inside the autophagosome to the lysosome. This hypothesis was verified by measuring the activation and subsequent fusion of the lysosomes with wild type and *ompA* deficient strains of *Salmonella.* Before infecting the cells, the lysosomes were loaded with Texas red ovalbumin, and their colocalization with the intracellular pathogens was evaluated. Compared to STM (WT), the enhanced colocalization of STM *ΔompA* with Texas red (trapped inside lysosomes) supported our hypothesis (**Figure 1G and 1H**). As a control, STM (WT): *LLO*, a wild type bacterial strain that leaves SCV because of the expression of pore-forming toxin listeriolysin O from *Listeria monocytogenes,*

was used. The reduced colocalization of STM (WT): *LLO* with lysosomes explicitly proves the role of OmpA to prevent lysosomal fusion with the cytosolic pool of wild type bacteria (**Figure 1G and 1H**). To prove the subsequent activation of autophagy and lysosomal degradation pathway in macrophages infected with STM *ΔompA*, the cells were loaded with Texas red ovalbumin before infection, and host syntaxin 17 was stained with the appropriate antibody. It was found that unlike the wild type *Salmonella* (**Figure S2A.1, S2A.3, and S2A.5**), STM *ΔompA* heavily colocalizes with both syntaxin 17 (**Figure S2A.2**) and lysosomes (**Figure S2A.4 and S2A.6**). This suggests that the syntaxin 17^+ autophagosome carrying STM *ΔompA* is finally targeted to the lysosome for degradation. The enhanced activity of lysosomes upon ingestion of any cargo can be estimated by measuring the activity of lysosomal marker enzymes such as acid phosphatases [24, 25]. To support the previous observation depicting the intense activity of lysosomes upon infection of macrophages with STM *ΔompA*, the activity of lysosomal acid phosphatases was measured. The improved activity of acid phosphatases from the cells infected with STM *ΔompA* (**Figure 1I**) supported our pervious observation. The lysosomal acid phosphatase activity was found impaired when the cells were infected with the wild type and complemented strains of *Salmonella* (**Figure 1I**). Taken together, our data proves that during the late phase of infection in murine macrophages, STM *ΔompA* reaches the cytosol of the host cell and activates host autophagy machinery. As a result, the mutant bacteria trapped inside syntaxin 17^+, $LC3B^+$ autophagosome is targeted to the lysosomal degradation pathway, which might be a reason behind the clearance of the mutant bacteria from macrophages.

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

Earlier, it was found that when the macrophage cells were infected with STM *ΔompA*, there was an activation of the host autophagy machinery. This observation was strengthened by inhibiting the autophagy pathway using bafilomycin A1, a macrolide antibiotic isolated from

Streptomyces gresius that inhibits autophagy. Bafilomycin A1 inhibits the acidification of lysosomes by abrogating the vacuolar H^+ ATPase pump [26]. In the absence of bafilomycin A1, we have seen that a significant population of STM *ΔompA* colocalizes with autophagy marker syntaxin17 (**Figure 2A and 2B**) and LC3B (**Figure 2C and 2D**) compared to the wild type bacteria, which is consistent with our previous findings. This suggests the formation of an active autophagosome which can trap the mutant bacteria and restrict the infection within macrophages. When the cells were treated with bafilomycin A1 (50 nM), an impeded recruitment of both the autophagy markers (syntaxin 17 and LC3B) around STM *ΔompA* (**Figure 2A and 2C**) was found. Bafilomycin A1 treatment significantly reduced the colocalization between syntaxin 17 with STM *ΔompA* compared to the untreated macrophages infected with STM *ΔompA* (**Figure 2A and 2B**). On the other hand, a drastic abrogation in the formation of LC3B puncta around STM *ΔompA* under bafilomycin A1 treatment (**Figure 2C and 2D**) was also noted. Normally, STM *ΔompA* has been found to be deficient in replication in murine macrophages compared to wild type bacteria (**Figure 2E**). When bafilomycin A1 was used, a significant recovery in the proliferation of STM *ΔompA* compared to the bafilomycin A1 untreated infected cells was observed. The acidification of SCV helps in the intracellular proliferation of wild type *Salmonella* by inducing SPI-2 gene expression. Bafilomycin A1 non-specifically inhibits the acidification of the cells' acidic compartments, including both SCV and lysosomes. Hence it can slow down the growth of wild type bacteria residing within SCV, which can be considered as the reason behind the unaltered proliferation of STM (WT) under bafilomycin A1 treatment (**Figure 2E**). Taken together, our data illustrate that *Salmonella* Typhimurium outer membrane protein A protects the bacteria against host autophagy machinery by improving the intracellular vacuolar life of the pathogen.

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

We further wanted to find out when the bacteria escape the SCV and come into the cytosol of macrophages. To answer this question, both RAW264.7 cells and PMA activated U937 cells were infected with wild type, *ompA* mutant, and complemented strains of *Salmonella* Typhimurium and investigated the vacuolar niche of the pathogen during early (2h post-infection) and middle (6h post-infection) stages of infection. It was found that compared to the STM (WT), the colocalization of STM *ΔompA* with LAMP-1 is significantly less at 2 hours post-infection in RAW 264.7 cells (**Figure 3A and 3B**), stating that the knockout bacteria leave the vacuole even before the early stage of infection in macrophages. The reduced colocalization between LAMP-1 and the mutant bacteria was restored in the complemented strain (**Figure 3A and 3B**). Similarly, the poor colocalization of STM *ΔompA* with LAMP-1 at 6 hours post-infection suggests that most of the *ompA* knockout bacteria that previously quit the vacuole at 2 hours post-infection remain in the cytoplasm during the middle stage of infection in macrophages (**Figure 3C and 3D**). To inspect whether this phenotype is cell type-specific or not, the same experiment was carried out in human monocyte-derived macrophages (U937 cells). In accordance with our previous observation in murine macrophages, here in U937 cells also the STM *ΔompA* has a higher propensity towards abandoning the SCV before the early stage of infection than STM (WT) (**Figure S3A – S3F**). Like murine macrophages, STM *ΔompA* could not maintain a consistent vacuolar niche during the middle and late phases of the disease in monocyte-derived human macrophages.

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

To discover the exact time point at which the bacteria abandon SCV while infecting macrophages, the pattern of LAMP-1 recruitment around the mutant and wild type bacteria was studied at 15- and 30-minutes post-infection in macrophages. The formation of SCV inside the host cell is a dynamic process. Immediately after entering the cells, the wild type *Salmonella* residing within early SCV attracts early endosome membrane markers such as early

endosome antigen-1 (EEA-1), Rab5, and transferrin receptors, which are replaced within 20 to 40 minutes post-infection by late endosome membrane markers like- LAMP-1/2/3, Rab7, and V-ATPase [5, 27]. As it was decided to study the intracellular behavior of the wild type and mutant bacteria during the immediate early stage of infection, our study was initiated by examining the recruitment of early endosomal markers on bacteria. The lack of significant difference between recruitment of EEA1 around STM (WT) and STM *ΔompA* at 15 minutes post-infection in activated U937 cells (**Figure 4A and 4B**) suggested that both the bacteria reside within EEA1 decorated vacuolar compartment during the immediate early stage of infection. In this experiment, PFA-fixed dead bacteria were used as a control. Taken together, this data also suggests that deletion of *ompA* from *Salmonella* Typhimurium does not hamper the SCV biogenesis during infection. Simultaneously, the colocalization of wild type and mutant bacteria with LAMP-1 was tested at 15-, 30- and 120 minutes post-infection in humans (**Figure 4C- 4F**) as well as murine macrophages (**Figure 4G- 4J**). At 15 minutes post-infection, STM *ΔompA* was found to be recruiting more LAMP-1 compared to the wild type bacteria in humans (**Figure 4C and 4F**) and murine macrophages (**Figure. 4G and 4J**). With an increase in time (at 30- and 120 minutes post-infection), the wild type *Salmonella* was found to be acquiring more LAMP-1 in both the cell lines (**Figure 4D- 4F and Figure 4H- 4J**). In contrast, STM *ΔompA* was unable to retain the acquired LAMP-1 around itself and gradually lost the SCV membrane from its surroundings. Taken together, our data demonstrated an essential role of outer membrane protein A of *Salmonella* to maintain a stable interaction with LAMP-1 (SCV marker). Earlier, it was found that wild type *Salmonella* uses SPI-1 encoded virulent factor SipC (*Salmonella* invasion protein C) to acquire LAMP-1 from Golgi in a host syntaxin 6 dependent manners [28]. Our study revealed that STM *ΔompA* is unable to receive LAMP-1 and escape SCV. Hence, we hypothesized that STM *ΔompA* is deficient in producing SipC. To test this hypothesis, we have infected RAW264.7 cells with wild type and *ompA*

knockout strains of *Salmonella* and checked their colocalization with SipC in intracellular conditions. Surprisingly, no signifiacntly difference in the colocalization of SipC with intracellular STM (WT) and STM *ΔompA* (**Figure S4A and S4B**) was observed. Simultaneously, the expression of *sipC* transcripts from intracellular wild type and mutant bacteria was quantified(**Figure S4C**). There was no significant difference between the expression of *sipC* in wild type and *ompA* knockout strains of *Salmonella* growing intracellularly in macrophage (**Figure S4C**), which is consistent with our previous findings. These data helped us to conclude that OmpA plays a SipC independent vital role in continuing the interaction with SCV marker LAMP-1.

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

Earlier, we have shown an enhanced expression of *ompA* transcript in wild type *Salmonella* Typhimurium growing intracellularly in murine macrophages at 9th and 12th hours post-infection, which suggested the requirement of OmpA for the intracellular survival of bacteria [15]. Our current study revealed that in the absence of OmpA, *Salmonella* cannot stay inside SCV and comes into the cytosol of host cells. Hence, we hypothesized that outer membrane protein A plays a direct role in stabilizing the SCV membrane by retaining LAMP-1. To prove the interaction between *Salmonella* OmpA with host LAMP-1, the macrophages were infected with wild type bacteria, and the interaction between OmpA and LAMP-1 during different time points was investigated. The intracellular bacteria were stained with anti-*Salmonella* OmpA antibody, and their colocalization with the LAMP-1 was checked. In RAW264.7 cells, the wild type bacteria use OmpA in maintaining a stable interaction with LAMP-1 at 2- and 6 hours post-infection (**Figure 5B and 5C**). This interaction was further increased during the late phase of infection (at 16 hours post-infection) (**Figure 5B and 5C**). In human monocyte-derived macrophages (activated U937 cells), the interaction between OmpA with the LAMP-1 was

found to be increasing significantly with time (**Figure 5D and 5E**). Taken together, our data suggested that indeed wild type *Salmonella* Typhimurium uses OmpA to retain the LAMP-1 around the SCV firmly. When OmpA is deleted, the bacteria cannot hold the LAMP-1 pool around the vacuole, and the bacteria quit the SCV. OmpA is embedded into *Salmonella* Typhimurium outer membrane with four extracellular loops exposed outside (**Figure 6A**), which are likely to be interacting with LAMP-1. Hence, it was decided to find out the role of these extracellular loops in establishing the interaction between the bacteria with LAMP-1. The *Salmonella* OmpA extracellular loop sequences were compared with *Escherichia coli* K1 (**Figure 6A**), another Gram-negative bacterial pathogen causing meningitis in neonates [29, 30]. Without hampering the membrane-embedded cylindrical structure of *Salmonella* Typhimurium OmpA, the conserved and unique domains of the four extracellular loops were altered by site-directed mutagenesis (**Figure 6A**), and six different loop mutants, namely L1-1, L1-2, L2-1, L2-2, L3-1, L4-1, were generated. The mutated versions of the gene were expressed in the *ompA* knockout background of *Salmonella* and used for infection. Out latest study demonstrated that the depletion of OmpA increases the permeability of the bacterial outer membrane [15]. The complementation of OmpA in the knockout background restored the outer membrane stability of *Salmonella.* To validate the proper folding of OmpA despite receiving mutations in the extracellular loop regions, the outer membrane permeability of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 was checked by $DiBAC_4$ staining (**Figure 6B and 6C**). In line with our expectations, enhanced uptake of $DiBAC_4$ was observed in STM *ΔompA* compared to the wild type and complemented strains of *Salmonella*. The complementation of the *ompA* knockout strain with mutated variants of *ompA* significantly recovered outer membrane

stability and reduced the entry of $DiBAC_4$ (**Figure 6B and 6C**). This also suggests that the mutation in the loop regions of OmpA does not alter its overall conformation.

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

To find out the role of the extracellular loops of OmpA in maintaining the interaction of the bacteria with LAMP-1, individual loop mutants were generated and used for infecting murine macrophages to study the recruitment of LAMP-1 at 6- and 16 hours post-infection. Altering the aminoacid sequences in each extracellular loop region of OmpA gives indistinguishable outcomes like STM *ΔompA*. Unlike the wild type and the complemented strains, all the loop mutants quit the vacuole at the 6th hour post-infection (**Figure S5A**). The cytosolic localization of the loop mutants was confirmed by quantifying their percent colocalization with LAMP-1, comparable to the *ompA* knockout bacteria (**Figure S5B**). As usual, most of the loop mutants that have come into the cytosol of macrophages at the 6th hour post-infection stay in the cytosol during the late phase of infection (16th hour post-infection) (**Figure 7A and 7B**). This suggests that the extracellular loops from the structure of *Salmonella* OmpA execute the most crucial role in maintaining the stability of the SCV membrane. These extracellular loops mediate the interaction between LAMP-1 with bacteria. Bacteria cannot retain the LAMP-1 pools around the SCV either in the absence of the entire OmpA protein (*ompA* knockout *Salmonella*) or because of the individual loop's structural and functional ineffectiveness. We further wanted to check the effect of the mutations in the loop region of OmpA on the intracellular survival of the bacteria. To answer this question, an intracellular survival assay was performed with the loop mutants in macrophages. Despite their cytosolic inhabitation, mutations in the loop region did not impact the intracellular survival of the bacteria. All the loop mutants were found to be surviving better than the STM *ΔompA* while infecting the macrophage (**Figure 7C**). Despite having mutations in the extracellular loop regions, the presence of intact OmpA in the mutant

bacteria might be the reason behind their better survival in the cytosol. We speculated that the cylindrical structure of OmpA, present in the outer membrane of the loop mutants, might be protecting the cytosolic bacteria from being targeted to the lysosome. To validate this observation, the colocalization of the intracellular loop mutants with the lysosomes was checked during the late phase of infection in murine macrophages (**Figure 8A and 8B**). Unlike the wild type and *ompA* complemented strains of *Salmonella*, STM *ΔompA* (which lacks the entire OmpA from the outer membrane) is engulfed by the lysosome during the late phase of infection in murine macrophages (**Figure 8A and 8B**). In accordance with our expectations, it was observed that the *ompA* variants having mutations in the extracellular loops are less prone to be captured by the lysosomes, which further explains their better survival within murine macrophages.

