Salmonella Typhimurium outer membrane protein A (OmpA) renders protection against nitrosative stress by promoting SCV stability in murine macrophage

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Abstract

Porins are highly conserved bacterial outer membrane proteins having β barrel structure and are majorly involved in the selective transport of charged molecules across the membrane. Despite having huge contributions in the pathogenesis of many gram-negative bacteria, their role remains elusive in salmonellosis. In this study, we have characterized the pathogenic role of porins majorly found on the outer membrane of *Salmonella* Typhimurium (such as OmpA, OmpC, OmpD, and OmpF) paying the utmost importance to OmpA. The outer membrane protein A (OmpA) of *Salmonella* Typhimurium has shown a multifaceted role in our study. We have observed that deletion of *ompA* from wildtype *Salmonella* has made it more prone to phagocytosis and weakly proliferative in macrophages. Whereas, in epithelial cells STM *ΔompA* was found to be invasion deficient and hyper-proliferative. The poor colocalization of STM *ΔompA* with LAMP-1 confirmed impaired stability of SCV membrane around the intracellular bacteria, which further resulted in the release of the knockout strain to the cytosol of macrophage where it is bombarded with reactive nitrogen intermediates (RNI). The cytosolic localization of STM *ΔompA* was found to be responsible for the downregulation of SPI-2 encoded virulent factor SpiC which is required for suppressing the activity of iNOS. The reduced recruitment of nitrotyrosine on wildtype *Salmonella* staying in the cytosol of macrophage by ectopically expressing Listeriolysin O (LLO) strongly proves the pro-bacterial role of OmpA against host nitrosative stress. The time-dependent increase in 405/ 488 ratio of STM *ΔompA* pQE60-Grx1-roGFP2 exposed to *in vitro* acidified nitrite suggested RNI dependent redox burst which further answered the reason for its enhanced sensitivity towards nitrosative stress. Our study further demonstrated loss of integrity and enhanced porosity in the bacterial outer membrane in absence of OmpA. The enhanced porosity of the bacterial outer membrane was further attributed to the upregulated expression of larger porins namely *ompC*, *ompD*, and *ompF*. In comparison with STM *ΔompA ΔompC* and *ΔompA ΔompF*, the enhanced

uptake of nitrite and greater recruitment of nitrotyrosine on intracellular STM *ΔompA ΔompD* demonstrate the involvement of OmpC and OmpF in the entry of excess nitrite in *ompA* deficient bacteria.

Keywords: *Salmonella* Typhimurium, Outer membrane protein A, *Salmonella* containing vacuole, Nitrosative stress, Reactive Nitrogen Intermediates.

Introduction

Salmonella enterica Serovar Typhimurium is a gram-negative, rod-shaped, flagellated, facultative anaerobic bacterium belonging to the family *Enterobacteriaceae* and major causative agent of typhoid fever-like symptoms in mice and self-limiting gastroenteritis in humans (Haraga et al., 2008, Galan, 2001). Typhoid fever is fatal if not treated in time and considered as one of the serious threats to global health. Despite having a significant worldwide reduction in incidence and fatality related to typhoid fever in the past two decades, it still remains a major cause of morbidity and mortality in developing nations. Globally, the occurrence of 14.3 million cases related to typhoid and paratyphoid fever along with 135.9 thousand deaths have been registered in 2017 (Bill and Foundation, 2019). *Salmonella* Typhimurium rapidly spread through the contaminated food and water while ingested by a healthy individual. After entering the host's body *Salmonella* eventually adheres and invades the intestinal epithelial cells of the small intestine and crosses the intestinal mucosal barrier through the M cells (microfold cells) of Peyer's patches. During the systemic phase of infection wildtype *Salmonella* inhabiting in macrophage, dendritic cells, polymorphonuclear cells can reach many parts of the body namely liver, spleen, mesenteric lymph node (MLN), brain, bone marrow, gall bladder, etc. (Gogoi et al., 2019) The infection caused by *Salmonella* is predominantly governed by two extensively characterized pathogenicity islands called SPI-1 and SPI-2 coding for a plethora of effector proteins and two distinct type 3 secretion systems (T3SS), which broadly regulate the adhesion, invasion and intracellular survival of the bacteria (Garai et al., 2012).

The pathogenesis of many gram-negative bacteria belonging to the family *Enterobacteriaceae* is immensely regulated by porins, commonly known as outer membrane proteins (or Omp). Porins are bacterial outer membrane-bound β barrel proteins with 8 to 24 antiparallel strands connected by loops and are well known for their role in the selective diffusion of ions and

solutes across the impermeable outer membrane of bacteria. (Vergalli et al., 2020) Despite their contribution to the maintenance of outer membrane stability, their pathogenic functions have also been well documented. OmpA, one of the most abundant porins of the bacterial outer membrane, is extensively utilized by *Klebsiella pneumoniae* to prevent the IL-8 dependent pro-inflammatory response in airway epithelial A549 cells. (March et al., 2011) The deletion of *ompC* and *ompF* from avian pathogenic *E. coli* not only impaired its invasion in bEnd.3 cells and but also reduced its virulence in *in vivo* mouse model. (Hejair et al., 2017) OprF an ortholog of OmpA present in the outer membrane of *Pseudomonas sp.* has been reported to function as a sensor of quorum sensing to induce the virulence of bacteria. (Krishnan and Prasadarao, 2012) OmpW of *E. coli* has been reported to have a significant role against phagocytosis and complement activation (Wu et al., 2013, Li et al., 2016).

The outer membrane of *Salmonella* Typhimurium, a member of the *Enterobacteriaceae* family, is densely populated with many porins such as OmpA, OmpC, OmpD, and OmpF. Unlike OmpA which has a massive role in the tight attachment of the outer membrane to the underlying peptidoglycan layer with the help of its periplasmic tail (Park et al., 2012), the other porins facilitate transportation of charged ions (Ipinza et al., 2014). The connection between the outer membrane porins possessed by wildtype *Salmonella* Typhimurium and its pathogenesis remains elusive due to the lack of detailed studies. Earlier Heijden *et al*. proposed a mechanism on OmpA and OmpC dependent regulation of outer membrane permeability in wildtype *Salmonella* in response to H_2O_2 stress and antibiotic (Heijden et al., 2016).

In the current study reported here, we have investigated the individual role of OmpA, OmpC, OmpD, and OmpF in the pathogenesis of *Salmonella* Typhimurium with a key focus on OmpA. We have found a strong dependence of wildtype *Salmonella* on OmpA for its survival in macrophages as well as in *in vivo* mouse model. Our study firmly illustrated a mechanism of strategic utilization of OmpA in comparison with other porins by wildtype *Salmonella* for

combatting the nitrosative stress of macrophages. Our study firmly endorses the importance of OmpA in the regulation of outer membrane stability of wild-type bacteria. To the best of our knowledge, this is the first study to demonstrate the impact of outer membrane porins in the maintenance of vacuolar stability of wildtype *Salmonella* in macrophage and epithelial cells.

Results

OmpA promotes the evasion of phagocytosis and intracellular survival of *Salmonella* in macrophages.

The transcriptomic analyses of intracellular *Salmonella* Typhimurium infecting J774-A.1 and HeLa cells have demonstrated almost 2 to 2.5 fold induction in the expression level of *ompA* during early (4 hours), middle (8 hours), and late (12 hours) stage of infection in comparison with other major membrane porins namely *ompC*, *ompD*, and *ompF* (Eriksson et al., 2003), (Hautefort et al., 2008). To validate this observation, we have isolated RNA from wild type *Salmonella* Typhimurium strain 14028S growing in nutritionally enriched Luria-Bertani broth, low magnesium minimal acidic F media (pH= 5.4) mimicking the nutrient-deprived acidic environment of *Salmonella* containing vacuole (SCV) (Deiwick et al., 1999), and RAW264.7 cells at different time points (3, 6, 9, 12 hours post-inoculation/ infection) and investigated the transcripts level of *ompA* (Figure S1A), *ompC* (Figure S1B), *ompD* (Figure S1C), and *ompF* (Figure S1D) by real-Time PCR. Unlike the other three major outer membrane porins- *ompC*, *ompD*, & *ompF*, whose expressions were steadily decreasing in wildtype bacteria growing in LB broth, F media and macrophage during all the indicated time points except *ompF* in macrophage at 9th hour post-infection (Figure S1D), we found a significant increase in the transcript level of *ompA* during the late phase of infection (9 and 12 hours post-infection) in macrophage (Figure S1A). Taken together, these results strongly demonstrate the preference of wildtype *Salmonella* to *ompA* over other membrane-bound larger porins to thrive inside the

141 macrophage and concomitantly satisfy the microarray data published by Eriksson *et al*., and
142 Hautefort *et al* (Eriksson et al., 2003, Hautefort et al., 2008). Because of their surface
143 localization, bacterial porins have a robust role in interaction with host immune cells. Earlier it
144 has been reported by multiple groups that, an eight stranded β barrel outer membrane porin
145 (*ompW*) of *Escherichia coli* helps the bacteria to evade phagocytosis and confers resistance
146 against alternative complement activation pathway mediated killing of the host (Wu et al.,
147 2013, Li et al., 2016). Another study carried out by March C *et al*. suggested that the *ompA*
148 mutant strain of *Klebsiella pneumoniae* is severely attenuated in the pneumonia mouse model
149 (March et al., 2011). In our study, we have found enhanced expression of *ompA* in wildtype
150 *Salmonella* Typhimurium growing in murine macrophage. Hence, we have hypothesized that
151 *ompA* may have a substantial role in the intracellular survival of wild-type *Salmonella.* To
152 verify our hypothesis, we have knocked out *ompA* from the genome of *Salmonella*
153 Typhimurium using one step chromosomal gene inactivation method demonstrated by
154 Datsenko and Wanner (Figure S2A and S2B) (Datsenko and Wanner, 2000). The wildtype
155 *ompA* gene (of size 1.3 kb) was replaced with a kanamycin-resistant gene cassette (of size 1.7
156 kb) (Figure S2A). The knockout was further confirmed with kanamycin-resistant gene cassette
157 specific internal primers (amplicon length 471 bp or .47 kb) (Figure S2B). The
158 complementation of the *ompA* gene in the pQE60 vector (size of recombinant plasmid 4.5 kb)
159 was primarily confirmed by dual restriction digestion which has released an insert of size 1.1
160 kb (empty vector size 3.4 kb) (Lane- DD, Figure S2C). Further, the knockout and
161 complementation were confirmed by RT PCR [almost 1.42 times induction of *ompA* in the
162 complemented strain compared to the wild type *S.* Typhimurium] (Figure S2D). It was also
163 found that knocking out of *ompA* from *Salmonella* does not alter the *in vitro* growth kinetics of
164 the bacteria in LB broth culture (Figure S2E and S2F). To find out the role of *S.* Typhimurium
165 *ompA* in phagocytosis, we have checked the phagocytosis of wildtype and knockout bacteria

166 in macrophages (Figure 1A). We have found that outer membrane protein A (OmpA) of *S.*
167 Typhimurium helps the bacteria to evade phagocytosis by RAW264.7 and activated U937 cells
168 (Figure 1A). The percent phagocytosis of STM *ΔompA* in both RAW 264.7 and U937 cells are
169 more compared to STM (WT). The complemented strain is less prone to be phagocytosed by
170 the macrophage-like wild type *S.* Typhimurium in both the cell lines. Adhesion of bacteria onto
171 the host cell surface takes place before they enter into the host cell, either by phagocytosis or
172 by invasion (Garai et al., 2016). Since we found enhanced phagocytosis of STM *ΔompA* in
173 macrophages in comparison to wild type bacteria, we decided to evaluate the bacterial
174 attachment on macrophage surface by *in vitro* adhesion assay (Figure 1B). We have found that
175 the adhesion of STM *ΔompA* on phagocytic RAW 264.7 cells is more compared to STM (WT)
176 (Figure 1B). The enhanced adhesion of the knockout strain was abrogated in the complemented
177 strain. To investigate the role of OmpA in the intracellular survival of bacteria, we have
178 performed an intracellular survival assay of STM (WT) and *ΔompA* strains in RAW264.7,
179 activated U937 cells respectively (Figure 1C). We have found that the intracellular growth of
180 STM *ΔompA* [fold proliferation in RAW264.7 cells- 11.45± 3.349, U937 cells- 5.075± 1.157]
181 is significantly attenuated in RAW 264.7 (Figure 1I) (by 2.75 folds) and activated U937 (Figure
182 1J) cells (by 3.08 folds) compared to the wild type bacteria [fold proliferation in RAW264.7
183 cells- 31.5± 5.347, U937 cells- 15.65± 3.981]. When both the cell lines were infected with the
184 complement strains the intracellular proliferation of bacteria was recovered (Figure 1C).
185 Hence, we have concluded that wildtype *Salmonella* utilizes OmpA as a double-edged sword
186 to protect itself from phagocytosis and concomitantly for its survival in macrophages. OmpA
187 is used as an essential appendage by a wide range of pathogens (nosocomial pathogen
188 *Acinetobacter* baumannii, enteric pathogen *Enterobacter sakazakii,* etc.) to invade epithelial
189 cells (Choi et al., 2008, Mohan Nair and Venkitanarayanan, 2007). *Salmonella* is an enteric
190 pathogen, once ingested with contaminated food or water, starts invading M cells of the Payer's

191 patch in the small intestine with the help of SPI-1 encoded type 3 secretion system. Hence, we
192 decided to check the role of *Salmonella* OmpA in bacterial invasion of non-phagocytic
193 epithelial cells (Figure S3A). It has been found that, in comparison with wild-type bacteria,
194 STM *ΔompA* has exhibited notable attenuation in the invasion of human colorectal
195 adenocarcinoma cell line- Caco-2 and human cervical cancer cell line- HeLa (Figure S3A).
196 The bacteria retrieved its invasion ability once the gene is complemented in the knockout
197 background (Figure S3A). To validate this observation, we further conducted *in vitro* adhesion
198 assay using HeLa cells as host models (Figure S3B). STM *ΔompA* has shown reduced
199 attachment on the surface of HeLa cells compared to the wild-type bacteria (Figure S3B). The
200 inefficiency of the knockout strain to attach the epithelial cell surface was revoked in the
201 complemented strain (Figure S3B). This observation was consistent with the result obtained
202 from the invasion assay and substantiated the fact of utilization OmpA by wildtype *Salmonella*
203 as an adhesion and invasion tool with paramount importance. We further verified the role of
204 OmpA in the maintenance of intracellular life of wild-type bacteria in an epithelial cell by
205 intracellular proliferation assay (Figure S3C). Surprisingly, STM *ΔompA* has exhibited
206 hyperproliferation in both Caco-2 and HeLa cells (Figure S3C) compared to wild-type bacteria.
207 The intracellular proliferation of complement strain was comparable to the wild-type bacteria
208 in both the cell lines. Since we have found opposite outcomes in the intracellular survival of
209 the *ompA* knockout strain of *Salmonella* in two different cell types (attenuation in macrophage
210 and hyper-proliferation in the epithelial cell), we decided to monitor the intracellular niche of
211 the bacteria in both cell types. After entering the host cells *Salmonella* resides inside a modified
212 phagosomal compartment called *Salmonella* Containing Vacuole (SCV), which is acidic in
213 nature. The intracellular life and proliferation of *Salmonella* depend upon the stability and
214 integrity of SCV. *Salmonella* recruits a plethora of host proteins to maintain the sustainability
215 of SCV. During the early stage of infection in macrophage and epithelial cells, SCV is

216 characterized by the presence of markers of early endocytic pathway such as EEA1, Rab5,
217 Rab4, Rab11 and transferrin receptors etc. These proteins are replaced by the markers of late
218 endosome such as LAMP-1, Rab7, vATPase etc within 15 to 45 minutes post infection (Garai
219 et al., 2012, Knuff and Finlay, 2017). Since we found an attenuation in the intracellular
220 proliferation of STM *ΔompA* in macrophage and hyperproliferation in epithelial cell, we
221 decided to check the intracellular niche of the bacteria using LAMP-1 as a marker of SCV in
222 RAW264.7 cells (Figure 1D) and Caco-2 cells (Figure S3D) 16 hours post-infection. It has
223 been observed that the percent colocalization of LAMP-1 with STM *ΔompA* is less compared
224 to STM (WT) in RAW264.7 cells (Figure 1D). The colocalization with LAMP-1 further
225 increased when the macrophage cells were infected with complement strain. The less
226 colocalization of STM *ΔompA* in comparison with wildtype and complemented strains of
227 *Salmonella* not only corroborates the gradual loss of SCV membrane from its surrounding but
228 also its enhanced cytosolic localization in macrophage (Figure 1D). The PFA fixed dead
229 bacteria lacking the ability to quit vacuole was used as positive control in this study (Figure
230 1D). We further proved the cytosolic inhabitation of *ompA* knockout bacteria in macrophage
231 using a pH sensitive dye called pH rhodo red succinimidyl ester whose fluorescence intensity
232 increases as the pH of the system decreases from neutral to acidic (Figure S4A). In comparison
233 with wildtype *Salmonella* staying in the acidic pH of SCV (pH= 5 - 5.4) (Chakraborty et al.,
234 2015), the reduction in the cumulative median fluorescence intensity of STM *ΔompA* labelled
235 with pH rhodo red succinimidyl ester confirms its cytosolic abundance where the pH is almost
236 neutral (pH= 7.2) (Figure S4A) (Canton et al., 2014). The successful systemic infection caused
237 by intracellular *Salmonella* depends upon the surface accumulation as well as secretion of SPI-
238 2 encoded effector proteins from bacteria into the host cell cytoplasm with the help of a needle
239 like complex called type III secretion system (T3SS). The formation of this needle complex is
240 further regulated by the secretion and assembly of SPI-2 encoded translocon proteins- SseB,

241 SseC, SseD on the surface of the intravacuolar bacteria inside the lumen of SCV (Chakravortty
242 et al., 2005, Hölzer and Hensel, 2010). Since cytosolic population of STM *ΔompA* lacks the
243 vacuolar membrane, we anticipated an interruption in the accumulation of these translocon
244 proteins on bacterial surface and their secretion into the host cytosol. In line with our previous
245 observations, we found a marked reduction in the accumulation and secretion of SseC (Figure
246 S4B, S4C and S4F) and SseD (Figure S4D, S4E and S4H) on or from the surface of STM
247 *ΔompA* in comparison with wildtype bacteria into the host cytosol. In continuation of this
248 observation we found dampened expression of *sseC* (Figure S4G) and *sseD* (Figure S4I) genes
249 in intracellularly growing STM *ΔompA*. To verify whether this is a cell type specific
250 phenomenon or not, we further investigated the intracellular niche of wildtype, knockout and
251 complement strains of *Salmonella* in epithelial Caco-2 cells. The quantitative percent
252 colocalization between LAMP-1 and all three bacterial strains (Figure S3D) clearly
253 demonstrated that STM *ΔompA* comes into the cytoplasm of Caco-2 cells after being released
254 from the SCV during late phase of infection, while wildtype and the complement strains
255 abstained themselves from doing so (Figure S3D). These findings were further supported by
256 chloroquine resistance assay of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ΔompA* in RAW264.7
257 (Figure 1E) and Caco-2 cells (Figure S3E). After being protonated inside SCV because of the
258 acidic pH, chloroquine can't quit the vacuole and kills the vacuolar population of *Salmonella*,
259 allowing the growth of cytosolic population. During late phase of infection (16 hours post
260 infection) in RAW264.7 cells (Figure 1E) and Caco-2 cells (Figure S3E), the cytosolic
261 abundance of STM *ΔompA* was found more compared to the wildtype and complement strain.
262 Taken together, all these results authenticate the fact that intravacuolar life of intracellular
263 *Salmonella* strongly depends upon OmpA irrespective of cell type.

264 **OmpA dependent protection of *Salmonella* against nitrosative stress inside RAW264.7**
265 **cells.**

266 Professional phagocytes like- macrophages, neutrophils, dendritic cells, etc. employ multiple
267 strategies to combat invading pathogens. The intracellular population of *Salmonella* is heavily
268 encountered by NADPH phagocytic oxidase-dependent oxidative burst and iNOS dependent
269 nitrosative burst during early and late stages of infection in macrophages respectively (Foster
270 et al., 2003, Umezawa et al., 1997). The SCV membrane protects the vacuolar niche of wildtype
271 *Salmonella* from all the potential threats present in the cytoplasm of macrophage in form of
272 Reactive Oxygen Species (ROS), Reactive Nitrogen Intermediates (RNI), antimicrobial
273 peptides (AMPs), etc (Heijden et al., 2015, Salcedo et al., 2002). Our study confirmed the
274 proneness of STM *ΔompA* to get released from SCV into the cytoplasm of macrophage and
275 epithelial cells during the late stage of infection. Hence, we comprehended the increasing
276 possibility of the mutant bacteria being bombarded with ROS and RNI in the cytoplasm of the
277 macrophage, which was assumed to be the probable reason for its attenuation of intracellular
278 proliferation. Our hypothesis was verified by measuring both nitrosative (Figure 2A – 2C) and
279 oxidative (Figure S5A – S5C) stress response of macrophages infected with wild-type,
280 knockout, and complement strains. We have quantified the extracellular (Figure 2A) [NO]
281 response produced by infected macrophages by Griess assay. It has been found that during the
282 late stage of infection (16 hours post-infection) the accumulation of nitrite in the culture
283 supernatant of RAW264.7 cells infected with STM *ΔompA* was significantly higher compared
284 to the wildtype. This heightened [NO] response was further revoked when the RAW264.7 cells
285 were infected with complement strain (Figure 2A). This result was further validated by
286 quantification of intracellular [NO] response using DAF2-DA (Figure 2B). Only (4.468 ±
287 0.774) % of wildtype bacteria infected macrophages produced [NO] (Figure 2B), which was
288 increased to (7.939 ± 1.056) % when the cells were infected with STM *ΔompA* (Figure 2B).
289 The percent population of cells producing [NO], after being infected with complement strains
290 (4.689 ± 0.5035) % was comparable to the wildtype infected cells (Fig 2B). During late stage