Discussion

While infecting the host cell, *Salmonella* Typhimurium stays inside a modified compartment called *Salmonella* containing vacuole (SCV) [3, 4]. The pH of the SCV is acidic. The low pH of the SCV triggers the activation of the PhoP/Q two-component system, which finally upregulates the expression of SPI-2 genes [31-33]. With the help of SPI-2 encoded T3SS2 and other virulent factors, *Salmonella* can inhibit the phagolysosome maturation and suppresses the lysosome biogenesis of the host cells [25, 34]. The SPI-2 genes of intracellular bacteria further impede the recruitment of iNOS and NADPH phagocytic oxidase around the SCV and ensure the successful proliferation of the bacteria within the host cells [35, 36]. The formation of SCV is a dynamic and complex process that employs a wide array of host and bacterial proteins. Immediately after entering the host cells, the bacteria-containing vacuole is decorated with early endosome membrane markers such as EEA1, transferrin receptors, and Rab5. With time, the early endosomal proteins are replaced with late endosome membrane markers such as LAMP-1, LAMP-2, Rab7, etc. [4]. *Salmonella* uses SPI-1 and SPI-2 encoded virulent factors

to regulate the biogenesis and stability of the SCV. SPI-1 encoded virulent factor SopB phosphatase can reduce the membrane charge of SCV and prevent the fusion of lysosomes with SCV [11]. *Salmonella* employs SPI-2 encoded protein SpiC to inhibit the fusion of lysosomes with SCV [34]. Another SPI-2 protein, SifA, forms *Salmonella*-induced filaments in epithelial cells to maintain the integrity of SCV by downregulating the recruitment of kinesins [37, 38]. SifA interacts with Rab9 to block the retrograde transport of mannose-6- phosphate receptors and MPR hydrolases to the Golgi apparatus, which ultimately inhibits lysosomal fusion with SCV [39]. Tampering (by point mutation or genomic deletion) the host and bacterial effectors, controlling the SCV stability, create a tremendous impact on the intracellular fate of the pathogen. Introducing point mutation in host Rab7 or deleting *sifA* from *Salmonella* released the bacteria from SCV into the host cytosol, making the bacteria hyper-virulent in epithelial cells and replication-deficient in macrophages [7, 8]. On the contrary, deletion of SPI-2 gene *sseJ* in *sifA* null background further restored the vacuolar status of the pathogen [9]. However, very little is known about the contribution of non-SPI virulent genes of *Salmonella* on the biogenesis and stability of SCV.

Our study revealed a novel role of *Salmonella* Typhimurium outer membrane protein A (OmpA) in maintaining SCV stability. Outer membrane protein A is an outer membrane-bound porin of *Salmonella* that protects the pathogen from oxidative and nitrosative stress [13, 15]. The deletion of OmpA compromises the intracellular vacuolar status of the bacteria. STM *ΔompA* was unable to recruit LAMP-1 (one of the significant SCV markers) around the SCV and gradually released into the host cytosol. The SCV quitting phenomenon of the pathogen was reversed using the complemented bacterial strain. Earlier, our group has shown that this cytosolic population of the bacteria becomes hyper-proliferative inside the epithelial cells, and their intracellular proliferation was significantly attenuated in macrophages. Our study further depicted that STM *ΔompA* is unable to recruit T3SS2 translocon proteins (SseC and SseD) on

its surface and is deficient in producing an SPI-2 effector [15]. Available literature suggests that *Salmonella* deficient in making active T3SS2 and with damaged SCV cannot suppress host autophagy machinery. Hence, we wanted to investigate the autophagy-inducing ability of the cytosolic population of STM *ΔompA*. Compared to the wild type bacteria, which stay within LAMP-1 decorated SCV, the cytosolic population of STM *ΔompA* colocalizes more with syntaxin 17 and LC3B. The inhibition of autophagy using bafilomycin A1 not only reduced the recruitment of autophagy markers (syntaxin 17 and LC3B) on STM *ΔompA* but also improved their intracellular life. It was speculated that the activation of the autophagy pathway would recruit lysosomes around the mutant bacteria. By Texas red ovalbumin pulse-chase experiment, an enhanced colocalization of lysosomes with STM *ΔompA* was observed in macrophages. When STM (WT): *LLO* (which stays in the macrophage cytosol with intact OmpA on its outer membrane) was used for infection, a reduced colocalization between the lysosome and bacteria was observed, suggesting that OmpA protects the cytosolic bacteria from being targeted to lysosomes. This proves that the syntaxin 17 and LC3B recruited around the cytosolic bacteria lacking OmpA can target them into the lysosomes. We confirmed this result by measuring the enhanced activity of lysosomal acid phosphatases in the cells infected with STM *ΔompA*. Earleir, it has been reported that *Salmonella* uses SPI-2 encoded virulent factor SpiC to inhibit phagosome-lysosome fusion [34]. STM *ΔompA* has been found to be deficient in producing SpiC, which might be the reason behind the enhanced colocalization between the bacteria with host lysosomes.

We further wanted to check at what time point the bacteria quit the vacuole in the absence of OmpA. A significant population of STM *ΔompA* escapes the SCV during the early stage of infection in macrophages. Once this bacterial population left the SCV, it remained in the cytosol during the rest of the infection. This result helped us speculate that the departure of STM *ΔompA* from SCV is happening even before the early stage of infection. Hence the

recruitment of LAMP-1 around the bacteria was examined at 15-, 30- and 120 minutes post-infection. It was found that, immediately after entering the macrophages, both the wild type and the mutant bacteria stay within EEA1^{+} early endosomes. Moreover, at this stage of infection (15 minutes p.i.), STM *ΔompA* recruits more LAMP-1 than wild type bacteria. With an increase in time, STM *ΔompA* was unable to retain the LAMP-1 pool around itself and gradually lost the SCV membrane, unlike the wild type bacteria. Wild type *Salmonella* uses SPI-1 effector protein SipC to recruit LAMP-1 from Golgi in host syntaxin 6 dependent manners [28]. As it was observed that STM *ΔompA* is unable to maintain the LAMP-1 pool around itself, we speculated that STM *ΔompA* is deficient in producing SipC. The comparable colocalization of SipC between the intracellular wild type and mutant bacteria proved that the OmpA deletion mutant works in a SipC independent manner. This conclusion was supported by measuring the unaltered *sipC* transcript level from intracellular wild type and mutant bacteria. This result motivated us to hypothesize that OmpA plays a structural role in maintaining the interaction of LAMP-1 with the bacteria confined within the SCV. The direct interaction between the LAMP-1 and the OmpA of wild type bacteria was measured to answer this question. The intracellular wild type *Salmonella* was stained with anti-*Salmonella* OmpA antibody, and the recruitment of LAMP-1 around the bacteria was estimated. We have seen that *Salmonella* Typhimurium OmpA maintains a stable interaction with the host LAMP-1. This interaction further increases during the late phase of infection in host macrophages.

Outer membrane protein A is embedded into the bacterial outer membrane with the help of its cylindrical β barrel structure. The anti-parallel β sheets that constitute the wall of this barrel structure are connected by four extracellular loops (L1, L2, L3, and L4), which are exposed outside. The externally exposed extracellular loops were thought to maintain the stability of SCV by directly interacting with host LAMP-1. Our speculation was validated by introducing mutations in the loop regions of *Salmonella* OmpA by site-directed mutagenesis. Before doing

that, a comparative study on the aminoacid sequences of the extracellular loops of OmpA was performed between *Salmonella* Typhimurium and *Escherichia coli* K1 [40]. The role of the extracellular loops of *E. coli* K1 OmpA has already been deciphered. The introduction of mutations in the loop regions of *E. coli* K1 OmpA made the bacteria proliferation deficient in immune cells and reduced their ability to cause meningitis in a neonatal mouse model [29, 41]. The unique and the conserved sequences of the extracellular loops of *Salmonella* OmpA were targeted to make them deformed structurally and functionally. The amino acid sequences of the loop region were changed to nonpolar amino acids like- alanine, leucine, and valine. It was found that each of these individual loop mutants plays a critical role in maintaining the interaction with LAMP-1 during the middle and late stages of infection in murine macrophages. All the OmpA loop mutants came into the cytosol of macrophages after abandoning the SCV. Surprisingly, our data suggested that the mutations in the loop regions do not hamper the intracellular proliferation of the bacteria within the cytosol of macrophages. To find out the reason behind the better survival of the *Salmonella* loop mutants in murine macrophages, their colocalization with the lysosomal compartments was examined. The reduced percent colocalization of the loop mutants with Texas red ovalbumin suggested poor recruitment of lysosomes on the cytosolic niche of *Salmonella* Typhimurium *ompA* variants. In the previous study, we have proved that the presence of intact OmpA maintains the integrity of the bacterial outer membrane and protects it from nitrosative stress. The final observation from this study indicated that despite mutations in the extracellular loops, the intact OmpA present on the bacterial outer membrane could protect the cytosolic bacteria from lysosomal degradation in murine macrophages.

Altogether, our study provides an OmpA dependent novel mechanism used by *Salmonella* Typhimurium to maintain the stability of the SCV. *Salmonella* uses the extracellular loops of OmpA to retain the LAMP-1 pool around the SCV. In the absence of OmpA, the bacteria fail

to hold the LAMP-1 and come into the cytosol after quitting the SCV. The cytosolic bacteria lacking OmpA further activate the host autophagy machinery. They cannot prevent the maturation of the phagolysosome, which further leads to the clearance of the bacteria by the lysosomal degradation pathway.

Materials and methods

Bacterial strains, media, and culture conditions

The wild type (WT) bacteria *Salmonella enterica* serovar Typhimurium strain 14028S used in this study was a generous gift from Professor Michael Hensel, Max Von Pettenkofer-Institute for Hygiene and Medizinische Mikrobiologie, Germany. The bacterial strains were revived from glycerol stock (stored in -80^{0}C) and plated either only on LB agar (purchased from HiMedia) (for the wild type *Salmonella*) or LB agar along with appropriate antibiotics like-kanamycin (50 µg/mL) (for the *ompA* knockout strains), ampicillin (50 µg/mL) (for the wild type *Salmonella* expressing mCherry/ RFP, GFP, and LLO), and kanamycin and ampicillin together (both 50 µg/mL), (for the complemented, loop mutants, mCherry and GFP expressing *ompA* knockout strains). *Salmonella- Shigella* agar was used for plate cell lysates/ cell suspensions to calculate the bacterial burden in infected cell lines. The complete list of strains and plasmids has been listed below. (Description in Table- 4.1) Dead bacteria used in several experiments were produced from viable wild type bacteria by treating the bacteria with 3.5% paraformaldehyde for 30 minutes.

Eukaryotic cell lines and growth conditions

The murine macrophage-like cell lines RAW 264.7 used in this study were maintained in Dulbecco's Modified Eagle's Media (Sigma-Aldrich) supplemented with 10% FCS (Fetal calf serum, Gibco) at 37^{0}C temperature in the presence of 5% CO_2. Human monocyte cell line U937

cells were maintained in Roswell Park Memorial Institute 1640 media (Sigma-Aldrich) supplemented with 10% FCS (Fetal calf serum, Gibco). Phorbol Myristate Acetate (Sigma-Aldrich) (concentration- 20 ng/ mL) was used for the activation of U937 cells for 24 hours at 37^0C temperature in the presence of 5% CO_2, followed by the replacement of the media carrying PMA with normal RPMI supplemented with 10% FCS and further incubating the cells for 24 hours before starting the experiments.

RNA isolation from intracellular bacteria and RT PCR

The RAW264.7 cells were infected with STM (WT) and *ΔompA* at MOI of 50. 12hours post-infection, the infected macrophages were lysed with TRIzol reagent (RNAiso Plus, Takara) and stored at -80^0C overnight. The lysed supernatants were further subjected to chloroform extraction followed by precipitation of total RNA by adding an equal volume of isopropanol. The pellet was washed with 70% RNA-grade ethanol, air-dried, and suspended in 20 µL of DEPC treated water. The RNA concentration was measured in nano-drop and analyzed on 1.5% agarose gel to assess RNA quality. To make cDNA, 3 µg of RNA sample was subjected to DNase treatment in the presence of DNase buffer (Thermo Fischer Scientific) at 37^0C for two hours. The reaction was stopped by adding 5mM Na_2EDTA (Thermo Fischer Scientific), then heating at 65^0C for 10 min. The samples were incubated with random hexamers at 65^0C for 10 minutes and then supplemented with 5X RT buffer, RT enzyme, dNTPs, and DEPC treated water at 42^0C for one hour. Quantitative real-time PCR was done using SYBR/ TB Green RT PCR kit (Takara Bio) in Bio-Rad real-time PCR detection system, and the expression level of target genes was measured using *Salmonella* Typhimurium *sipC* specific RT primers (Table- 4.2). 16S rRNA transcript level was used to normalize the expression levels of the target genes.

Intracellular proliferation assay

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

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

In some sets of experiments, the fold proliferation of STM (WT) and *ΔompA* in the macrophages (RAW 264.7) was measured in the presence of autophagy inhibitor bafilomycin A (50 nM). Bafilomycin A was added to the cells infected with STM (WT) and *ΔompA* along with 25 µg/ mL of gentamycin solution and incubated till the lysis. As usual, the cells were lysed with 0.1% triton-X-100 at 2 hours and 16 hours post-infection. The lysates were plated on *Salmonella- Shigella* Agar, and the corresponding CFU at 2 hours and 16 hours were calculated to determine Fold proliferation.

Confocal microscopy

RAW 264.7 or U937 cells were seeded at a 1.5 to 2 X 10^5 cells density per sterile glass coverslips. U937 cells were activated using PMA (as mentioned earlier in 4.2.2). The cells were infected with appropriate bacterial strains at MOI 20. The cells were washed thrice with

PBS and fixed with 3.5% paraformaldehyde for 15 minutes at indicated time points post-infection. The cells were first incubated with specific primary antibody raised against- wild type *Salmonella* Typhimurium (rabbit raised anti- *Salmonella* O antigen), mouse lysosome-associated membrane protein-1 (LAMP-1) (rat raised anti-mouse LAMP-1), human LAMP-1 (mouse raised anti-human LAMP-1), mouse LC3B (rabbit raised anti-mouse LC3B), mouse syntaxin 17 (rabbit raised anti-mouse syntaxin 17), *Salmonella* Typhimurium SipC (mouse raised anti-*Salmonella* SipC), *S.* Typhimurium OmpA (rabbit raised anti-*Salmonella* OmpA antibody) and human EEA1 (mouse raised anti-human EEA1) as per the requirements of experiments. The primary antibodies were diluted in 2.5% BSA and 0.01% saponin (dilution 1: 100, duration 6 to 8 hours at 4^0C temperature). This was followed by incubating the cells with appropriate secondary antibodies conjugated with fluorophores (dylight 488, alexa fluor 647, and cy3) (dilution 1: 200, duration 1 hours at room temperature). The coverslips were mounted with anti-fade reagent and fixed on a glass slide with transparent nail paint. Samples were imaged by confocal laser scanning microscopy (Zeiss LSM 710) using a 63X oil immersion objective lens. The images were analyzed with ZEN Black 2009 software provided by Zeiss.

Texas red ovalbumin pulse-chase experiment

1.5 to 2 X 10^5 RAW 264.7 cells were seeded on the top of sterile glass coverslips in the wells of a 24 well plate. The cells were fed with a 50 µg/ mL concentration of Texas red ovalbumin (resuspended in DMEM media) for 30 minutes at 37^0C in the presence of 5% CO_2. After this, the labeling media was removed, and the cells were washed with sterile PBS. The cells were further incubated for 30 minutes with fresh DMEM media and infected with overnight grown 10- 12 hours old stationary phase culture of STM (WT), STM (WT): *GFP*, *ΔompA*: *GFP*, and STM (WT): *LLO* at MOI of 20. PFA-fixed dead bacteria were used for infection as control at MOI of 25. The cells were washed thrice with PBS and fixed with 3.5% paraformaldehyde for

15 minutes at indicated time points post-infection. Rabbit-raised anti-*Salmonella* primary antibody stained the STM (WT): *LLO* and PFA fixed dead bacteria. The coverslips were mounted with anti-fade reagent and fixed on a glass slide with transparent nail paint to visualize the lysosomal arrangements in the infected macrophage cells. Samples were imaged by confocal laser scanning microscopy (Zeiss LSM 710) using a 63X oil immersion objective lens. The images were analyzed with ZEN Black 2009 software provided by Zeiss.

Acid phosphatase assay

The protocol of acid phosphatase assay has been followed as mentioned earlier [25, 42, 43]. 1.5 to 2 X 10^5 RAW 264.7 cells were seeded into the wells of a 24 well plate and infected with overnight grown stationary phase culture of STM (WT), *ΔompA, ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, and STM (WT): *LLO* at MOI of 10. PFA-fixed dead bacteria were used as a control for the infection (MOI-20). The infected cells were incubated for 12 hours under gentamycin treatment (as mentioned earlier). At the end of the incubation period, the cells were washed with PBS and incubated for 4 hours at 37^0C with a buffer containing a 0.1 (M) sodium acetate of pH= 5, 0.1% triton-X-100, 5mM of *p*-nitrophenyl phosphate (pNPP). The absorbance of the supernatant was measured at 405 nm using a microplate reader. The non-enzymatic hydrolysis of pNPP (negligible) was measured media control without macrophage cells.

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

The protocol for site-directed mutagenesis was followed, as mentioned earlier [44]. Primer pairs (from Table- 4.2) carrying mutations in the desired locations of the four extracellular loops of *Salmonella* Typhimurium OmpA (Figure 4.12.B) were used to amplify the pQE60-*ompA* recombinant plasmid (Size- 4.5 kb) using Phusion high fidelity DNA polymerase (NEB). The reaction mixture was heated at 95^0C for 10 minutes for the plasmid's denaturation, followed by 35 amplification cycles at 95^0C for 30 seconds, 54^0C for 60 seconds, and 72^0C for

2 minutes 30 seconds with a final extension time of 10 minutes. The amplified PCR product was then digested with DpnI and transformed into *E. coli* TG1. The transformants were then selected on ampicillin plates. The plasmids were isolated from the transformant colonies, and the mutations were verified by sequencing. Once the confirmation was done, the recombinant plasmids carrying desired mutations were transformed into STM *ΔompA* by electroporation to create specific loop mutant *Salmonella*. These loop mutants were used for infecting RAW264.7 cells.

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

Outer membrane porosity of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 grown in low magnesium acidic F medium (pH= 5.4) for 12 hours was measured using a dye called bis-(1,3-dibutyl barbituric acid)-trimethylene oxonol (Invitrogen) ($DiBAC_4$). Briefly, 4.5 X 10^7 CFU of each bacterial strain was incubated with 1 µg/ml of DiBAC4 for 30 minutes in a 37^0C shaker incubator. The $DiBAC_4$ treated bacterial cells were analyzed by flow cytometry (BD FACSVerse by BD Biosciences-US) to evaluate the porosity of the bacterial outer membrane.

Statistical analysis

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

Abbreviations

STM: *Salmonella* Typhimurium

OmpA: Outer membrane protein A

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

Stx17: Syntaxin 17

LLO: Listeriolysin O

SCV: *Salmonella* containing vacuole

LAMP-1: Lysosome associated membrane protein-1

EEA1: Early endosome antigen 1

RFP: Red fluorescent protein

GFP: Green fluorescent protein

SipC: *Salmonella* invasion protein C

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
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

Table 4.2. Primers used in this study (5'-3')

sipC forward- GACCTGGGGTTGAGTCCTAC

sipC reverse- ACGGCACTGGAAGACATTC

16S rRNA forward-CGGACGGGTGAGTAATGT

16S rRNA reverse-TGCTTCTTCTGCGGGTAA

ompA site directed mutagenesis primers (5'-3')

ompA Loop 1-1 forward- GCTTCATTCACAATGTGGTGGTGACTCATGAAAACC

ompA Loop 1-1 reverse- TGGTTTTCATGAGTCACCACCACATTGTGAATGAAGCCG

ompA Loop 1-2 forward- ACAATGATGGCCCGGCGGCGGCGAACCAACTGGGCG

ompA Loop 1-2 reverse-TGCGCCCAGTTGGTTCGCCGCCGCCGGGCCATCATTGTG

ompA Loop 2-1 forward- GTTAGGCCGTATGGCGGCGGCGGGCGACAACATC

OmpA Loop 2-1 reverse- TGATGTTGTCGCCCGCCGCCGCCATACGGCCTAACC

OmpA Loop 2-2 forward- ATGCCGTACAAAGTGGTGAACATCAATGGCGC

OmpA Loop 2-2 reverse- AGCGCCATTGATGTTCACCACTTTGTACGGC

OmpA Loop 3-1 forward- AGACACCAAGTCTCTGGCGCTGGGCGGCCCGTCTAC

OmpA Loop 3-1 reverse- TAGACGGGCCGCCCAGCGCCAGAGACTTGGTGTC

OmpA Loop 4-1 forward- ACTAACAACATCGTGGTGGCCAACACCATC

OmpA Loop 4-1 reverse- CCGATGGTGTTGGCCACCACGATGTTGTTAG

ompA SDM confirmatory primer- ATGAAAAAGACAGCTATCGC

Author Contributions

ARC and DC conceived the study and designed the experiments. ARC performed all the experiments, analyzed the data, and wrote the original draft of the manuscript. DH performed

the experiments with ARC, participated in proofreading and editing of the manuscript. DC supervised the study and reviewed the manuscript. All the authors have read and approved the manuscript.

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Availability of data and materials

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

Declarations

Ethics statement

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare to have no conflict of interest.

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Figure Legends

Figure 1.

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

(A) RAW264.7 cells were infected with STM (WT): RFP, *ΔompA*: RFP, and *ΔompA*: pQE60-*ompA* at MOI of 20. Cells were fixed at 16 hours post-infection & LAMP-1 was labeled with anti-mouse LAMP-1 antibody. To stain the complemented strain anti-*Salmonella* antibody was used. (B) The quantification of LAMP-1 recruitment on bacteria in RAW 264.7 cells has been

represented in a graph. Percent colocalization was determined after analyzing more than 60 different microscopic stacks from two independent experiments. Scale bar = 5μm, [n≥60, N=2]. (C-F) RAW264.7 cells were infected with STM (WT): *RFP* and *ΔompA*: *RFP* at MOI of 20. Cells were fixed at 16 hours post-infection. Two autophagy markers, (C) syntaxin 17 and (E) LC3B, were stained with rabbit-raised anti-mouse syntaxin 17 and LC3B-II primary antibodies, respectively. The quantification of (D) syntaxin 17 and (F) LC3B recruitment on STM (WT): *RFP* and *ΔompA*: *RFP* has been represented in the form of two graphs. (D) The percent colocalization of syntaxin 17 with bacteria was determined after analyzing 100 microscopic stacks from three independent experiments (n=100. N=3). (F) The percent colocalization between the bacteria and LC3B was determined after analyzing more than 60 different microscopic stacks from two independent experiments. Scale bar = 5μm, [n≥60, N=2]. To stain the lysosomes, RAW 264.7 cells were pre-treated with Texas red ovalbumin for 30 minutes. (G) The cells are washed thrice with PBS after that and infected with STM (WT): *GFP*, *ΔompA*: *GFP*, and STM (WT): *LLO*, respectively, at MOI of 20. PFA-fixed dead bacteria were used for infection at MOI of 25. To stain STM (WT): *LLO* and PFA fixed dead bacteria rabbit-anti *Salmonella* O primary and anti-rabbit dylight 488 secondary antibodies were used. (H) The colocalization of lysosomes with bacteria has been represented in the form of a graph. The percent colocalization between Texas red and the bacteria was determined after counting 50 microscopic stacks from two independent experiments (n=50, N=2). Scale bar = 5μm. (I) To measure the acid phosphatase activity of lysosomes, RAW 264.7 cells were infected with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, STM (WT): *LLO*, and PFA fixed dead bacteria at MOI of 10. Twelve hours post-infection, the cells were washed with PBS and further incubated for 4 hours at 37^0C with a buffer containing sodium acetate, triton-X-100, and *p*-nitrophenyl phosphate (pNPP). The absorbance of the supernatant was measured at 405 nm using a microplate reader (n=6, N=2).

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

Figure 2.

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

RAW264.7 cells were infected with STM (WT): *RFP* and *ΔompA*: *RFP* at MOI of 20. One set of cells infected with STM *ΔompA*: *RFP* were treated with bafilomycin A (50 nM). Cells were fixed at 16 hours post-infection. Two autophagy markers, (A) syntaxin 17 and (C) LC3B, were stained with rabbit-raised anti-mouse syntaxin 17 and LC3B-II primary antibodies, respectively. The quantification of (B) syntaxin 17 and (D) LC3B recruitment on STM (WT): *RFP*, *ΔompA*: *RFP*, and *ΔompA*: *RFP* under bafilomycin A treatment have been represented in the form of two graphs. (B) The percent colocalization of syntaxin 17 with bacteria was determined after analyzing 100 microscopic stacks from three independent experiments (n=100. N=3). (D) The percent colocalization between the bacteria and LC3B was determined after analyzing more than 60 different microscopic stacks from two independent experiments. Scale bar = 5μm, [n≥60, N=2]. (E) Intracellular survival of STM (WT) and *ΔompA* (MOI-10) in RAW264.7 cells (16 hours post-infection) in presence and absence of autophagy inhibitor bafilomycin A (50 nM). The bacteria's fold proliferation was calculated by normalizing the CFU at 16 hours to CFU at 2 hours (n=3, N=2).

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

Figure 3.