291 of infection (16 hours post infection) in macrophage the intracellular ROS and extracellular
292 H_2O_2 level were estimated using H_2DCFDA (Figure S5A and S5B) and phenol red assay
293 (Figure S5C) respectively. We did not find any considerable change in the percent population
294 of cells producing ROS, infected with wildtype (2.157 ± 0.1611) %, knockout (2.192 ± 0.2955)
295 % and complement strains (2.61 ± 0.2244) % of *Salmonella* (Figure S5A and S5B). To validate
296 this observation, we further went ahead to check the accumulation of H_2O_2 in the culture
297 supernatants of infected RAW264.7 cells. But there was hardly any difference in H_2O_2
298 production (Figure S5C). The [NO] produced from cellular pool of L- arginine by the catalytic
299 activity of inducible nitric oxide synthase (iNOS) is further oxidized into [NO]-adducts
300 (NONOates, peroxynitrite, nitrite etc.) with the help of the superoxide ions which have higher
301 oxidation potential and bactericidal activity (Chakravortty et al., 2002). The damage that is
302 caused to bacteria by peroxynitrite ($ONOO^-$) can be monitored by the recruitment of
303 nitrotyrosine on the surface of intracellular bacteria using specific anti-nitrotyrosine antibody
304 by confocal microscopy (Figure 2C). During the late stage of infection in RAW264.7 cells, the
305 STM *ΔompA* strain has shown higher surface recruitment of nitrotyrosine residues and greater
306 percent colocalization in comparison with the wildtype bacteria (Figure 2C), indicating
307 massive damage caused by the bactericidal function of peroxynitrite. *Listeria monocytogenes*
308 produce listeriolysin O (LLO) a pore-forming toxin to degrade the vacuolar membrane to
309 escape lysosomal fusion (Ruan et al., 2016). Despite having intact OmpA on its surface,
310 wildtype *Salmonella* residing in the cytoplasm due to the ectopic expression of listeriolysin O
311 (LLO) showed poor colocalization with nitrotyrosine (Figure 2C). This suggests the ability of
312 OmpA to impart defense machinery to the cytosolic population of wildtype *Salmonella* against
313 the bombardment of RNI. The greater recruitment of nitrotyrosine on STM *ΔompA*: *LLO* which
314 is comparable to STM *ΔompA* further suggests that LLO doesn't play any role in modulating
315 the activity of iNOS. Consistent with this result, it has been found that the intracellular survival

316 of STM (WT): *LLO* in macrophage is better than STM *ΔompA* and comparable to that of wild-
317 type bacteria (Figure 2D). Taken together, we firmly confer that OmpA protects intracellular
318 wild-type *Salmonella* Typhimurium against the nitrosative stress of macrophages. While
319 proliferating in macrophage wildtype *Salmonella* utilizes SPI-2 encoded virulent factor SpiC
320 to downregulate the activity of inducible nitric oxide synthase in SOCS-3 dependent manner
321 (Uchiya and Nikai, 2008, Uchiya and Nikai, 2005). To verify whether the OmpA dependent
322 downregulation of the activity of iNOS happens in SpiC dependent manner the transcript level
323 of *spiC* was measured from wild-type and mutant bacteria growing in macrophages (Figure
324 2E). It was found that STM *ΔompA* is unable to produce *spiC* within macrophage, unlike the
325 wildtype *Salmonella*. The reduced expression of *spiC* by STM (WT): *LLO* and bafilomycin A
326 treated cells infected with wild-type bacteria not only proves the requirement of the acidic pH
327 of SCV for the expression of *spiC* but also indirectly authenticates the cytosolic localization of
328 STM *ΔompA*. To validate this observation the promoter activity of *spiC* was measured in STM
329 (WT) and *ΔompA* growing in stationary phase in LB media and macrophage (Figure 2F) by
330 beta-galactosidase assay. No significant change in the activity of the promoter of *spiC* was
331 observed in wild-type and mutant bacteria when they were grown in LB broth (Figure 2F)
332 where there was no significant change in the pH. Inside macrophage where there exists a
333 notable difference between the localization of wildtype and *ompA* deficient bacteria, a
334 significant drop in the activity of the *spiC* promoter was observed in intracellularly growing
335 STM *ΔompA* (Figure 2F). The localization of STM *ΔompA* in the cytosol of macrophage where
336 the pH is relatively higher than SCV can be held accountable for the abrogated expression of
337 *spiC*. We observed a higher 488 nm/ 405 nm values of STM *ΔompA* labelled with 20 μM
338 concentration of BCECF-AM resuspended in phosphate buffer of acidic pH (range 5.5, 6, and
339 6.5) in comparison with STM (WT) and the complemented strain (*ΔompA*: pQE60-*ompA*)
340 (Figure 2G). Surprisingly when all these bacterial strains were incubated separately in

phosphate buffer of neutral pH (pH= 7) we found comparable 488 nm/ 405 nm values, which suggests the indispensable role of OmpA in the acidification of the cytosol of *S.* Typhimurium in response to extracellular acidic stress (Figure 2G).

Inhibition of the activity of iNOS improves the survival of STM *ΔompA* in *in vitro* and *in vivo* infection model.

To authenticate the pro-bacterial role of *Salmonella* OmpA against the nitrosative stress of macrophage, we have decided to tamper the activity of inducible nitric oxide synthase (iNOS) the key enzyme involved in the production of [NO] using an irreversible inhibitor of iNOS 1400W dihydrochloride (at 10 µM concentration) (Figure 3A, 3C, and 3D) and mouse IFN-ɣ that upregulates the expression of iNOS (at 100 U/ mL concentration) (Figure 3B, 3C, and 3E). Inhibition of the activity of iNOS using 1400W completely restored the intracellular proliferation of STM *ΔompA* (43.61 ± 6.837) in comparison with STM *ΔompA* infected RAW264.7 cells without 1400W treatment (19.32 ± 3.913) (Figure 3A). Consistent with this finding STM *ΔompA* has shown poor colocalization with nitrotyrosine under 1400W treatment (Figure 3C and 3D), which can be attributed to the poor biogenesis of RNI due to the inhibition of iNOS. Augmenting the iNOS activity of macrophage using mouse IFN-ɣ hindered the intracellular proliferation of STM *ΔompA* (12.52 ± 1.334) to a greater extent as compared to STM *ΔompA* infected (19.64 ± 2.11) IFN-ɣ untreated RAW264.7 cells (Figure 3B). The enhanced biogenesis and colocalization of nitrotyrosine with STM *ΔompA* under IFN-ɣ treatment as demonstrated by the confocal image can be taken into consideration for its higher attenuation in intracellular proliferation (Figure 3C and 3E). In contrary to the wild-type bacteria the reduced CFU burden of *ompA* deficient *Salmonella* in the liver, spleen, and MLN of C57BL/6 mice strongly supports the role of OmpA in bacterial pathogenesis (Figure 3G). In our *ex vivo* studies, we have established the role of OmpA in protecting the wild-type *Salmonella* against the nitrosative stress of macrophages. To extrapolate that result in *in vivo*

366 system we have used *iNOS*$^{-/-}$ C57BL/6 mice and further treated the wildtype C57BL/6 mice
367 with a pharmacological inhibitor of iNOS called aminoguanidine hydrochloride at a dose of 10
368 mg/ kg of body weight from 0th day (on the day of infecting the mice) to 5th day (the day of
369 sacrificing and dissecting the mice) (Figure 3G) (Pearse et al., 2003). We found a comparable
370 CFU burden of wildtype and *ompA* deficient bacteria in the liver, spleen, and MLN of *iNOS*$^{-/-}$
371 and wildtype C57BL/6 mice administered with the inhibitor. The higher bacterial burden of
372 STM *ΔompA* from the *iNOS*$^{-/-}$ and aminoguanidine-treated mice in comparison with PBS
373 treated mice (Figure 3G) concomitantly authenticates our results obtained from *ex vivo*
374 experiments. We have used *gp91phox*$^{-/-}$ mice that are unable to produce ROS upon bacterial
375 infection and observed comparable CFU burden of wildtype and *ΔompA* in the liver, spleen,
376 and MLN. The significantly higher bacterial burden of *ompA* deficient *Salmonella* from the
377 liver of *gp91phox*$^{-/-}$ in comparison with STM *ΔompA* burden from wildtype C57BL/6 mice
378 indicates the inability of *gp91phox*$^{-/-}$ mice to generate peroxynitrite due to dampened
379 production of superoxide ions (ROS) (Figure3G). However, the comparable bacterial burden
380 of STM *ΔompA* in the spleen and MLN of *gp91phox*$^{-/-}$ mice concerning the CFU of STM
381 *ΔompA* obtained from wildtype C57BL/6 mice proves that ROS alone can't clear the *in vivo*
382 infection of STM *ΔompA*. Earlier we have found that the infection of RAW264.7 cells with
383 STM *ΔompA* doesn't induce the level of intracellular ROS. To prove the role of *ompA* in *in*
384 *vivo* infection of *Salmonella* Typhimurium, we have challenged 4- 6 weeks old adult BALB/c
385 and C57BL/6 mice (Figure 3H) with a lethal dose (10^8 CFU of bacteria/ animal) of wildtype
386 and knockout strains by oral gavaging. Almost 80% of BALB/c mice infected with STM
387 *ΔompA* survived compared to the group of mice infected with wild-type bacteria (Figure 3H).
388 On the other side, the C57BL/6 mice infected with STM *ΔompA* have shown better survival
389 and retarded death compared to the wildtype infected mice, suggesting the paramount role of
390 *ompA* in *in vivo* infection of *Salmonella*.

OmpA dependent regulation of outer membrane permeability in *Salmonella* controls cytoplasmic redox homeostasis in response to *in vitro* nitrosative stress.