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

(A-D) RAW264.7 cells were infected with STM (WT): RFP, *ΔompA*: RFP, and *ΔompA*: pQE60-*ompA* at MOI of 20. Cells were fixed at (A) 2 hours (early phase) and (C) 6 hours

(middle phase) post-infection & LAMP-1 was labeled with anti-mouse LAMP-1 antibody. To stain the complemented strain and the PFA fixed dead bacteria anti-*Salmonella* antibody was used. The quantification of LAMP-1 recruitment on bacteria in RAW 264.7 cells at (B) 2 hours and (D) 6 hours post-infection has been represented in the form of two graphs. (B) During the early stage of infection (2 hours post-infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 50 different microscopic stacks from two independent experiments [n≥50, N=2]. (D) During the middle stage of infection (6 hours post-infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 40 different microscopic fields from two independent experiments [n≥40, N=2]. Scale bar = 5μm.

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

Figure 4.

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

PMA activated U937 cells were infected with STM (WT), *ΔompA*, and PFA fixed dead bacteria at MOI of 25. (A) Cells were fixed at 15 minutes post-infection (immediate early stage of infection). The bacteria have been stained with rabbit-raised anti-*Salmonella* O primary antibody. Early endosome antigen (EEA1) was labeled with an anti-human EEA1 antibody raised in the mouse. (B) The quantification of EEA1 recruitment on bacteria in U937 cells has been represented in the form of three graphs. During the immediate early stage of infection (15 minutes post-infection), the percent colocalization of bacteria with EEA1 was determined after analyzing more than 30 different microscopic stacks from two independent experiments [n≥30, N=2]. PMA activated U937 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at MOI of 20. Cells were fixed at (C) 15 minutes (immediate early phase), (D) 30 minutes, and (E) 2 hours post-infection & LAMP-1 was labeled with anti-human LAMP-1 antibody. (F) The

quantification of LAMP-1 recruitment on bacteria in U937 cells at 15 minutes, 30 minutes, and 2 hours post-infection has been represented in the form of a graph. (F) The percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 50 different microscopic stacks from two independent experiments [n≥50, N=2]. Scale bar = 5μm. RAW264.7 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at MOI of 20. Cells were fixed at (G) 15 minutes (immediate early phase), (H) 30 minutes, and (I) 2 hours post-infection & LAMP-1 was labeled with anti-mouse LAMP-1 antibody. (J) The quantification of LAMP-1 recruitment on bacteria in RAW264.7 cells at 15 minutes, 30 minutes, and 2 hours post-infection has been represented in the form of a graph. (J) The percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 60 different microscopic stacks from two independent experiments [n≥60, N=2]. Scale bar = 5μm.

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

Figure 5.

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

(A) The structure of *Salmonella* Typhimurium OmpA obtained with the help of SWISS-MODEL software (B) RAW 264.7 cells were infected with STM (WT) at MOI of 20. Cells were fixed at 2 hours (early phase), 6 hours (middle phase), and 16 hours (late phase) post-infection. Intracellular *Salmonella* was stained with rabbit-raised anti-*Salmonella* OmpA primary antibody. In RAW264.7 cells, LAMP-1 was labeled with rat-raised anti-mouse LAMP-1 antibody. (C) The quantification of LAMP-1 recruitment on bacteria in RAW264.7 cells at 2 hours, 6 hours, and 16 hours post-infection has been represented in the form of a graph. (C) The percent colocalization of bacteria with LAMP-1 was determined after analyzing 50 different microscopic stacks from two independent experiments [n=50, N=2]. (D) PMA

activated U937 cells were infected with STM (WT) at an MOI of 20. Cells were fixed at 2 hours (early phase), 6 hours (middle phase), and 16 hours (late phase) post-infection. Intracellular *Salmonella* was stained with rabbit-raised anti-*Salmonella* OmpA primary antibody. In U937 cells, LAMP-1 was labeled with an anti-human LAMP-1 antibody. (E) The quantification of LAMP-1 recruitment on bacteria in U937 cells at 2 hours, 6 hours, and 16 hours post-infection has been represented in the form of a graph. (E) The percent colocalization of bacteria with LAMP-1 was determined after analyzing 50 different microscopic stacks from three independent experiments [n=50, N=3]. Scale bar = 5μm.

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

Figure 6.

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

(A) The outer membrane-embedded β barrel structure of *Salmonella* Typhimurium OmpA with extracellular loops (Loop1, Loop2, Loop3, and Loop4). Comparison between the extracellular loop sequences of *Escherichia coli* K1 and *Salmonella* Typhimurium. Two different mutations were introduced in loop1 (L1- L1-1 and L1-2) and loop2 (L2- L2-1 and L2-2) separately. Two distinct single mutations (L3-1 and L4-1) were introduced in loop3 (L3) and loop4 (L4). (B-C) Measurement of membrane porosity of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1, and *ΔompA*: pQE60-*ompA*-L4-1 in acidic F media (12 hours post-inoculation) using DiBAC4 (final concentration- 1 μg/ mL) by flow cytometry. Unstained bacterial cells were used as control (Autofluorescence). Both dot plots (SSC-A vs. DiBAC4) and histograms (Count vs. DiBAC4) have been represented. The percent

population of DiBAC4 positive bacterial cells has been represented here. Data are represented as mean ± SEM (n=5, N=2).

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

Figure 7.

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

RAW264.7 cells were infected with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 at MOI of 20. Cells were fixed at (A) 16 hours (late phase) post-infection. Intracellular *Salmonella* was stained with rabbit-raised anti-*Salmonella* O primary antibody. LAMP-1 was labeled with rat-raised anti-mouse LAMP-1 primary antibody. The quantification of LAMP-1 recruitment on bacteria in RAW 264.7 cells at 16 hours post-infection has been represented in a graph. (B) During the late stage of infection (16 hours post-infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing 100 different microscopic fields from three independent experiments [n=100, N=3]. Scale bar = 5μm. (C) Intracellular survival of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 (MOI-10) in RAW264.7 cells (16 hours post-infection). The bacteria's fold proliferation was calculated by normalizing the CFU at 16 hours to CFU at 2 hours (n=3, N=3).

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

Figure 8.

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

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

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

Figure 9.

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

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

syntaxin 17 and LC3B, a significant fraction of cytosolic STM *ΔompA* is targeted to lysosomal degradation.

Supplementary Figures

Figure S1.

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

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

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

Figure S2.

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

To stain the lysosomes, RAW 264.7 cells were pre-treated with Texas red ovalbumin for 30 minutes and infected with STM (WT): *GFP* and *ΔompA*: *GFP*, respectively, at MOI of 20. Host syntaxin 17 was labeled with rabbit-raised anti-mouse syntaxin 17 primary and anti-rabbit alexa fluor 647 secondary antibodies, respectively. (H) The colocalization of bacteria with lysosomes and syntaxin 17 has been represented in the form of a graph. The percent colocalization of the bacteria with texas red and syntaxin 17 were determined after counting

more than 50 microscopic stacks from two independent experiments (n≥ 50, N=2). Scale bar = 5μm.

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

Figure S3.

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

PMA activates U937 cells were infected with STM (WT): RFP, and *ΔompA*: RFP at MOI of 20. Cells were fixed at (A) 2 hours (early phase), (C) 6 hours (middle phase), and (E) 16 hours (late phase) post-infection & LAMP-1 were labeled with anti-human LAMP-1 antibody. The quantification of LAMP-1 recruitment on bacteria in U937 cells at (B) 2 hours, (D) 6 hours, and (F) 16 hours post-infection has been represented in the form of three graphs. (B) During the early stage of infection (2 hours post-infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 40 different microscopic stacks from two independent experiments [n≥40, N=2]. (D) During the middle stage of infection (6 hours post-infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 30 different microscopic stacks from two independent experiments [n≥30, N=2]. (F) During the late stage of infection (16 hours post-infection), the percent colocalization of bacteria with LAMP-1 was determined after analyzing more than 50 different microscopic stacks from two independent experiments [n≥50, N=2]. Scale bar = 5μm.

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

Figure S4.

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

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

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

Figure S5.

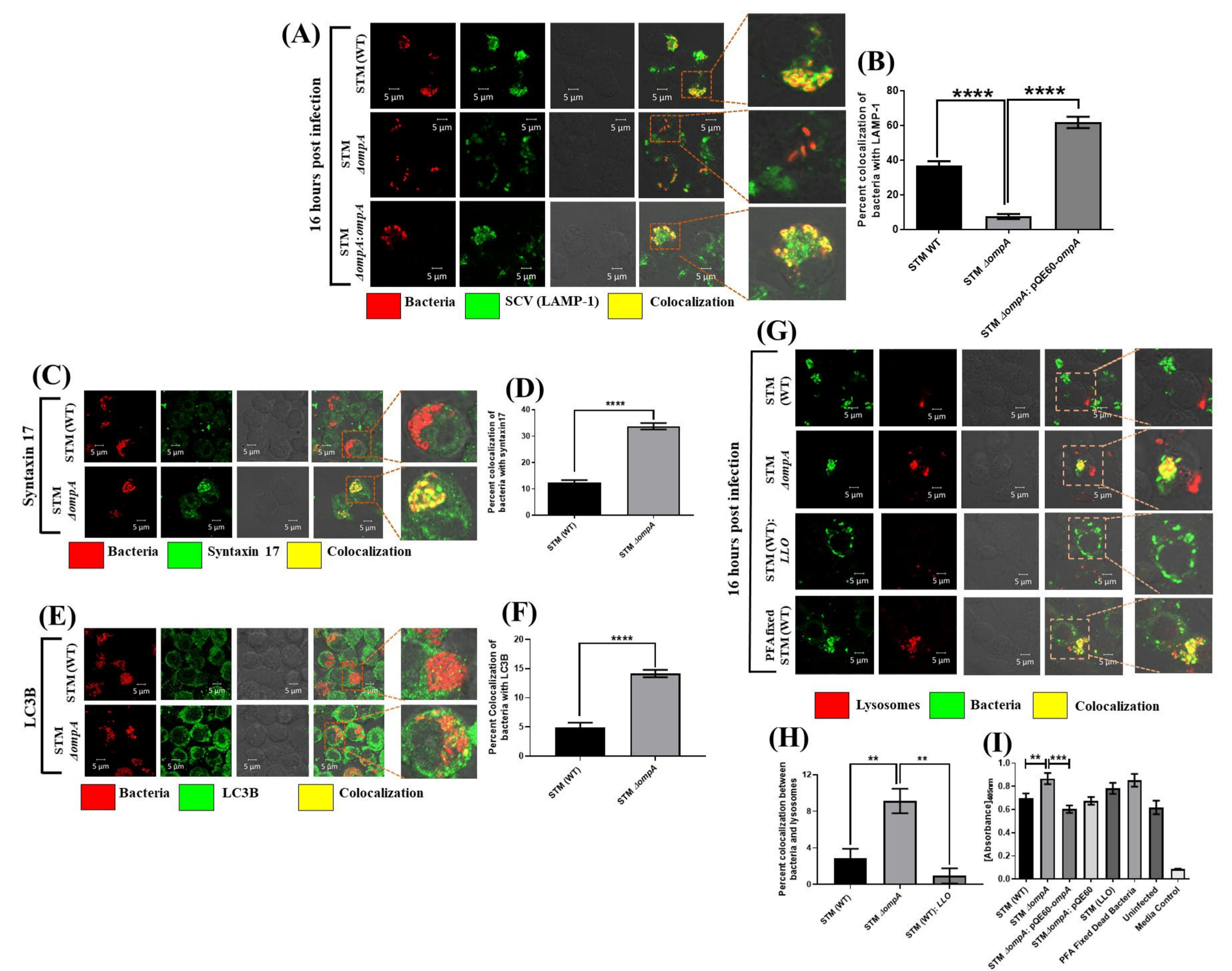
Mutation in the extracellular loops of *Salmonella* Typhimurium OmpA reduces the retention of LAMP-1 around the bacteria in murine macrophages.

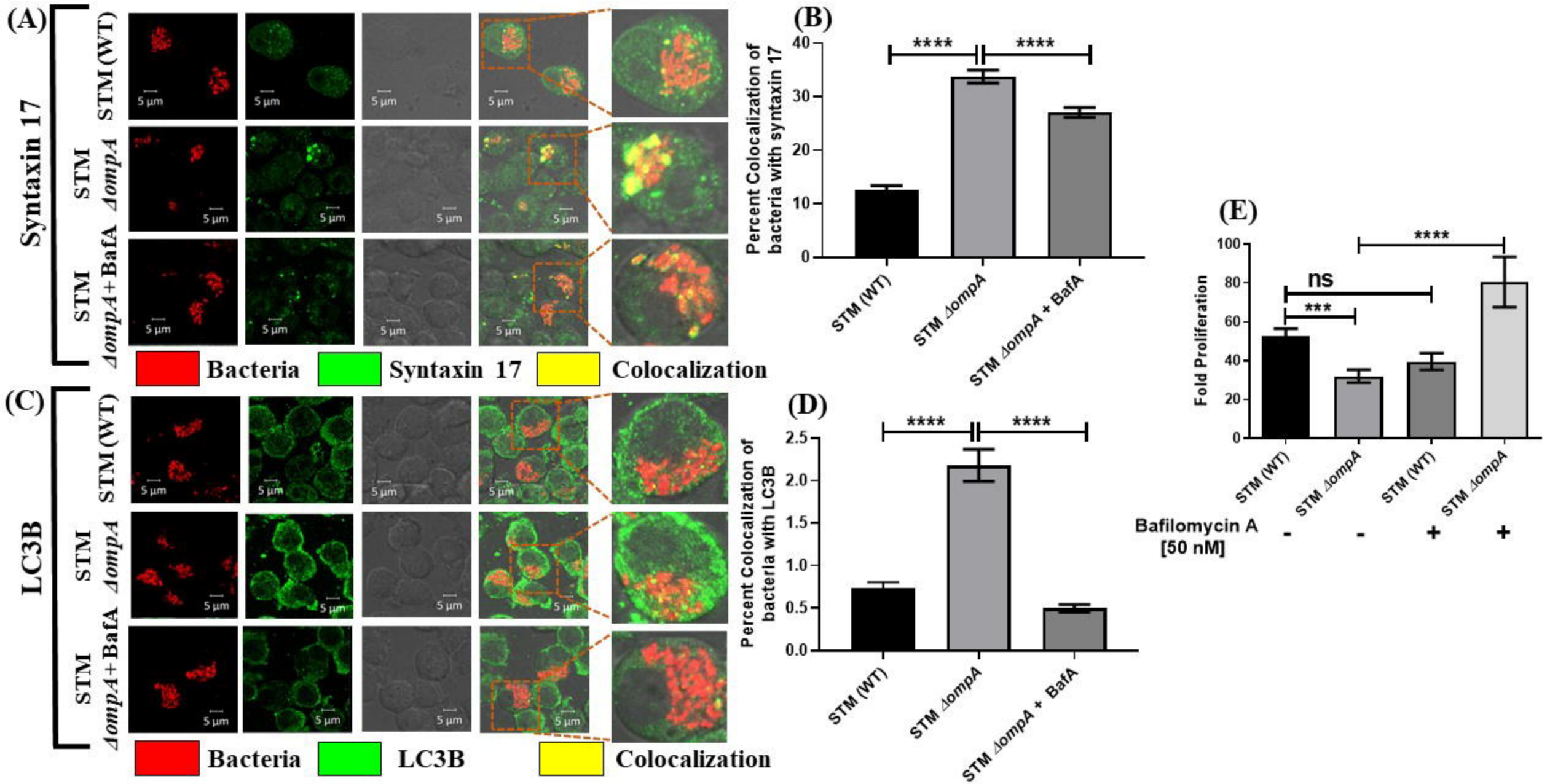
RAW264.7 cells were infected with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60-*ompA*-L1-1, *ΔompA*: pQE60-*ompA*-L1-2, *ΔompA*: pQE60-*ompA*-L2-1, *ΔompA*: pQE60-*ompA*-L2-2, *ΔompA*: pQE60-*ompA*-L3-1 and *ΔompA*: pQE60-*ompA*-L4-1 at MOI of 20. Cells were fixed at (A) 6 hours (late phase)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