In a mildly acidic environment (pH= 5 – 5.5) $NaNO_2$ dissociates to form nitrous acid (HNO_2), which undergoes dismutation reaction upon oxidation and eventually generates a wide range of reactive nitrogen intermediates (RNI) such as, nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), nitric oxide (NO), etc (Heaselgrave et al., 2010). After entering the bacterial cells RNIs cause irreparable deleterious effects targeting multiple subcellular components such as nucleic acids (cause deamination of nucleotides), proteins (disruption of Fe-S clusters, heme group; oxidation of thiols and tyrosine residues), lipids (cause peroxidation), etc, and finally kill the pathogen (Fang). To investigate the role of *Salmonella* OmpA against *in vitro* nitrosative stress, we decided to check *in vitro* sensitivity of the wildtype and *ompA* knockout strains in presence of a varying concentration of H_2O_2 (Figure S5D), $NaNO_2$ (Figure S5E), and a combination of H_2O_2 & $NaNO_2$ (Figure S5F) by CFU and resazurin test. Both the strains were exposed to increasing concentrations of (range of 0 - 5 mM; as mentioned in the figures) H_2O_2, $NaNO_2$, and a combination of H_2O_2 & $NaNO_2$ for 12 hours. In comparison with wild-type bacteria, STM *ΔompA* did not show any significant difference in viability when exposed to peroxide (Figure S5D). The knockout strain displayed a strong reduction in viability compared to the wild-type bacteria at 800 μM concentration when they were incubated with $NaNO_2$ alone (Figure S5E). The sensitivity of the knockout strain towards acidified nitrite further increased when H_2O_2 was added to $NaNO_2$ (where the growth inhibition started at 600 μM concentration) (Figure S5F). To strongly ensure OmpA dependent protection of wildtype *Salmonella* against the damage caused by *in vitro* nitrosative stress, we performed a death kinetics experiment with wildtype and knockout bacteria in presence of 800 μM concentration of acidified nitrite (Figure 4A). Consistent with our previous observations, the knockout strain has shown a notable hindered growth compared to the wild type at 12 hours post-inoculation. Taking the impeded

416 growth of the mutant strain in presence of *in vitro* nitrosative stress into consideration, we
417 further hypothesized higher entry of nitrite into the *ΔompA* strain compared to the wildtype. To
418 verify this, we proceeded to perform nitrite uptake assay with the wildtype, knockout,
419 complement, and empty vector strain in MOPS-NaOH buffer with 200 µM initial concentration
420 of nitrite. We noticed a greater time-dependent uptake of nitrite by the *ΔompA* and empty vector
421 strain in comparison with wild-type and complement bacteria (Figure 4B). To verify OmpA
422 dependent redox homeostasis of *Salmonella* in response to *in vitro* nitrosative stress, we have
423 exposed both wildtype and knockout strains harboring pQE60-Grx1-roGFP2 plasmid to 800
424 µM, 1 mM, and 5 mM (Figure 4C) concentration of acidified nitrite for 15, 30, 45 and 60
425 minutes. Glutathione (GSH) a low molecular weight thiol of gram-negative bacteria is mainly
426 required to maintain the reduced state of the cytoplasm. In presence of external ROS/ RNI
427 stress cytosolic GSH pool is oxidized to form glutathione disulfide (GSSG) (Tung Ngoc et al.,
428 2018). The redox-sensitive GFP2 (roGFP2) encoded by pQE60-Grx1-roGFP2 construct,
429 having two redox-sensitive cysteine residues at 147th and 204th positions, (which form disulfide
430 bond upon oxidation) can absorb at two wavelengths 405 nm and 488 nm depending upon its
431 oxidized and reduced state respectively. It has a fixed emission at 510 nm (Hans Reuter et al.,
432 2019). The glutaredoxin protein (Grx1) fused to the redox-sensitive GFP2 can reversibly
433 transfer electrons between the cellular (GSH/GSSG) pool and the thiol groups of roGFP2 at a
434 much faster rate. The ratio of fluorescence intensity of Grx-roGFP2 measured at 405 nm and
435 488 nm demonstrates the redox status of the cytoplasm of bacteria (Tung Ngoc et al., 2018). In
436 all three concentrations of acidified nitrite {800 µM, 1 mM, 5 mM (Figure 4C)}, we have found
437 a time-dependent steady increase in the 405/ 488 ratio of Grx1-roGFP2 possessed by STM
438 *ΔompA* strain in comparison with STM (WT) suggesting a heightened redox burst in the
439 cytoplasm of *ompA* knockout strain in response to RNI *in vitro*. Taken together, our data clearly
440 indicates the importance of OmpA in maintaining the cytosolic redox homeostasis of wildtype

441 *Salmonella*. Earlier, we have noticed a significant downregulation in the transcripts level of
442 outer membrane bound larger porins (*ompC*, *ompD*, and *ompF*) compared to *ompA* in wildtype
443 *Salmonella* growing in acidic F media and RAW264.7 cells. Our previous study depicted
444 enhanced uptake of nitrite by STM *ΔompA* strain in comparison to STM (WT), which further
445 indicates increased permeability of the bacterial outer membrane. Hence, we decided to check
446 the expression of larger porins in STM *ΔompA* strain growing in *in vitro* and *ex vivo* conditions.
447 Surprisingly, in contrast to the wildtype bacteria, we have found elevated expression of *ompC*,
448 *ompD*, and *ompF* in STM *ΔompA* strain growing in nutritionally enriched LB media, nutrient-
449 depleted acidic F media (pH= 5.4), and RAW264.7 macrophage cells (Figure 4D). This
450 heightened expression of larger porins was revoked in the complemented strain. In this regards
451 the enhanced outer membrane polarity of STM (WT) and *ΔompA* growing in acidic F media
452 was further tested (during stationary phase) using a membrane-permeant negatively charged
453 dye called $DiBAC_4$ (Figure 4E) (Marathe et al., 2010). Because of the enhanced outer
454 membrane permeability when the negative charge of the bacterial cytosol (called polarised
455 state) gets diminished by the inflow of cations (called depolarization), the cell allows the entry
456 of $DiBAC_4$ which starts fluorescing after being bound to the cell membrane proteins and lipids.
457 In contrary to the wildtype and the complemented strain, the higher $DiBAC_4$ positive
458 population and greater median fluorescence intensity of $DiBAC_4$ corresponding to STM
459 *ΔompA* and the empty vector strain (Figure 4E) proves our hypothesis to be true. To strengthen
460 this observation, we used another porin specific DNA binding fluorescent dye called
461 bisbenzimide (Coldham et al., 2010), which has given higher fluorescence intensity after being
462 incubated with the *ompA* knockout strain compared to the wild-type bacteria (Figure 4F). In
463 comparison with the wild-type bacteria greater fluorescence intensity of bisbenzimide
464 corresponding to STM *ΔompA* grown intracellularly in murine macrophage for 12 hours (Fig
465 4G) firmly endorsed the result obtained from the *in vitro* experiment. Hence, we have

concluded that in absence of *ompA* the enhanced outer membrane porosity of the bacteria depends upon elevated expression of larger porins such as *ompC*, *ompD*, and *ompF*.

The maintenance of the integrity of the SCV membrane inside RAW264.7 macrophage solely depends upon OmpA, not on other larger porins such as OmpC, OmpD, and OmpF.

To specify the sole contribution of larger porins in the increment of outer membrane porosity of STM *ΔompA*, we decided to knockout *ompC*, *ompD*, and *ompF* individually in kanamycin-resistant *ΔompA* background of *Salmonella*. We have employed one step chromosomal gene inactivation method as demonstrated earlier by Datsenko and Wanner, using chloramphenicol resistant gene cassette to finally obtain STM *ΔompA ΔompC* (Figure S6A), STM *ΔompA ΔompD* (Figure S6B), and STM *ΔompA ΔompF* (Figure S6C). We further investigated the intracellular niche of these double knockout bacterial strains in RAW264.7 cells during the late phase of infection (16 hours post-infection). In line with our previous finding, contrary to the wild-type bacteria STM *ΔompA* has shown poor colocalization with SCV marker LAMP-1 (Figure 5A and 5B). Surprisingly, we have found drastic loss of SCV membrane from the surroundings of STM *ΔompA ΔompC*, *ΔompA ΔompD*, and *ΔompA ΔompF* as demonstrated by their poor colocalization with LAMP-1 (Figure 5B), indicating the cytosolic localization of these double knockout strains in macrophage during the late phase of infection. The reduced colocalization of STM (WT) expressing LLO (which usually stays in the cytoplasm) with LAMP-1 (Figure 5A and 5B) strongly authenticates the cytosolic phenotype of the double knockout bacterial strains. To reject the counter-argument that the cytosolic confinement of the double knockout bacterial strains namely STM *ΔompA ΔompC*, *ΔompA ΔompD*, and *ΔompA ΔompF* (Figure 5A and 5B) is not only because of the absence of OmpA but also due to the lack of OmpC, OmpD, and OmpF respectively, we further decided to construct single knockout strains of *Salmonella* lacking *ompC* (Figure S6D), *ompD* (Figure S6E), and *ompF* (Figure S6F)

491 respectively. In line with our expectations, it has been observed that in contrast to the STM
492 *ΔompA*, the STM *ΔompC*, *ΔompD*, and *ΔompF* strains are showing higher colocalization with
493 LAMP-1, which is comparable to that of the wildtype bacteria (Figure 5C and 5D) and indicates
494 the presence of intact SCV membrane around them while thriving within the murine
495 macrophage. Hence, it can be firmly concluded that the maintenance of the vacuolar life of
496 wildtype *Salmonella* strongly depends on OmpA, but not on other membrane porins such as
497 OmpC, OmpD, and OmpF. The lower recruitment of nitrotyrosine on STM *ΔompC, ΔompD*,
498 and *ΔompF* in comparison with STM *ΔompA* growing intracellularly in RAW264.7 cells
499 further prove the presence of undamaged SCV membrane around them (Figure S7A). The *in*
500 *vitro* sensitivity of STM *ΔompC*, *ΔompD*, and *ΔompF* against the acidified nitrite was further
501 checked and compared to STM (WT) and *ΔompA* (Figure S7B). It was observed that, unlike
502 the *ompA* deficient strain of *Salmonella*, the ability of STM *ΔompC*, *ΔompD*, and *ΔompF* to
503 withstand the bactericidal effect of acidified nitrite is comparable to the wildtype bacteria
504 (Figure S7B). To strengthen this observation, we proceeded further to check the consumption
505 of nitrite by STM *ΔompC*, *ΔompD*, and *ΔompF* (Figure S7C). It was found that in comparison
506 to STM *ΔompA* strain, *ΔompC*, *ΔompD*, and *ΔompF* are more efficient in restricting the entry
507 nitrite (Figure S7C) which can be attributed to their better survival in the presence of acidified
508 nitrite. From this study, it can be further anticipated that in contrast to *ompA* negative strain of
509 *Salmonella*, the STM *ΔompC*, *ΔompD*, and *ΔompF* will have an improved intracellular life
510 inside macrophage because of the presence of intact SCV membrane and showing better
511 survival in acidified nitrite (Study under progress).