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

1008

1009


(A)
16 hours post infection
STM (WT)
STM ΔompA
STM ΔompA: ompA
5 µm
Bacteria
SCV (LAMP-1)
Colocalization
(B)
Percent colocalization of bacteria with LAMP-1
****
****
STM WT
STM ΔompA
STM ΔompA: pQE60-ompA
(C)
Syntaxin 17
STM (WT)
STM ΔompA
Bacteria
Syntaxin 17
Colocalization
(D)
Percent colocalization of bacteria with syntaxin17
****
STM (WT)
STM ΔompA
(E)
LC3B
STM (WT)
STM ΔompA
Bacteria
LC3B
Colocalization
(F)
Percent Colocalization of bacteria with LC3B
****
STM (WT)
STM ΔompA
(G)
16 hours post infection
STM (WT)
STM ΔompA
STM (WT): LLO
PFA fixed STM (WT)
Lysosomes
Bacteria
Colocalization
(H)
Percent colocalization between bacteria and lysosomes
**
**
STM (WT)
STM ΔompA
STM (WT): LLO
(I)
[Absorbance]405nm
**
***
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60
STM (LLO)
PFA Fixed Dead Bacteria
Uninfected
Media Control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Syntaxin 17
STM (WT)
STM ΔompA
STM ΔompA+BafA
5 µm
Bacteria
Syntaxin 17
Colocalization
(B)
Percent Colocalization of bacteria with syntaxin 17
****
****
STM (WT)
STM ΔompA
STM ΔompA + BafA
(C)
LC3B
STM (WT)
STM ΔompA
STM ΔompA+BafA
Bacteria
LC3B
Colocalization
(D)
Percent Colocalization of bacteria with LC3B
****
****
STM (WT)
STM ΔompA
STM ΔompA + BafA
(E)
Fold Proliferation
ns
***
****
STM (WT)
STM ΔompA
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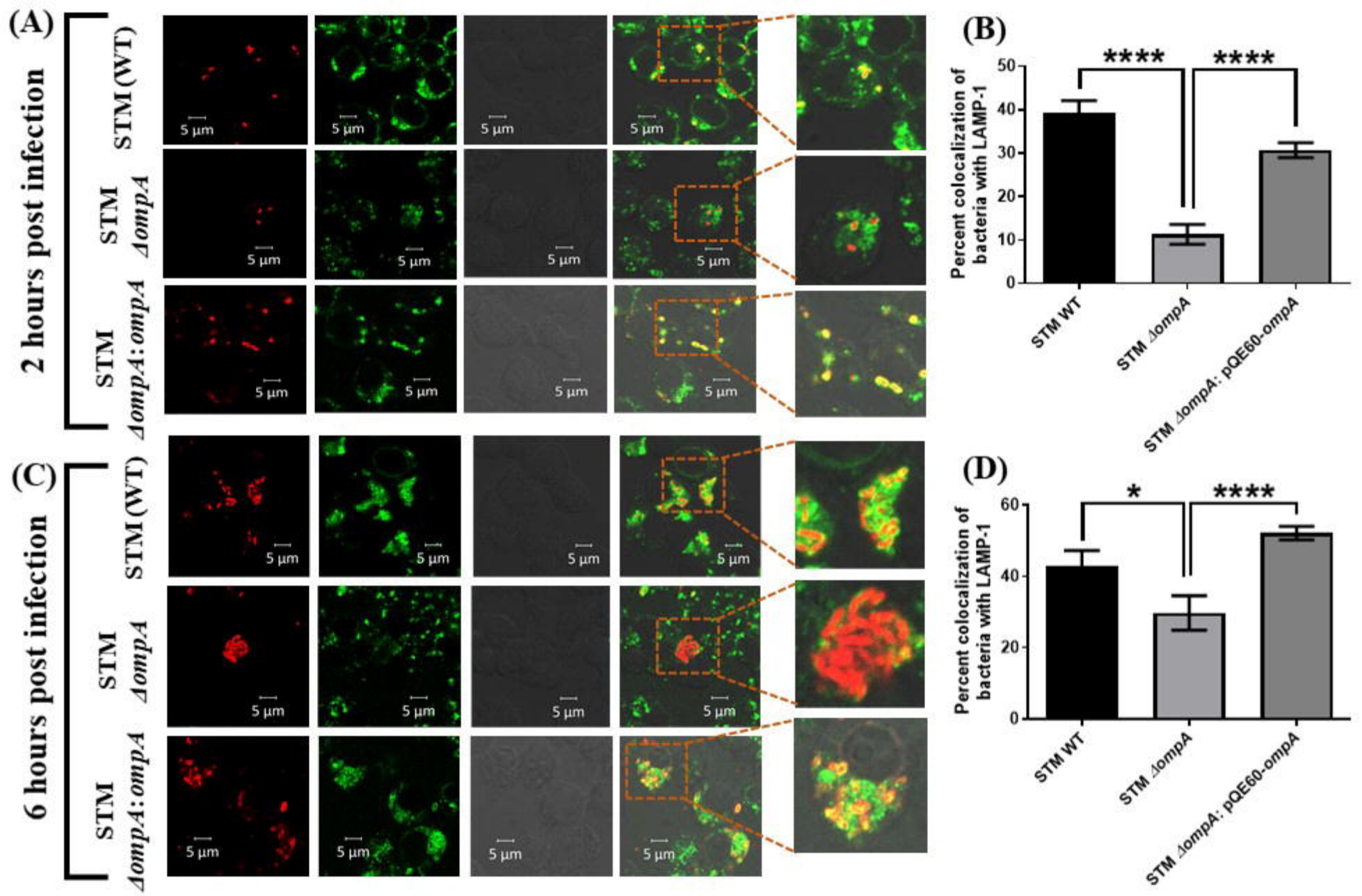
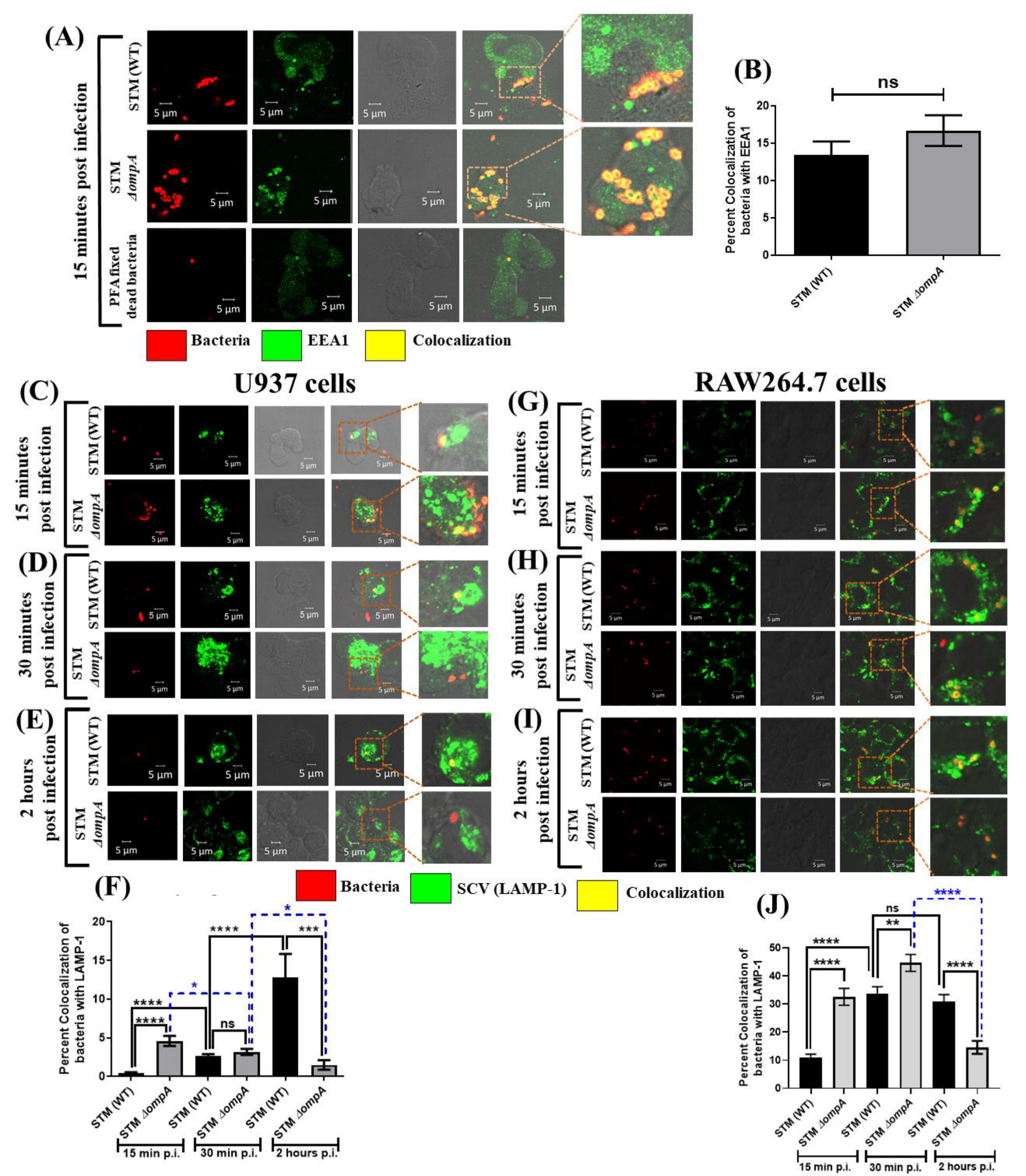
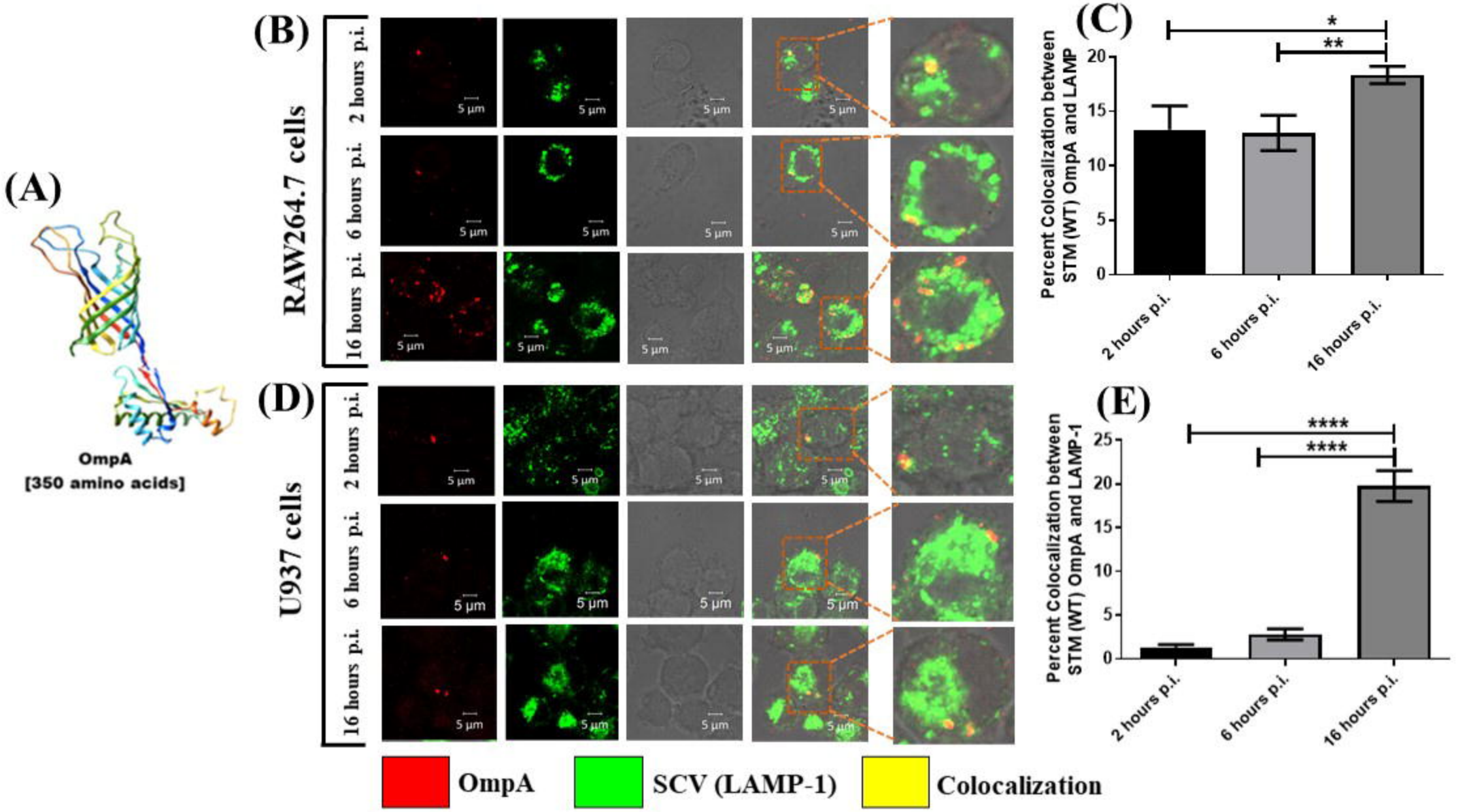
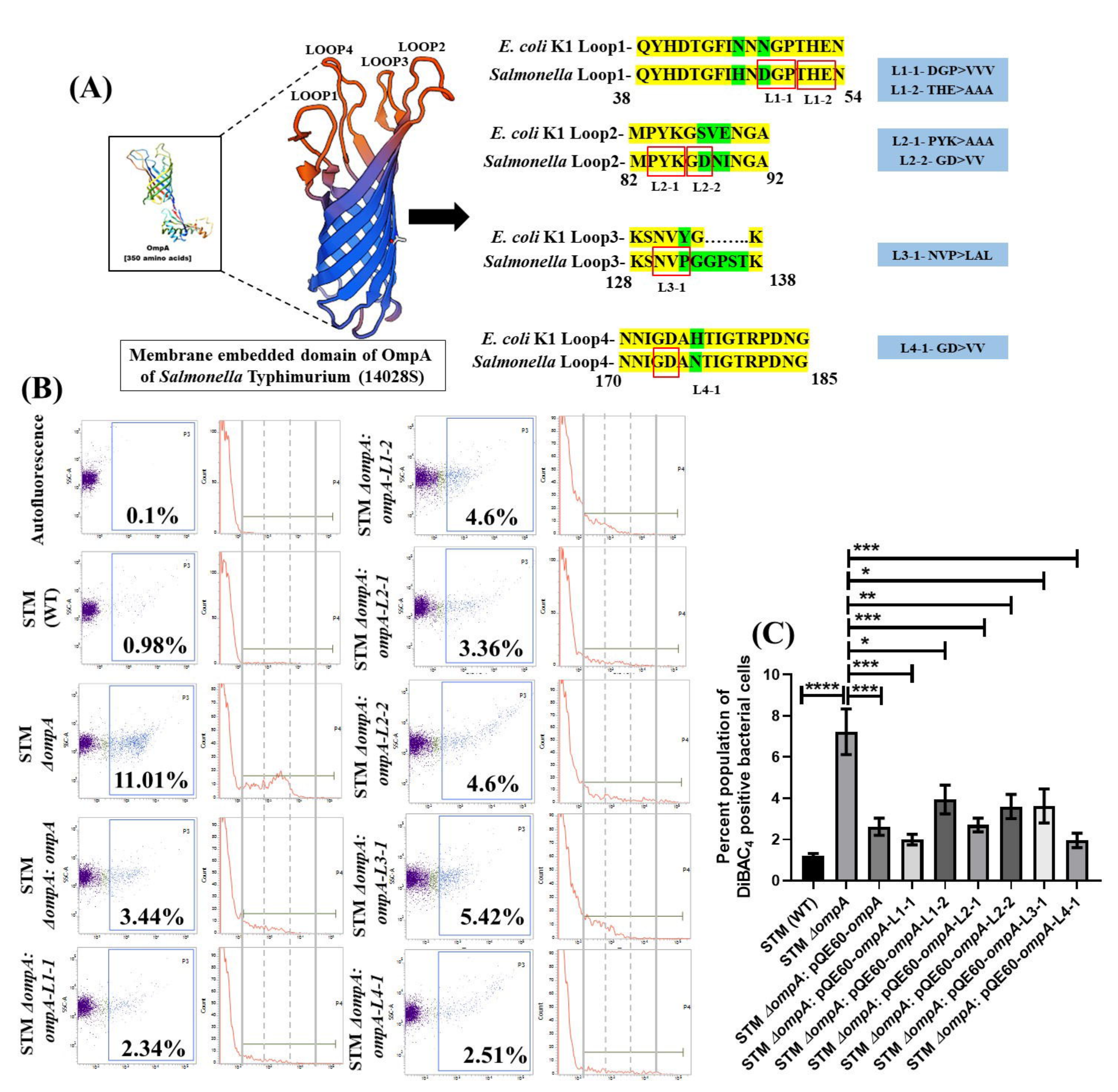
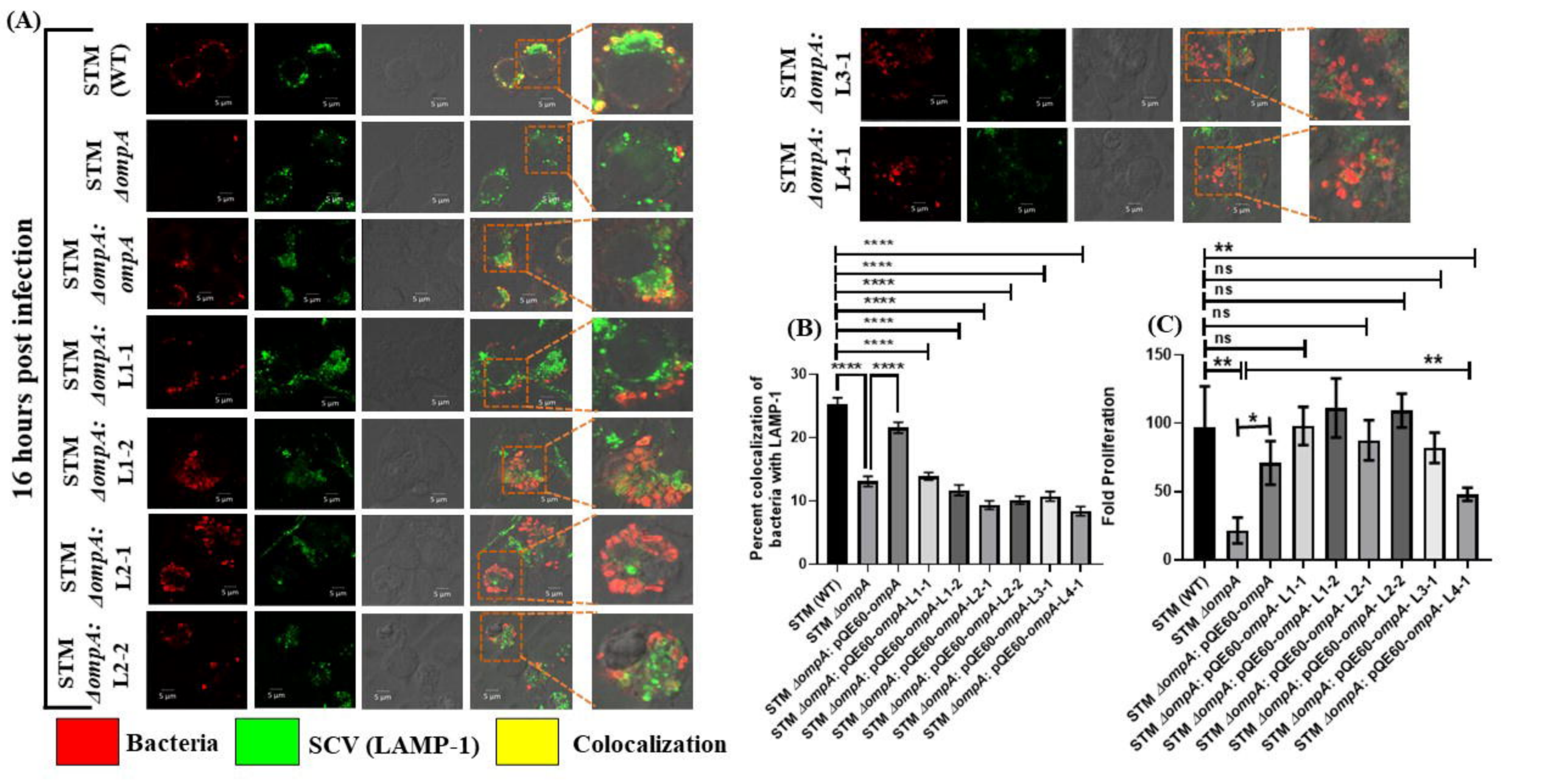
Bafilomycin A [50 nM]
- - + +

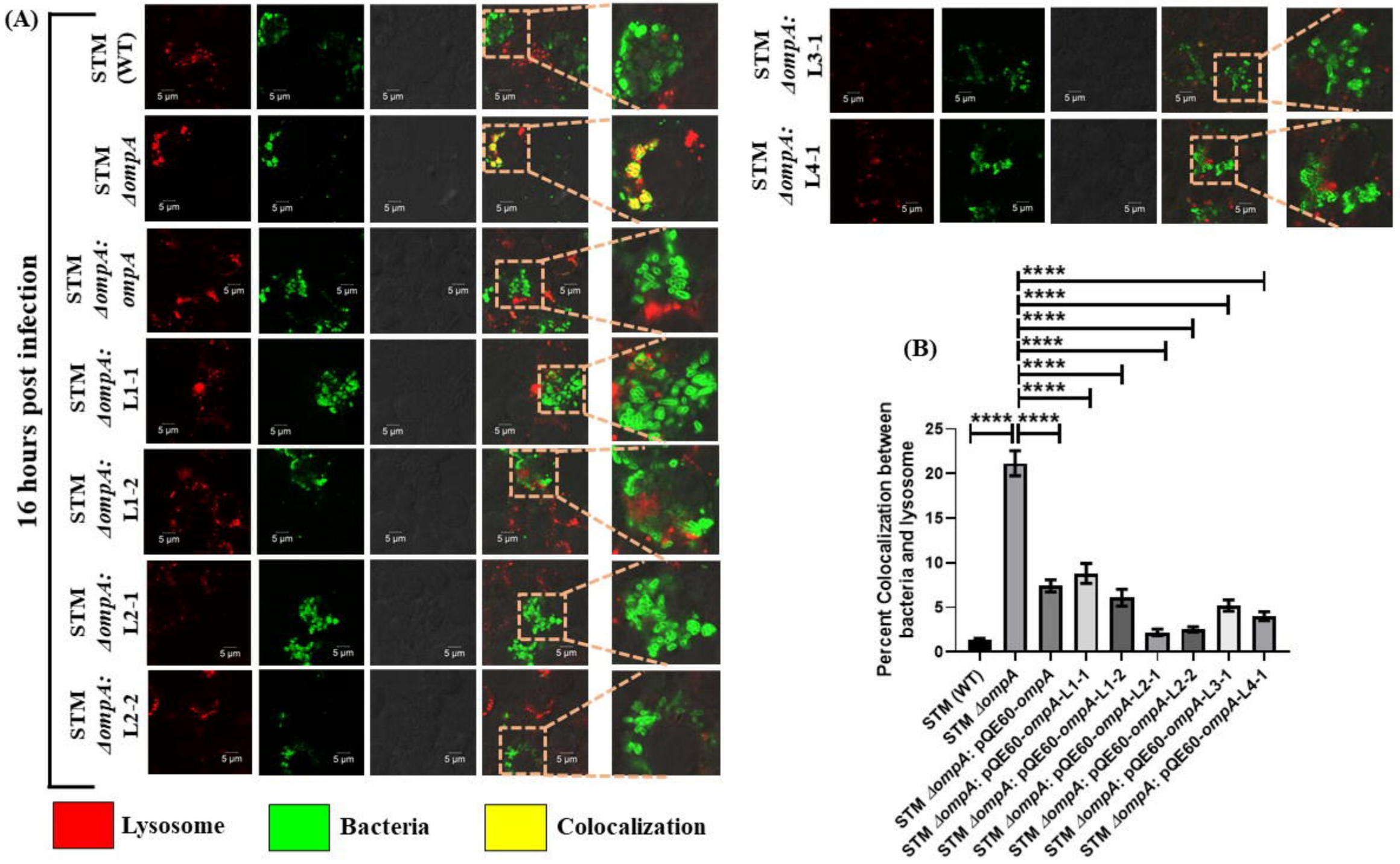

(A)
2 hours post infection
STM (WT)
STM ΔompA
STM ΔompA: ompA
5 µm
(B)
Percent colocalization of bacteria with LAMP-1
****
****
STM WT
STM ΔompA
STM ΔompA: pQE60-ompA
(C)
6 hours post infection
STM (WT)
STM ΔompA
STM ΔompA: ompA
5 µm
(D)
Percent colocalization of bacteria with LAMP-1
*
****
STM WT
STM ΔompA
STM ΔompA: pQE60-ompA