512 **In absence of OmpA, porins OmpC and OmpF enhance the susceptibility of *Salmonella***
513 **against nitrosative stress of RAW264.7 cells.**

514 To dissect the role of each larger porin namely OmpC, OmpD, and OmpF in the entry of nitrite
515 in bacteria during the absence of OmpA, we performed nitrite uptake assay using the double

516 knockout bacterial strains STM *ΔompA ΔompC*, *ΔompA ΔompD*, and *ΔompA ΔompF* (Figure
517 6A). In comparison with the wildtype bacteria, the rapid disappearance of nitrite which
518 corroborates substantial nitrite uptake from the media by STM *ΔompA* and *ΔompA ΔompD*
519 demonstrates the involvement of OmpC and OmpF (acquired by both STM *ΔompA* and *ΔompA*
520 *ΔompD* strains) in this entry process (Figure 6A). This result was further validated by the
521 measurement of the reduced *in vitro* percent viability of STM *ΔompA* and *ΔompA ΔompD* in
522 comparison with wildtype, *ΔompA ΔompC* and *ΔompA ΔompF* in presence of acidified nitrite
523 (800 μM) (Figure 6B). We further extrapolated these *in vitro* findings to the *ex vivo* system by
524 checking the nitrosative burst in murine macrophage induced by the double knockout strains
525 (Figure 6C) and their ability of intracellular survival (Figure 6D). In line with our expectation,
526 during the late stage of infection in murine macrophage, we have found enhanced recruitment
527 of nitrotyrosine on STM *ΔompA* and *ΔompA ΔompD* as depicted by their higher percent
528 colocalization in comparison with wildtype, *ΔompA ΔompC*, and *ΔompA ΔompF* (Figure 6C).
529 The intracellular nitrosative burst of macrophage infected with STM (WT), *ΔompA, ΔompA*
530 *ΔompC, ΔompA ΔompD*, and *ΔompA ΔompF* was tested using DAF2DA (Figure 6E). Only
531 7.35% of macrophage infected with wild-type bacteria produced [NO], which is comparable to
532 STM *ΔompA ΔompC* (6.12%) and *ΔompA ΔompF* (3.4%) infected macrophage population
533 producing [NO]. The enhanced median fluorescence intensity of DAF2DA and greater percent
534 population of infected macrophage corresponding to STM *ΔompA* (12.79%) and *ΔompA*
535 *ΔompD* (13.99%) (Figure 6E) strengthens the conclusion obtained from confocal data (Figure
536 6C). This data was further verified by the attenuated intracellular proliferation of STM *ΔompA*
537 and *ΔompA ΔompD* inside RAW264.7 cells unlike STM (WT), *ΔompA ΔompC*, and *ΔompA*
538 *ΔompF* whose improved intracellular survival can be attributed to reduced recruitment of
539 nitrotyrosine (Figure 6C and 6D). Taken together, it can be concluded that in absence of OmpA,
540 the elevated expression of larger porins OmpC and OmpF on the bacterial outer membrane

helps in the entry of nitrite from outside within the bacterial cytoplasm and makes the bacteria highly susceptible to the intracellular nitrosative burst.

Discussion

Wildtype *Salmonella* Typhimurium a gram-negative enteric bacterial pathogen belonging to the family *Enterobacteriaceae* experiences a wide range of environmental stresses during its extracellular as well as, intracellular life cycle. In standard laboratory conditions, upon nutrient depletion, wildtype *Salmonella* growing in LB broth culture reaches the stationary phase. While infecting a human host the bacteria are exposed to different kinds of adverse environments such as acid stress in the stomach, bile salt stress in the duodenum, short-chain fatty acid, and oxygen depletion stress in the ileum, cationic antimicrobial peptides in the intestinal epithelial cell linings, etc. All these challenges are brilliantly handled by *Salmonella* either by using EnvZ/OmpR, PhoPQ two-component systems or by global stress response regulators RpoS, Fur proteins (Rychlik and Barrow, 2005).

While infecting macrophage or epithelial cells, wild-type *Salmonella* staying in *Salmonella* containing vacuole (SCV) undergoes nutrient limitation, ion depletion (Mg^{2+}, Fe^{3+}, PO_4^{3-}), and low pH stress respectively (Eriksson et al., 2003, Hautefort et al., 2008). By inducing significant changes in their metabolic status such as upregulation of Enter-Duodoroff (which prefers alternative carbon source such as gluconate) and aromatic amino acid biosynthesis pathways, downregulation of purine/ pyrimidine biosynthesis pathway (because of the slow rate of replication in SCV), blocking the expression of H+/ATPase pumps (to stop the influx of H+ ions) and by increasing the synthesis of specific ion transporters on its surface wildtype *Salmonella* survives efficiently in SCV (Eriksson et al., 2003). During systemic infection in macrophage and epithelial cells, *Salmonella* is further challenged by cationic antimicrobial peptides, reactive oxygen, and nitrogen species respectively (Gogoi et al., 2019, Balakrishnan

565 et al., 2016, Chakraborty et al., 2014), which are successfully encountered by the bacteria with
566 the impermeable membranous barrier of SCV and SPI-2 encoded effectors proteins (Heijden
567 et al., 2015, Das et al., 2009). Along with that, the compositions, arrangements, and
568 heterogeneity of the outer membrane lipids and proteins play a very crucial role in the
569 maintenance of the pathogenesis of gram-negative bacteria. Recent studies have suggested that
570 bacterial pathogens (such as *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella*
571 *pneumoniae* etc) can restrict the entry of toxic molecules such as antibiotics and cationic
572 antimicrobial peptides either by changing the outer membrane permeability (Smani et al., 2014,
573 Ghai and Ghai, 2018), or by augmenting omptins/ proteases that degrade the antimicrobial
574 peptides (Kukkonen and Korhonen, 2004). The alteration in the outer membrane permeability
575 as well as integrity of gram-negative pathogen is strictly regulated by the core oligosaccharide
576 composition of outer membrane lipopolysaccharide and differential expression of outer
577 membrane porins (Ebbensgaard et al., 2018, Iyer et al., 2018, Choi and Lee, 2019).

578 OmpA, OmpC, OmpD, OmpF, and PhoE, the predominantly found porins on the outer
579 membrane of wildtype *Salmonella* Typhimurium, are involved in a wide range of functions.
580 OmpA tightly holds the outer membrane of the bacteria to the peptidoglycan layer of the cell
581 wall and hence, deletion of OmpA aggravates the biogenesis of outer membrane vesicles
582 (OMV) (Park et al., 2012, Nevermann et al., 2019). OmpD, the most abundant porin on
583 *Salmonella* outer membrane, is involved in the uptake of H_2O_2 (Calderón et al., 2011). OmpC
584 in conjunction with OmpF contributes to the acquisition of cations, whereas PhoE majorly
585 helps in the uptake of negatively charged phosphate groups (Ipinza et al., 2014). The correlation
586 between outer membrane porins and *Salmonella* pathogenesis has been poorly understood due
587 to the lack of extensive studies. Earlier Heijden *et al.* have proved that $HpxF^-$ a strain of
588 *Salmonella* altered outer membrane permeability by reciprocally regulating the expression of
589 OmpA and OmpC when exposed to H_2O_2 (Heijden et al., 2016). OmpA deficient *Salmonella*

590 was found to be incapable of reaching the mouse brain (Chaudhuri et al., 2018). Deletion of
591 *ompD* from the genome of *Salmonella* made it hyper-proliferative in RAW264.7 macrophage
592 and BALB/c mouse models (Ipinza et al., 2014). In this study, we delineated the individual
593 contributions of OmpA, OmpC, OmpD, and OmpF in *Salmonella* pathogenesis. Our study not
594 only revealed the role of porins in the maintenance of the intravacuolar life of wildtype
595 *Salmonella* Typhimurium but also deciphered their role in the regulation of outer membrane
596 permeability and bacterial resistance to the nitrosative stress of macrophage.

597 We have found a time-dependent steady decrease in the transcripts level of *ompC*, *ompD*, and
598 *ompF* (Figure S1B, S1C, S1D) in wildtype *Salmonella* growing in RAW264.7 cells. On
599 contrary, the consistently elevated expression of *ompA* during the late phase of infection in
600 RAW264.7 cells (9 hours and 12 hours post-infection) (Figure S1A) suggested the importance
601 of this porin in bacterial survival inside the macrophage. Interestingly, our observations
602 strongly corroborated the findings of Eriksson *et al.*, and Hautefort *et al* (Eriksson et al., 2003,
603 Hautefort et al., 2008). Unlike other major porins, OmpA has a small pore size and a unique
604 periplasmic domain (Figure S1B, S1D, 1F, and 1H) which can act as a gate to restrict the entry
605 of many toxic molecules (Heijden et al., 2016). Hence it can be concluded that wildtype
606 bacteria growing in a nutrient-depleted stressed environment of SCV within the macrophage,
607 where the bacterial growth is severely challenged by acidic pH, would always prefer OmpA a
608 porin with a smaller pore size and periplasmic gating mechanism to be expressed on its surface
609 to restrict the entry of excess H+ ions. Outer membrane protein A (OmpA), one of the most
610 abundant porins of the outer membrane of *E. coli* K1, the causative agent of neonatal
611 meningitis, is highly conserved in the family of *Enterobacteriaceae* (Krishnan and Prasadarao,
612 2012). Besides its structural role in the outer membrane, OmpA of *E. coli* K1 and *Enterobacter*
613 *sakazakii* has been reported to be involved in the invasion of hBMEC cells, and INT407 cells
614 by multiple studies (Mohan Nair and Venkitanarayanan, 2007, Mittal et al., 2011, Singamsetty

615 et al., 2008). OmpA of *E. coli* K1 not only augments complement resistance by recruiting C4
616 binding protein (C4BP) on the surface of the bacteria (Abreu and Barbosa, 2017) but also
617 abrogates their intracellular survival in murine and human macrophages (Sukumaran et al.,
618 2003). In our study, the OmpA deficient strain of *Salmonella* has shown a higher inclination
619 towards phagocytosis and severe attenuation in intracellular proliferation in macrophages. In
620 comparison with untreated STM *ΔompA,* the higher phagocytosis of STM *ΔompA* pre-treated
621 with mouse complement sera proves the role of OmpA of *Salmonella* Typhimurium against
622 complement activation. Surprisingly on the other hand deletion of *ompA* makes *Salmonella*
623 invasion deficient and hyper-proliferation in epithelial cells. The successful systemic infection
624 of *Salmonella* in macrophages as well as in epithelial cells depends upon its intravacuolar
625 inhabitation. The acidification inside the SCV is a prerequisite for the synthesis and secretion
626 of SPI-2 encoded virulent proteins which are required for the successful proliferation of
627 wildtype *Salmonella* (Chakraborty et al., 2015). Mild tampering with the integrity of SCV may
628 create unusual outcomes in the bacterial burden of the cells. Earlier it has been reported that
629 the intracellular proliferation of *sifA* mutant of *Salmonella,* which comes into the cytoplasm
630 after quitting SCV, was severely abrogated in macrophages and aggravated in the cytosol of
631 epithelial cells (Salcedo et al., 2002). The introduction of point mutations in the Rab5 or Rab7
632 proteins (markers of SCV) can also trigger the release of wild-type bacteria from SCV to the
633 cytosol of epithelial cells (Brumell et al., 2002). Our immunofluorescence microscopy data
634 along with the result from chloroquine resistance assay proved the cytosolic localization of
635 *ompA* deficient strain of *Salmonella* in macrophage as well as in epithelial cells. STM *ΔompA*
636 that stay into the neutral pH of cytoplasm has shown reduced colocalization with SseC and
637 SseD, two translocon proteins, whose synthesis, surface accumulation, and secretion into the
638 host cell cytoplasm depend upon the acidic pH of SCV and SPI-2 encoded T3SS needle
639 complex (Chakravortty et al., 2005), further proved our observation to be true. Surprisingly,

640 the result obtained from intracellular proliferation assay of STM *ΔompA* in macrophage and
641 epithelial cells and the immunofluorescence microscopy data on vacuolar/ cytosolic niche fell
642 in line with the available literatures. Hence, to best of our knowledge we are assuming that this
643 is the first report commenting on the ability of OmpA to maintain the integrity and stability of
644 *Salmonella* containing vacuole.