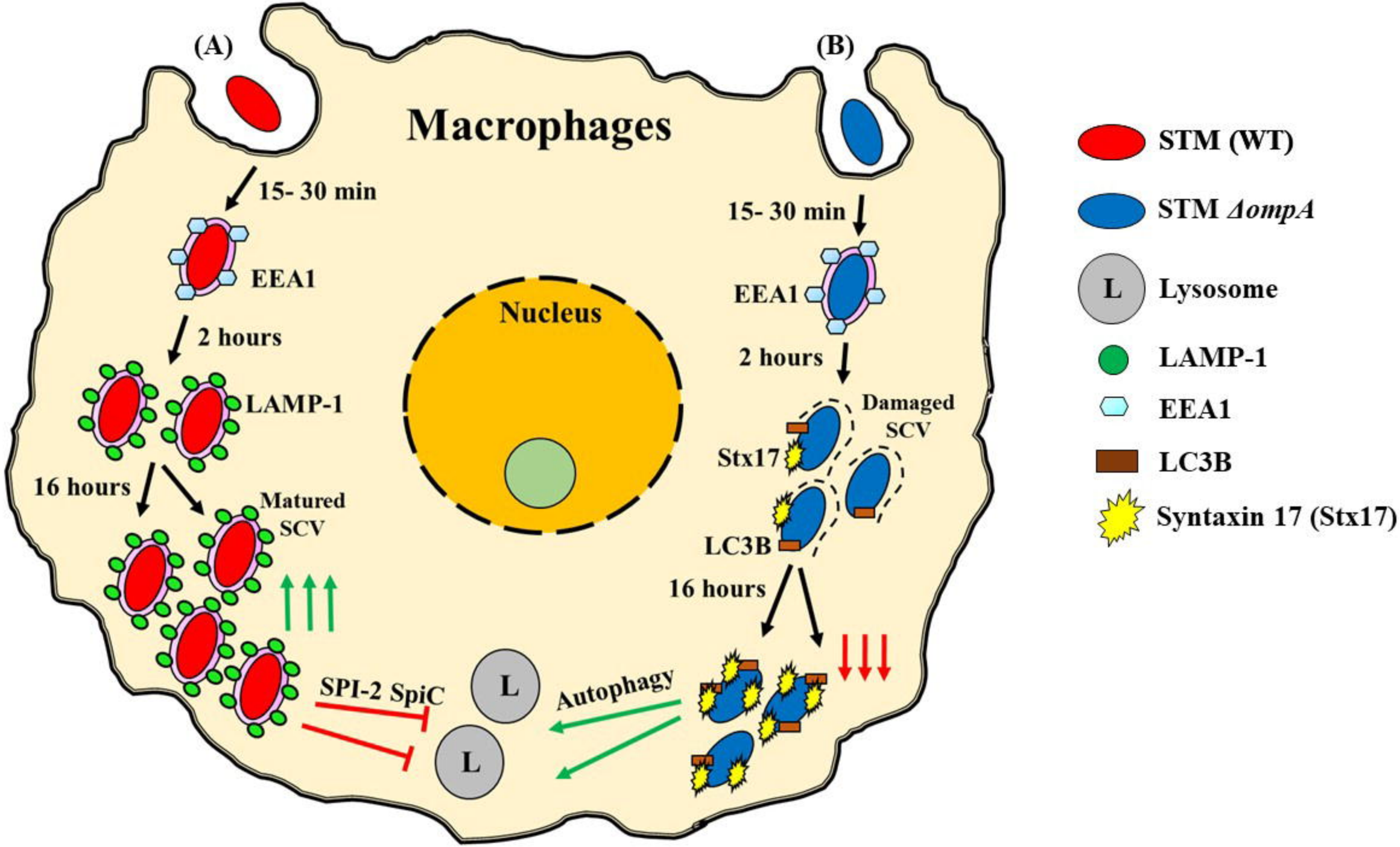

(A)
15 minutes post infection
STM (WT)
STM ΔompA
PFA fixed dead bacteria
5 µm
Bacteria
EEA1
Colocalization
(B)
ns
Percent Colocalization of bacteria with EEA1
STM (WT)
STM ΔompA
U937 cells
RAW264.7 cells
(C)
(D)
(E)
(F)
(G)
(H)
(I)
(J)
15 minutes post infection
30 minutes post infection
2 hours post infection
SCV (LAMP-1)
Percent Colocalization of bacteria with LAMP-1
****
***
**
*
ns
15 min p.i.
30 min p.i.
2 hours p.i.

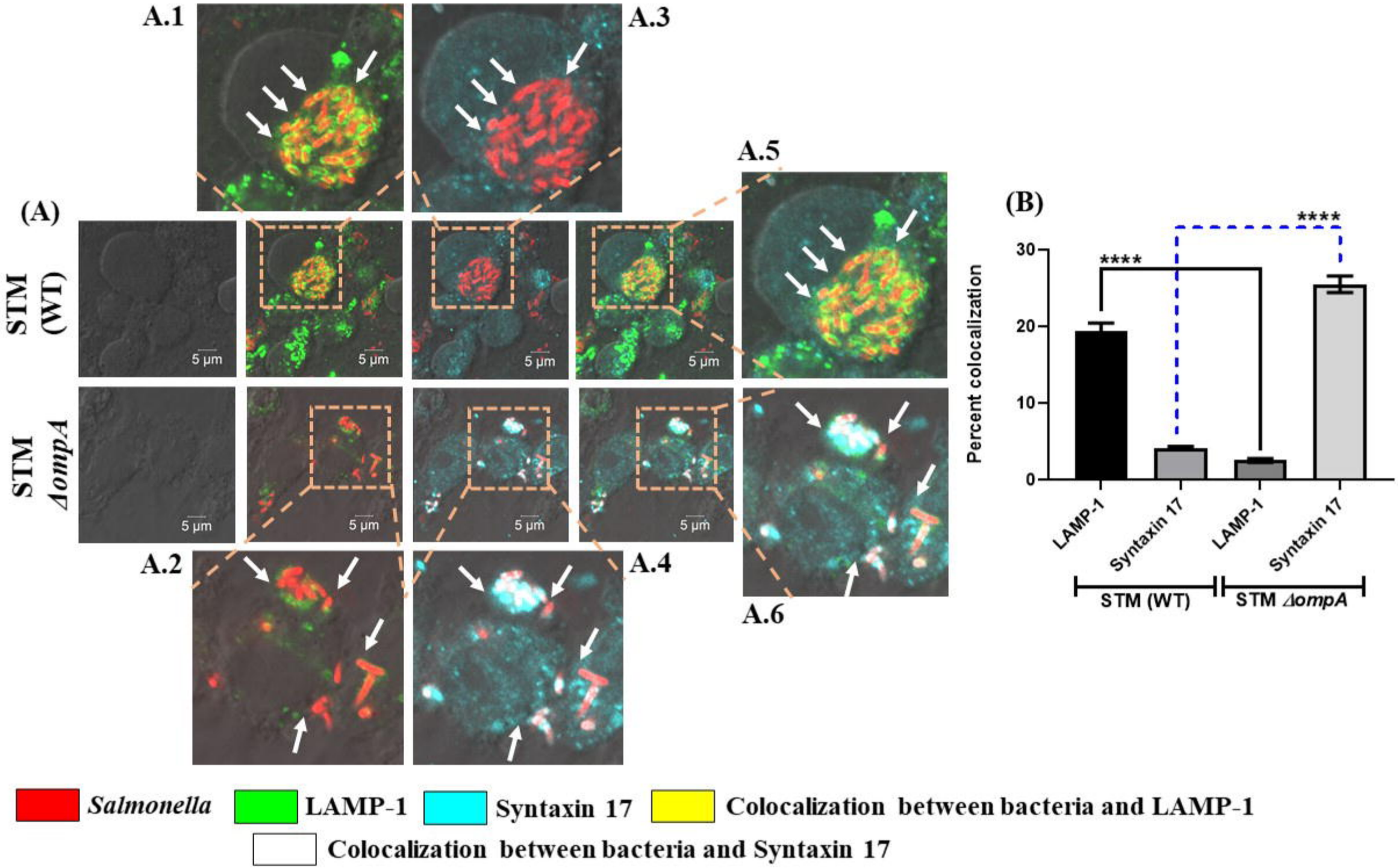

(A)
OmpA
[350 amino acids]
(B)
RAW264.7 cells
2 hours p.i.
6 hours p.i.
16 hours p.i.
5 µm
(C)
Percent Colocalization between STM (WT) OmpA and LAMP
*
**
2 hours p.i.
6 hours p.i.
16 hours p.i.
(D)
U937 cells
(E)
Percent Colocalization between STM (WT) OmpA and LAMP-1
****
OmpA
SCV (LAMP-1)
Colocalization

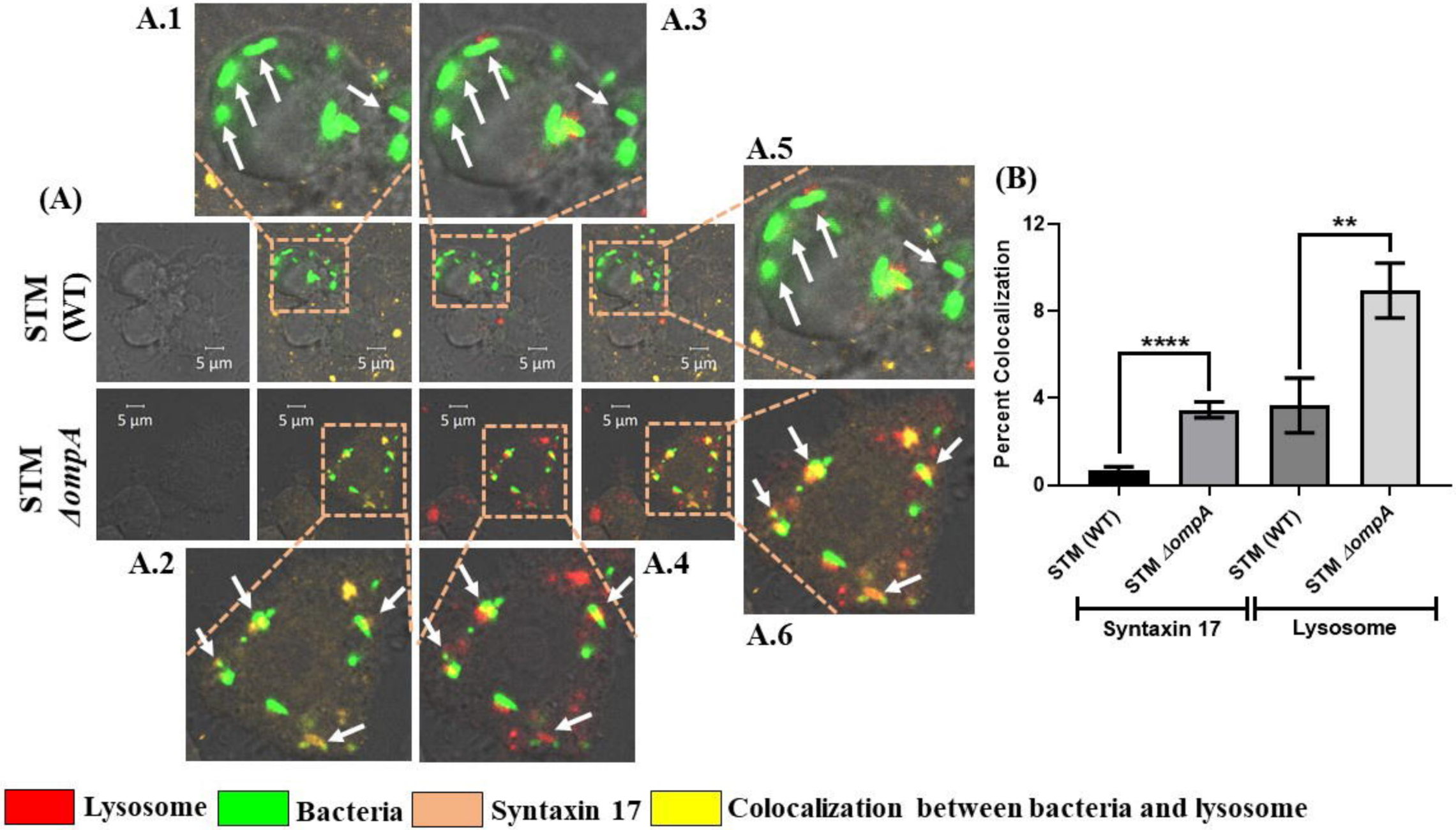

(A)
LOOP4
LOOP2
LOOP3
LOOP1
OmpA
[350 amino acids]
Membrane embedded domain of OmpA of Salmonella Typhimurium (14028S)
E. coli K1 Loop1- QYHDTGFINNNGPTHEN
Salmonella Loop1- QYHDTGFIHNDGPTHEN
38
L1-1
L1-2
54
L1-1- DGP>VVV
L1-2- THE>AAA
E. coli K1 Loop2- MPYKGSVENGA
Salmonella Loop2- MPYKGDNINGA
82
L2-1
L2-2
92
L2-1- PYK>AAA
L2-2- GD>VV
E. coli K1 Loop3- KSNVYG.....K
Salmonella Loop3- KSNVPGGPSTK
128
L3-1
138
L3-1- NVP>LAL
E. coli K1 Loop4- NNIGDAHTIGTRPDNG
Salmonella Loop4- NNIGDANTIGTRPDNG
170
L4-1
185
L4-1- GD>VV
(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%
(C)
Percent population of DiBAC4 positive bacterial cells
0
2
4
6
8
10
****
***
*
**
***
*
***
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