645 The SCV membrane functions as a protective barrier around the wild-type bacteria. Once the
646 intactness of the SCV membrane is lost, the bacteria will eventually be exposed to the threats
647 present in the cytosol in form of ROS and RNI (Heijden et al., 2015). Hence, the oxidative and
648 nitrosative burst of macrophage infected with wildtype and *ompA* knockout strain of
649 *Salmonella* was checked. Surprisingly, we found an outstanding rise in the level of intracellular
650 as well as, extracellular [NO] of macrophages infected with STM *ΔompA*, clearly indicating
651 the protective role of OmpA against the nitrosative stress of macrophages. In continuation with
652 the previous observation, STM *ΔompA* which has already quit the SCV and staying in cytosol
653 has shown greater colocalization with nitrotyrosine when compared with the wildtype bacteria
654 which is protected inside the vacuole. The less recruitment of nitrotyrosine on STM (WT): *LLO*
655 staying in the cytoplasm of macrophage due to ectopic expression of listeriolysin O and their
656 better survival in macrophage in comparison with STM *ΔompA* further strengthen the previous
657 conclusion. The alteration in the proliferation of STM *ΔompA* in macrophages and the
658 recruitment profile of nitrotyrosine upon manipulation of iNOS activity using specific
659 irreversible inhibitor 1400W dihydrochloride and activator IFN-ɣ fell in line with the previous
660 results. Wildtype *Salmonella*, with the help of *Salmonella* pathogenicity island (SPI)- 2
661 encoded virulent factor SpiC activates suppressor of cytokine signaling 3 (SOCS-3) which
662 inhibit IFN-ɣ signaling and thus eventually represses the activation of iNOS. [44, 58] As
663 discussed earlier, the acidification of the SCV compartment acidifies the cytosol of wildtype
664 *Salmonella* and is essential for the synthesis and secretion of SPI-2 effector proteins into the

665 cytoplasm of the host cell (Chakraborty et al., 2015). We have also found that STM *ΔompA*
666 which stays in the neutral pH of cytoplasm in macrophage after quitting SCV is unable to
667 produce SpiC. We further checked the promoter activity of *spiC* in wild-type and mutant
668 bacteria growing both extracellularly as well as intracellularly by β galactosidase assay.
669 Compared to the intracellularly growing STM *ΔompA* the higher activity of *spiC* promoter in
670 wildtype *Salmonella* simultaneously proves the cytosolic inhabitation of STM *ΔompA* and
671 answers the reason behind the nitrosative burst of macrophage. The downregulation of SPI-2
672 encoded gene *spiC* in STM *ΔompA* can also be attributed to its incompetence to acidify its
673 cytosol in response to extracellular acidic response which was determined by a higher 488nm/
674 405nm ratio of BCECF-AM. But strikingly, STM *ΔompA* was unable to induce the production
675 of intracellular and extracellular ROS while infecting macrophages. NADPH phagocytic
676 oxidase is the key enzyme of macrophages involved in the synthesis of superoxide ions. It is a
677 multimeric protein consisting of two membrane-bound subunits such as gp91^{phox}, p22^{phox}, and
678 four cytosolic subunits such as p47^{phox}, p40^{phox}, p67^{phox,} and RacGTP. (Panday et al., 2015) In
679 continuation of our previous observations, we have found very poor colocalization of gp91^{phox}
680 with STM (WT) and *ΔompA* in macrophages during the late phase of infection (data not
681 shown). Wildtype *Salmonella* can inhibit the recruitment of NADPH oxidase on the surface of
682 the SCV membrane with the help of the SPI-2 encoded type 3 secretion system. (Gallois et al.,
683 2001) The restriction in the recruitment of NADPH oxidase on the surface of the *ompA*
684 knockout bacteria lacking SCV membrane and staying in the cytosol is the probable reason
685 behind the dampened oxidative stress inside the infected macrophage. On contrary, the ability
686 of iNOS to maintain its uninterrupted catalytic activity, despite being recruited on the cortical
687 actin of macrophage (Webb et al., 2001), is the most probable reason behind elevated
688 production of RNI in RAW264.7 cells infected with STM *ΔompA*. To investigate the role of
689 OmpA in the establishment of *in vivo* infection by wildtype *Salmonella* Typhimurium we have

690 used 4 to 6 weeks old *Nramp*$^{-/-}$ C57BL/6 and BALB/c mice. The better survival of the mice
691 infected with *ompA* deficient strains of *Salmonella*, which was attributed to the reduced
692 bacterial burden in the liver, spleen, and MLN, finally endorsed the essential role of OmpA in
693 the *in vivo* infection of *Salmonella*. Administration of iNOS inhibitor aminoguanidine
694 hydrochloride by intraperitoneal injection or direct oral infection of *iNOS*$^{-/-}$ mice diminished
695 the *in vivo* attenuation of STM *ΔompA* suggesting OmpA dependent protection of wildtype
696 bacteria against nitrosative stress *in vivo*. The comparable CFU burden of STM (WT) and
697 *ΔompA* in *gp91phox*$^{-/-}$ C57BL/6 mice, which is unable to produce ROS, can be accounted for
698 the abrogated peroxynitrite response (Zielonka et al., 2016, Vlahos R, Stambas J, Bozinovski
699 S, Broughton BRS, Drummond GR, 2011). Acidified nitrite generating a wide range of reactive
700 nitrogen intermediates (RNI) such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), nitric
701 oxide (NO), etc, are extensively used for assessing *in vitro* viability of bacteria and fungi
702 (Heaselgrave et al., 2010, Weller et al., 2001). After entering the bacterial cells RNIs cause
703 enormous irreparable damages to multiple subcellular components such as nucleic acids (cause
704 deamination of nucleotides), proteins (disruption of Fe-S clusters, heme group; oxidation of
705 thiols and tyrosine residues), lipids (cause peroxidation), etc, and eventually destroy the
706 pathogen. (Fang) The enhanced sensitivity of STM *ΔompA* against acidified nitrite and
707 combination of acidified nitrite with peroxide which generates peroxynitrite, (Kono, Y.,
708 Shibata, H., Adachi, K., Tanaka) suggested increased entry of nitrite in the knockout strain
709 comparison with wildtype bacteria. The faster depletion of nitrite from the media having STM
710 *ΔompA* strongly supported our hypothesis. The time-dependent gradual increase in 405/ 488
711 ratio of pQE60-Grx1-roGFP2 harboured by STM *ΔompA* under all the tested concentrations of
712 acidified nitrite proved the loss of redox homeostasis in STM *ΔompA*.

713 As discussed earlier, the outer membrane of gram-negative bacteria acts as an impermeable
714 barrier to many noxious compounds (including antibiotics, bile salts, cationic antimicrobial

715 peptides, reactive oxygen species, abnormal pH, osmotic stress, etc.) and protect the bacteria
716 from many environmental threats during its extracellular and intracellular survival. In absence
717 of OmpA, the alleviation in outer membrane integrity was speculated by the enhanced entry of
718 nitrite. The enhanced uptake of $DiBAC_4$ (measure the cell membrane polarity) and
719 bisbenzimide (binds to the DNA) by the *ompA* deficient strain of *Salmonella* further indicated
720 our speculation to be true. To reach a rationale for the enhanced uptake of fluorescent probes
721 by STM *ΔompA* we further checked the expression of larger porins such as *ompC*, *ompD*, and
722 *ompF* in wildtype, mutant, and complemented strains of *Salmonella* growing in LB broth, F
723 media, and RAW264.7 macrophage. We have found a remarkable increase in the expression
724 level of the larger porins in the knockout strain (lacking OmpA) in comparison with wildtype
725 and complement strain. Hence, we have concluded that OmpA regulates the outer membrane
726 integrity of wildtype *Salmonella*. When OmpA is deleted, the increased expression of larger
727 porins such as OmpC, OmpD, and OmpF reduces the intactness and stability of the outer
728 membrane of bacteria and further makes it permeable to small molecules like nitrite. This
729 conclusion can further be extrapolated to enlighten the reason behind enhanced recruitment of
730 nitrotyrosine on STM *ΔompA* in comparison with STM (WT): *LLO* in macrophage during the
731 late phase of infection. Despite having cytosolic niche STM (WT): *LLO* has intact OmpA in its
732 outer membrane unlike STM *ΔompA*, which maintains the integrity and stability of the outer
733 membrane and prohibits the entry of peroxynitrite.

734 To ascertain the individual contribution of the larger porins such as *ompC*, *ompD*, and *ompF* in
735 the entry of nitrite in *ompA* deficient bacteria, we have constructed *ompC*, *ompD*, and *ompF*
736 single and double knockout strains in wildtype and $ompA^-$ background of *Salmonella*
737 respectively. The cytosolic localization of double knockout strains namely STM *ΔompA*
738 *ΔompC*, *ΔompA ΔompD*, and *ΔompA ΔompF* and vacuolar imprisonment of the single knockout
739 strains such as STM *ΔompC*, *ΔompD*, and *ΔompF* provided strong support to our previous

740 conclusion on the dependence of SCV integrity and stability on OmpA. Under the *in vitro*
741 challenge of acidified nitrite, the better survival of STM *ΔompC*, *ΔompD*, and *ΔompF* in
742 comparison with *ΔompA* not only suggested the abrogated consumption of nitrite but also
743 indisputably established the paramount importance of OmpA in the maintenance of the outer
744 membrane permeability of wildtype *Salmonella.* STM *ΔompA ΔompD* which possesses intact
745 OmpC and OmpF on their outer membrane not only showed enhanced consumption of nitrite
746 but also higher sensitivity towards *in vitro* nitrosative stress (800 μM of acidified $NaNO_2$). In
747 comparison with STM *ΔompA ΔompC* and *ΔompA ΔompF* greater recruitment of nitrotyrosine
748 on STM *ΔompA ΔompD* because of its cytosolic localization and significant loss of outer
749 membrane stability is considered as the sole reason behind their poor proliferation in murine
750 macrophage. These results were further supported by the inability of STM *ΔompA ΔompC* and
751 *ΔompA ΔompF* to induce heightened [NO] response while staying inside the macrophage,
752 unlike STM *ΔompA ΔompD*. These results collectively endorse the pivotal role of OmpC and
753 OmpF in the entry of nitrite in *ompA* deficient *Salmonella* Typhimurium by increasing the
754 permeability and worsening the integrity of the bacterial outer membrane.

755 To summarize, our study claims Outer membrane protein A (OmpA) of wildtype *Salmonella*
756 Typhimurium to be a versatile protein with a multitude of activities. The deletion of *ompA* from
757 *Salmonella* not only interrupts the stability of SCV but also imposes outstanding paradoxical
758 consequences on the intracellular proliferation of bacteria. We further proposed an OmpA
759 dependent unique mechanism that deals with the integrity and permeability of the outer
760 membrane and cleverly employed by wildtype *Salmonella* to fight against the nitrosative stress
761 of macrophage.

762

763 **Abbreviations**

STM: *Salmonella* Typhimurium

OmpA: Outer membrane protein A

OmpC: Outer membrane protein C

OmpD: Outer membrane protein D

OmpF: Outer membrane protein F

LLO: Listeriolysin O

SCV: *Salmonella* containing vacuole

LAMP-1: Lysosome associated membrane protein-1

iNOS: Inducible nitric oxide synthase

RNI: Reactive nitrogen intermediates

ROS: Reactive oxygen species

RFP: Red fluorescent protein

roGFP2: Redox sensitive green fluorescent protein

Materials and Methods

Bacterial strains, media, and culture conditions

The wild type (WT) bacteria *Salmonella enterica* serovar Typhimurium [STM- (WT)] strain 14028S used in this study was a generous gift from Professor Michael Hensel, Max Von Pettenkofer-Institute for Hygiene und 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) or LB agar along with appropriate antibiotics like- kanamycin (50 µg/mL), ampicillin (50 µg/mL), Chloramphenicol (25 µg/mL), kanamycin and ampicillin (both

50 µg/mL), Kanamycin and Chloramphenicol (Kanamycin= 50 µg/mL, Chloramphenicol= 25 µg/mL) for wild type, knockout (single and double), complement and mCherry expressing strains respectively. *Salmonella- Shigella* agar was used for plating of cell lysates/ cell suspensions to calculate the bacterial burden in infected cell lines and several organs of infected mice respectively. Bacterial LB broth cultures were grown in a shaker incubator at 180 rpm either 37^0C for normal wild type and knockout strains or 30^0C for the strains having temperature-sensitive pKD46 plasmid or the strains harboring pQE60-Grx1-roGFP2 plasmid and undergoing IPTG (concentration= 500 µM) treatment. For growth curve experiments and *in vitro* RNA extraction studies, a single colony was inoculated in 5mL of LB broth and grown overnight with or without antibiotics at 37^0C. Overnight-grown stationary phase bacteria were sub-cultured at a ratio of 1: 100 in freshly prepared LB or minimal N salt media and kept in a 37^0C shaker incubator. At different time intervals aliquots were taken for RNA isolation, serial dilution, plating, and measurement of $[OD]_{600nm}$ by TECAN 96 well microplate reader. The complete list of strains and plasmids have been listed below. (Description in Table-1) Dead bacteria used in several sets of experiments were produced from viable wild-type bacteria either heating at 65^0C for 20 minutes or by treating the bacteria with 3.5% paraformaldehyde for 30 minutes.

Eukaryotic cell lines and growth conditions

The murine macrophage-like cell line RAW 264.7, human cervical adenocarcinoma cell line HeLa, human colorectal adenocarcinoma cell line Caco-2, were maintained in Dulbecco's Modified Eagle's Media (Sigma-Aldrich) supplemented with 10% FCS (Fetal calf serum, Gibco) at 37^0C temperature in 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). For polarizing the Caco-2 cells, DMEM media was further supplemented with 1% non-essential amino acid solution (Sigma-Aldrich). 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 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.

Construction of *ompA, ompC, ompD,* & *ompF* knockout strains of *Salmonella*

The knockout strains (mentioned above) of Salmonella *enterica* serovar Typhimurium (strain 14028S) were made using one step chromosomal gene inactivation method demonstrated by Datsenko and Wanner (Datsenko and Wanner, 2000). Briefly, STM (WT) was transformed with pKD46 plasmid, which has a 'lambda red recombinase system' under arabinose inducible promoter. The transformed cells were grown in LB broth in presence of ampicillin (50µg/mL) and 50 mM arabinose at 30^0C incubator until the $[OD]_{600nm}$ reaches 0.35 to 0.4. Electrocompetent STM pKD46 cells were prepared after washing the bacterial cell pellet thrice with double autoclaved chilled Milli Q water and 10% glycerol respectively. Finally, the electrocompetent STM pKD46 cells were resuspended in 50 µL of 10% glycerol. Kanamycin resistant gene cassette (Kan^R -size- 1.6kb- for knocking out *ompA, ompD*) and chloramphenicol resistant gene cassette (Chl^R – size- 1.1 kb- for knocking out *ompC, ompD* & *ompF*) were amplified from pKD4 and pKD3 plasmids respectively using knockout primers having a stretch of oligos at the 5' end homologous to the flanking region of the target genes (Description in Table-2). The amplified Kan^R and Chl^R gene cassettes were subjected to phenol-chloroform extraction and further transformed into STM (WT) pKD46 by electroporation. The transformed cells were plated on LB agar with kanamycin (50 µg/mL) for selection of *ΔompA, ΔompD* strains and LB agar with chloramphenicol (25 µg/mL) for selection of *ΔompC, ΔompD,* & *ΔompF* strains. The plates were further incubated overnight at 37^0C. The knockout colonies were confirmed by confirmatory and kanamycin/ chloramphenicol internal primers (Description in Table-2).

Construction of *ompA* complemented strain of *Salmonella*

For making the complemented strain, the first *ompA* gene was amplified with *ompA* cloning primers (Description in Table-2) by colony PCR. The amplified PCR product which was purified by phenol-chloroform extraction was subjected to restriction digestion by NcoI (NEB) and HindIII (NEB) in CutSmart buffer (NEB) at 37^0C for 2-3 hours along with the empty pQE60 vector backbone. Double digested insert and vector were subjected to ligation by a T_4 DNA ligase in 10X ligation buffer (NEB) overnight at 16^0C. The ligated product, as well as the empty vector, were transformed into STM *ΔompA* strain separately to form complemented and empty vector strains. Complementation was initially confirmed by colony PCR with cloning and *ompA* internal primers (data not shown) and finally by restriction digestion of recombinant plasmid. The expression level of *ompA* in the knockout, complemented, and empty vector strains were further confirmed by RT PCR using *ompA* specific RT primers (Description in Table-2).

Construction of *ΔompA ΔompC, ΔompA ΔompD,* & *ΔompA ΔompF* double knockout strains of *Salmonella*

The double knockout strains (mentioned above) of Salmonella *enterica* serovar Typhimurium (strain 14028S) were made by slightly modifying one step chromosomal gene inactivation strategy demonstrated by Datsenko and Wanner (Datsenko and Wanner, 2000). Briefly, STM *ΔompA*, where the *ompA* gene has already been replaced with a kanamycin-resistant gene cassette, was transformed with pKD46 plasmid. The transformed cells were grown in LB broth in presence of ampicillin (50μg/mL) and 50 mM arabinose at 30^0C incubator until the $[OD]_{600nm}$ reaches 0.35 to 0.4. Electrocompetent STM *ΔompA* pKD46 cells were made using the protocol mentioned above. Chloramphenicol resistant gene cassette (Chl^R – size- 1.1 kb) was amplified from pKD3 plasmid using knockout primers having a stretch of oligos at the 5’

end homologous to the flanking region of the target genes- *ompC*, *ompD*, *ompF*. (Description in Table-2) The amplified Chl^R gene cassette was subjected to phenol-chloroform extraction and further transformed into STM *ΔompA* pKD46 by electroporation. The transformed cells were plated on LB agar with kanamycin (50 µg/mL) and chloramphenicol (25 µg/mL) both for selection of double knockout strains (*ΔompA ΔompC*, *ΔompA ΔompD*, & *ΔompA ΔompF*). The plates were further incubated overnight at 37^0C. The knockout colonies were confirmed by confirmatory primers (Description in Table-2).

RNA isolation and RT PCR

In this study the temporal expression of *ompA*, *ompC*, *ompD*, *ompF* were measured in wild type *Salmonella* Typhimurium growing in nutritionally enriched LB broth, low magnesium acidic F media (pH= 5.4), which mimics the internal environment of *Salmonella* Containing Vacuole (SCV) [25, 44] and RAW264.7 macrophage cells by Real-Time PCR. Briefly, the bacterial cells from LB and F media were pelleted down by centrifugation at 12000 rpm for 5 minutes. The pellet was lysed with TRIzol reagent (RNAiso Plus, Takara) and stored at -80^0C overnight. Similarly, RAW264.7 cells infected with STM (WT) at MOI 50, were lysed with TRIzol reagent and stored at -80^0C overnight. The lysed supernatants were further subjected to chloroform extraction, which was immediately followed by an equal volume of isopropanol treatment. The pellet obtained from isopropanol treatment was finally washed with 70% RNA grade ethanol. The final pellet was air-dried and suspended in 20 µL of DEPC water. The concentration of RNA was measured in nano-drop and it was run on 1.5% agarose gel to assess the quality of RNA. To make cDNA from RNA, 3 µg of RNA sample was subjected to DNase treatment in presence of DNase buffer (Thermo Fischer Scientific) at 37^0C for 2 hours. The reaction was stopped with the addition of EDTA (Thermo Fischer Scientific), followed by heating the sample at 65^0C for 10 minutes. The cDNA was made by incubating the RNA initially with random hexamer at 65^0C for 10 minutes and then incubating the same template

with 5X RT buffer, RT enzyme, dNTPs, and DEPC water at 42^0C for 1 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 specific RT primers (Description in Table-2). 16S rRNA gene of *Salmonella* was used to normalize the expression level of target genes.