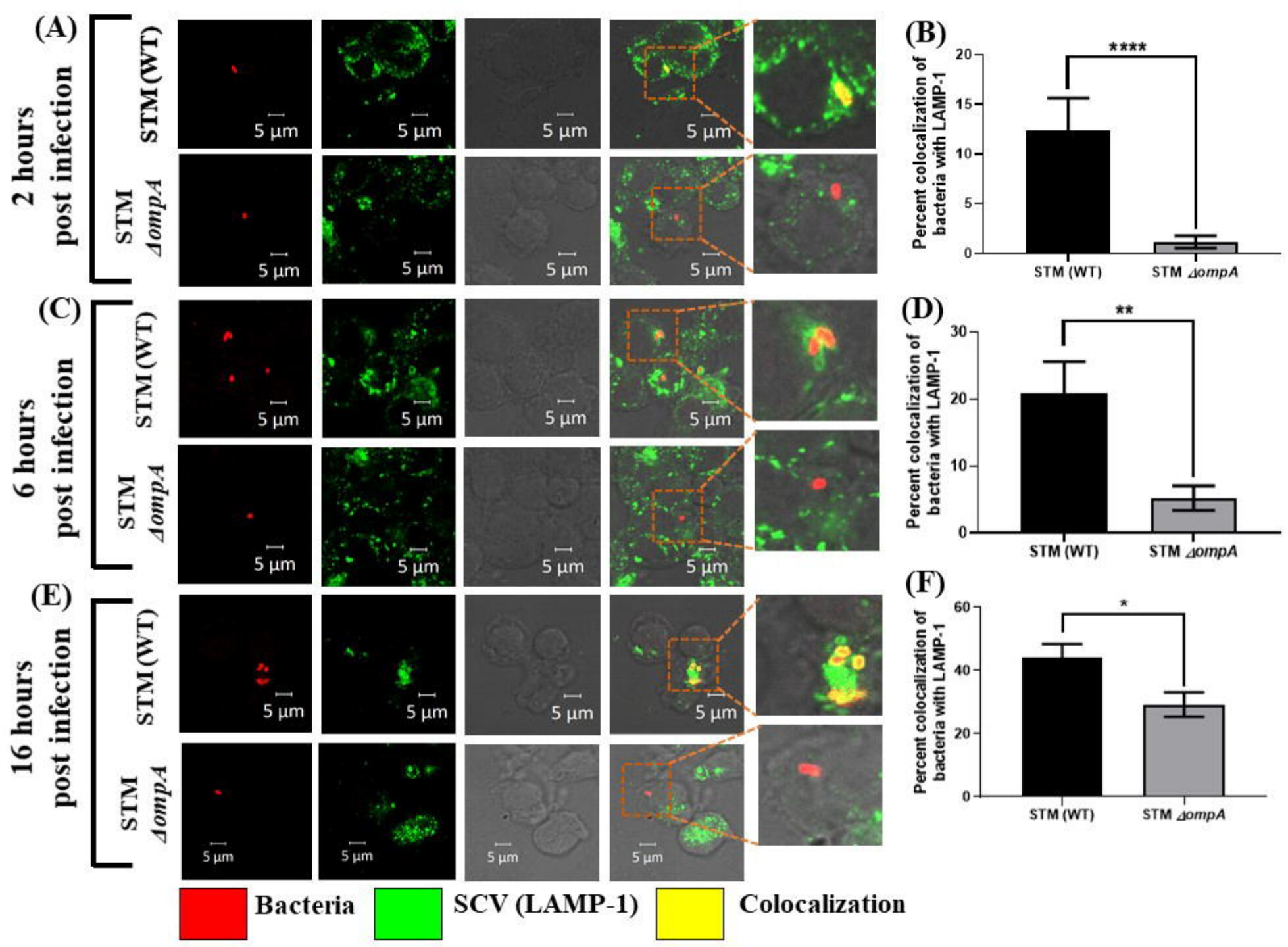

(A)
16 hours post infection
STM (WT)
STM ΔompA
STM ΔompA: ompA
STM ΔompA: L1-1
STM ΔompA: L1-2
STM ΔompA: L2-1
STM ΔompA: L2-2
STM ΔompA: L3-1
STM ΔompA: L4-1
5 µm
Bacteria
SCV (LAMP-1)
Colocalization
(B)
Percent colocalization of bacteria with LAMP-1
0
10
20
30
****
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
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
*

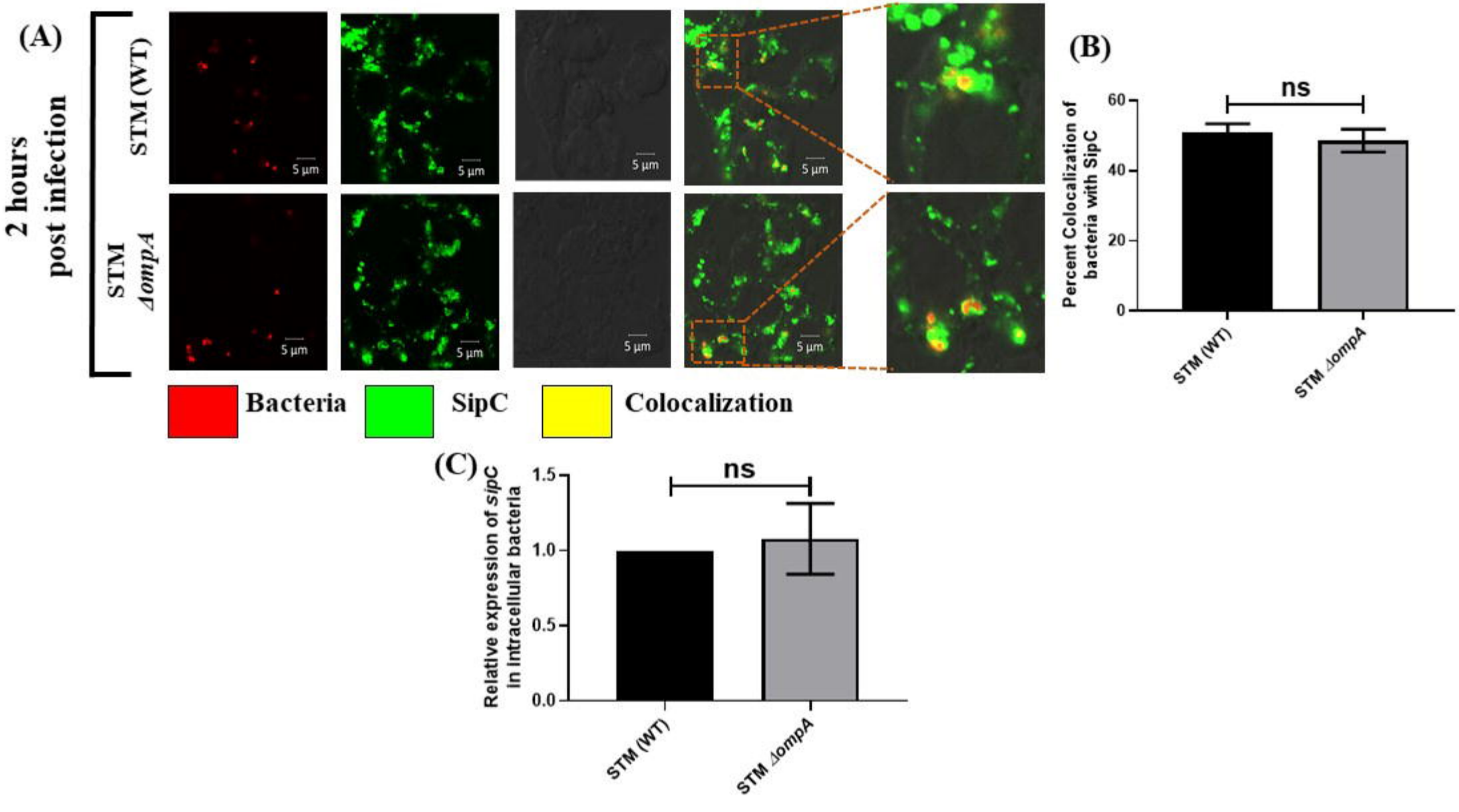

(A)
16 hours post infection
STM (WT)
STM ΔompA
STM ΔompA: ompA
STM ΔompA: L1-1
STM ΔompA: L1-2
STM ΔompA: L2-1
STM ΔompA: L2-2
STM ΔompA: L3-1
STM ΔompA: L4-1
5 μm
Lysosome
Bacteria
Colocalization
(B)
Percent Colocalization between bacteria and lysosome
0
5
10
15
20
25
****
STM (WT)
STM ΔompA
STM ΔompA: 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

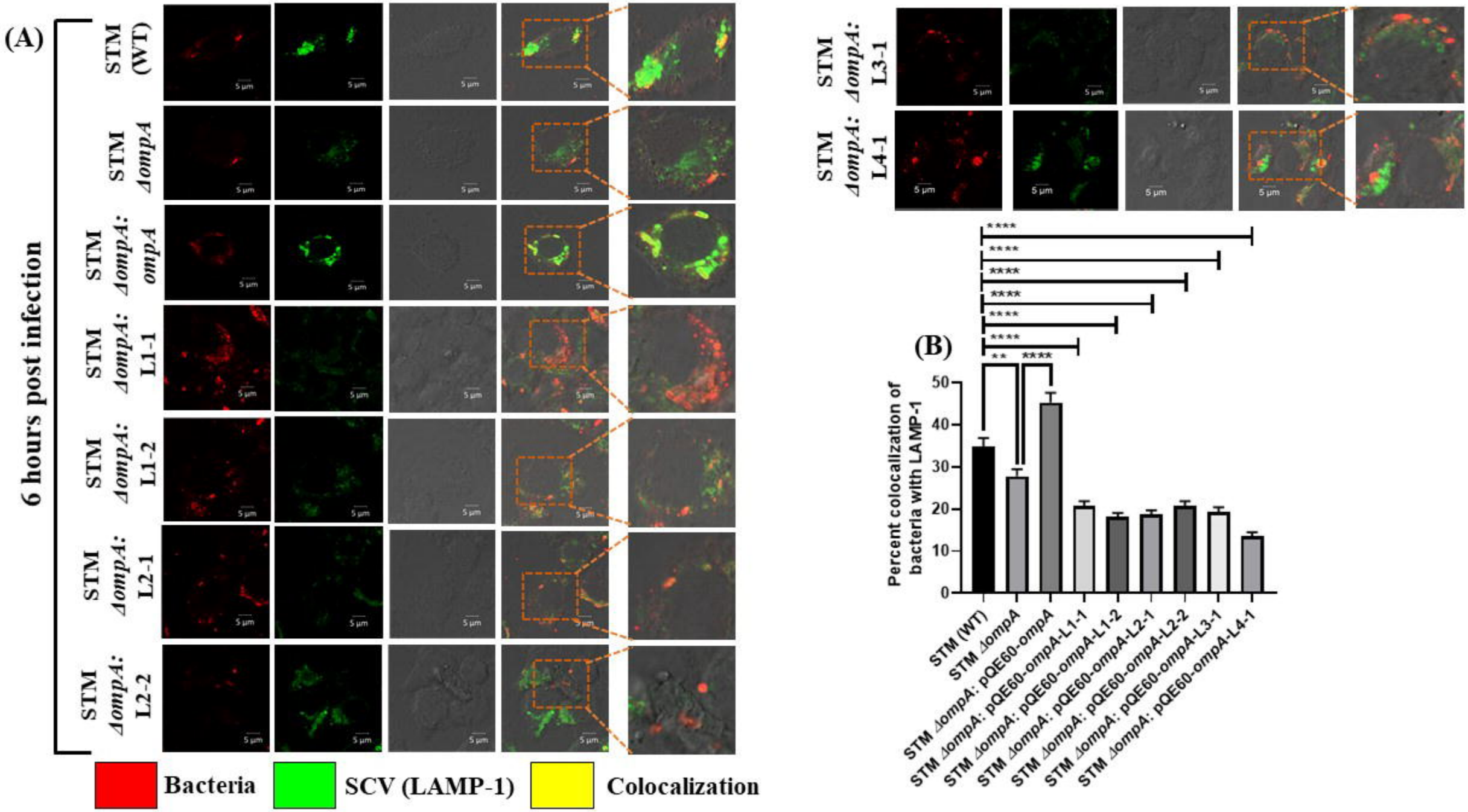

(A)
(B)
Macrophages
15- 30 min
EEA1
2 hours
LAMP-1
16 hours
Matured SCV
Nucleus
15- 30 min
EEA1
2 hours
Damaged SCV
Stx17
LC3B
16 hours
SPI-2 SpiC
L
L
Autophagy
STM (WT)
STM ΔompA
L
Lysosome
LAMP-1
EEA1
LC3B
Syntaxin 17 (Stx17)


A.1
A.3
A.5
(A)
STM (WT)
STM ΔompA
5 µm
A.2
A.4
A.6
(B)
Percent colocalization
30
20
10
0
****
****
LAMP-1
Syntaxin 17
LAMP-1
Syntaxin 17
STM (WT)
STM ΔompA
Salmonella
LAMP-1
Syntaxin 17
Colocalization between bacteria and LAMP-1
Colocalization between bacteria and Syntaxin 17


A.1
A.3
A.5
(A)
(B)
STM (WT)
STM ΔompA
5 µm
A.2
A.4
A.6
Percent Colocalization
12
8
4
0
****
**
STM (WT)
STM ΔompA
STM (WT)
STM ΔompA
Syntaxin 17
Lysosome
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
(B)
ns
Percent Colocalization of bacteria with SipC
60
40
20
0
STM (WT)
STM ΔompA
(C)
ns
Relative expression of sipC in intracellular bacteria
1.5
1.0
0.5
0.0
STM (WT)
STM ΔompA


(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
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
40
50
**
****
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