Percent phagocytosis calculation/ Invasion assay

1.5 to 2 X 10^5 cells (RAW264.7, U937, Caco-2, and HeLa) were seeded in the wells of 24 well plates and incubated for 6-8 hours at 37^0C temperature in presence of 5% CO_2. The protocol for calculating percent phagocytosis by macrophage cells has been modified a little, as demonstrated earlier (Balakrishnan et al., 2016). Briefly, the phagocytic macrophage cells (RAW264.7 and activated U937 cells) were infected with overnight grown 10- 12 hours old stationary phase culture of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* and *ΔompA*: pQE60 at MOI 10. For phagocytosis assay with complement proteins, the bacterial strains were incubated with 10% mouse complement sera for two hours before the experiment, and MOI 50 was used for the infection. Non-phagocytic epithelial cells (Caco-2 and HeLa cells) were infected with 3 to 4 hours old log phase culture of all four bacterial strains at the same MOI. The attachment of bacteria to the cell surface was increased by centrifuging the cells at 800 rpm for 5 minutes, followed by incubating the infected cells at 37^0C temperature in presence of 5% CO_2 for 25 minutes. Next, the cells were washed thrice with PBS to remove all the unattached bacteria and subjected to 100 µg/ mL and 25 µg/ mL concentration of gentamycin treatment for 1 hour each. 2 hours post-infection, the cells were lysed with 0.1% triton-X-100. The lysate was plated on *Salmonella- Shigella* Agar and the corresponding CFU. Percent phagocytosis (for phagocytic macrophage cells)/ Percent invasion (for non-phagocytic epithelial cells) was determined using the following formula-

Percent phagocytosis/ Percent invasion= [CFU at 2 hours]/ [CFU of pre-inoculum] X 100%

Adhesion assay

The protocol of adhesion assay has been performed as described before (Garai et al., 2016). Briefly, 1.5 to 2 X 10^5 cells (RAW264.7 and HeLa) were seeded on the top of sterile glass coverslips. Phagocytic macrophage cells (RAW264.7) were infected with overnight grown 10-12 hours old stationary phase culture of STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* and *ΔompA*:pQE60 at MOI 50. Non-phagocytic epithelial cells (HeLa) were infected with 3 -4 hours old log phase culture of all four bacterial strains at the same MOI. After centrifuging the cells at 800 rpm for 5 minutes, the infected cells were incubated at 37^0C temperature in presence of 5% CO_2 for 15 minutes (for RAW264.7 cells) and 25 minutes (for HeLa cells), respectively. After the incubation period, the cells were washed twice with sterile PBS and fixed with 3.5% PFA. To visualize the externally attached bacteria, the cells were primarily treated with anti-*Salmonella* antibody raised in rabbit (dilution 1: 100, duration 6 to 8 hours at 4^0C temperature), which was followed by the treatment of the cells with secondary antibody conjugated to an appropriate fluorophore (dilution 1: 200, duration 1 hour at room temperature), dissolved in 2.5% BSA solution without saponin. Images were obtained by confocal laser scanning microscopy (Zeiss LSM 710) using a 63X oil immersion objective lens. The number of bacteria adhering per cell was calculated by dividing the total number of bacteria adhered by the total number of host cells in a single microscopic field. The counting and analysis were done with the help of ZEN Black 2009 software provided by Zeiss.

Intracellular proliferation assay

The protocol of intracellular proliferation assay has been followed as demonstrated earlier (Chaudhuri et al., 2018). Briefly, the seeded RAW264.7, U937, Caco-2, and HeLa cells (1.5 to

2 X 10^5 cells per well) were infected with STM (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* and *ΔompA*:pQE60 at MOI 10, as mentioned earlier in this study. After centrifuging the cells at 800 rpm for 5 minutes, the infected cells were incubated at 37^0C temperature in 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 and 25 µg/ mL concentration of gentamycin treatment for 1 hour each. 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 set of experiments, the fold proliferation of STM (WT) and *ΔompA* in the macrophage (RAW 264.7) was measured in the presence of iNOS inhibitor 1400W dihydrochloride [10µM] and activator mouse IFN-γ [100U/ mL]. Both inhibitor (1400W) and activator (IFN-γ) were added to the cells infected with STM (WT) and *ΔompA* along with 25 µg/ mL of gentamycin solution. 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.

Chloroquine resistance assay

To estimate the number of intracellular bacteria localized in the cytoplasm of macrophage and epithelial cells chloroquine resistance assay was performed using a modified protocol as described previously (Wrande et al., 2016, Knodler et al., 2014). Briefly, the seeded RAW264.7 and Caco-2 cells (density- 1.5 to 2 X 10^5 cells per well) were infected with STM- (WT), *ΔompA*, and *ΔompA*: pQE60-*ompA* at MOI 10, as mentioned earlier in this study. After centrifuging the cells at 800 rpm for 5 minutes, the infected cells were incubated at 37^0C temperature in

presence of 5% CO_2 for 25 minutes. Next, the cells were washed with PBS and subjected to 100 µg/ mL and 25 µg/ mL of gentamycin treatment respectively. The old DMEM (having 25 µg/ mL gentamycin) was replaced from the wells with freshly prepared DMEM, supplemented with 25 µg/ mL gentamycin and 50 µg/ mL chloroquine two hours before lysis (14 hours post-infection). The cells were lysed with 0.1% triton-X-100 at 16 hours post-infection. The lysates were plated on *Salmonella- Shigella* agar and the corresponding CFU at 16 hours were determined. Percent abundance of cytosolic and vacuolar bacteria was obtained after dividing the CFU from chloroquine treated set with chloroquine untreated set.

Percentage of cytosolic bacteria= [CFU at 16 hours with chloroquine]/ [CFU at 16 hours without chloroquine] X 100%

Percentage of vacuolar bacteria= (100 - percentage of cytosolic bacteria) %

Confocal microscopy

For immunofluorescence study, RAW 264.7 or Caco-2 cells seeded at a density of 1.5 to 2 X 10^5 cells per sterile glass coverslip, were infected with appropriate bacterial strains at MOI 20. The cells were washed thrice with PBS and fixed with 3.5% paraformaldehyde for 15minutes at indicated time points post-infection. The cells were first incubated with specific primary antibody raised against *Salmonella* SseC/ SseD proteins, mouse lysosome-associated membrane protein-1 (LAMP-1) (rat anti-mouse LAMP-1), mouse nitrotyrosine (mouse anti-mouse nitrotyrosine), and/or S. Typhimurium (anti- Salmonella O antigen) as per the requirements of experiments, 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 the incubation of the cells with appropriate secondary antibody conjugated with fluorophores (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.

Griess assay to measure extracellular nitrite concentration

Extracellular nitrite from infected macrophage cells was measured using a protocol described earlier (Das et al., 2009). 3.13 μM, 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM standard $NaNO_2$ solutions were prepared from main stock [0.1 (M) $NaNO_2$] by serial dilution in deionized distilled water. The $[OD]_{545nm}$ of the standard solutions were measured after adding and incubating them with Griess reagent and the standard curve was drawn. Culture supernatants were collected from RAW264.7 cells infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, and heat-killed bacteria at 16 hours post-infection and subjected to nitrite estimation by adding Griess reagents. To 50 μL of culture supernatant, 50 μL of 1% sulphanilamide (made in 5% phosphoric acid) and 50 μL of 0.1% NED (N-1-naphthyl ethylene diamine dihydrochloride) were added and incubated for 10 minutes in darkness at room temperature. The $[OD]_{545nm}$ was measured within 30 minutes of the appearance of a purple-colored product.

Measurement of intracellular nitric oxide

The level of intracellular nitric oxide of infected macrophage was measured using cell membrane-permeable fluorescent nitric oxide probe 4, 5- diaminofluorescein diacetate (DAF2-DA) (Lahiri et al., 2008a). Briefly, RAW264.7 cells were infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA ΔompC*, *ΔompA ΔompD*, *ΔompA ΔompF* & LLO at MOI 10 as described before. 16 hours post-infection the culture supernatants were replaced with DMEM media, supplemented with 5 μM concentration of DAF2-DA, which was followed by further

incubation of the infected cells at 37^0C temperature in presence of 5% CO_2 for 30 minutes. The cells were washed with sterile PBS and acquired immediately for analysis by flow cytometry (BD FACSVerse by BD Biosciences-US) using 491 nm excitation channel and 513 nm emission channel.

Measurement of the activity of the *spiC* promoter

The activity of *spiC* promoter in STM (WT) and *ΔompA* was measured by mild alteration of a protocol described earlier (Das et al., 2009). Briefly, 1.5 mL of overnight grown stationary phase culture of STM (WT) and *ΔompA* carrying pHG86 *spiC-lacZ* construct were centrifuged at 6000 rpm for 10 minutes and the pellet was resuspended in 500 µL of Z-buffer (Na2HPO4, 60 mM; NaH2PO4, 40 mM; KCl, 10 mM; MgSO4.7H2O, 1mM). The OD of the Z-buffer was measured at 600 nm after resuspension. The cells were permeabilized by adding 5 µL of 0.1% SDS and 20 µL of chloroform and incubated at room temperature for 5 minutes. 100 µL of 4 mg/ mL of o-nitrophenyl *β*-D galactopyranoside was added in dark and incubated till the color appeared. The reaction was stopped using 250 µL 1 M Na_2CO_3. The reaction mixture was centrifuged at 6000 rpm for 10 minutes and the OD of the supernatant was measured at 420 nm and 550 nm on a flat bottom transparent 96 well plate. STM (WT) and *ΔompA* harboring promoter-less empty pHG86 *LacZ* construct were used as control. The activity of the *spiC* promoter was measured in Miller Unit using the following formula

$$\textbf{Miller Unit (MU)} = 1000[\, OD_{420nm} - OD_{550nm} * 1.75]/\, T*V*OD_{600nm}$$

OD_{420nm}= Absorbance by o-nitrophenol and light scattering by cell debris

OD_{550nm}= light scattering by cell debris

OD_{600nm} = bacterial cell density in the washed media

T= Time of reaction in minutes

V= Volume of the culture in mL.

Measurement of the activity of the *spiC* promoter from intracellular bacteria

RAW264.7 cells were infected with STM (WT): pHG86 *spiC-LacZ* and *ΔompA*: pHG86 *spiC-LacZ* at MOI 50. After centrifuging the cells at 800 rpm for 5 minutes, the infected cells were incubated at 37^0C temperature in 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 and 25 µg/ mL concentration of gentamycin treatment for 1 hour each. The cells were lysed with 0.1% triton-X-100 at 12 hours post-infection. The lysates were centrifuged at 14,000 rpm for 30 minutes and the pellet was resuspended in 500 µL of Z-buffer. The OD of the Z-buffer was measured at 600 nm after resuspension. The cells were permeabilized by the addition of 5 µL of 0.1% SDS and 20 µL of chloroform and incubated at room temperature for 5 minutes. 100 µL of 4 mg/ mL of o-nitrophenyl *β*-D galactopyranoside was added in dark and incubated till the color appeared. The reaction was stopped using 250 µL 1 M Na_2CO_3. The reaction mixture was centrifuged at 6000 rpm for 10 minutes and the OD of the supernatant was measured at 420 nm and 550 nm on a flat bottom transparent 96 well plate. The activity of the *spiC* promoter was measured in Miller Unit using the formula mentioned earlier.

Measurement of the cytosolic acidification of the bacteria using BCECF-AM

The acidification of the cytosol of the bacteria in presence of *in vitro* acidic stress was measured using a cell-permeable dual excitation ratiometric dye called 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM). 4.5 X 10^7 CFU of STM (WT), *ΔompA,* and *ΔompA*: pQE60-*ompA* from 12 hours old overnight grown stationary phase culture was resuspended in phosphate buffer (of pH 5.5, 6, 6,5, and 7 respectively) separate microcentrifuge tubes and incubated for 2 hours in a shaker incubator at 37^0C temperature. 30 minutes before the flow cytometry analysis BCECF-AM (1mg/ mL) was added in each tube so

that the final concentration becomes 20 µM. At the end of the incubation period of 2 hours, the bacterial cells were analyzed in flow cytometry (BD FACSVerse by BD Biosciences-US) using 405 nm and 488 nm excitation and 535 nm emission channel. The median fluorescence intensity (MFI) of the bacterial population at 488 nm and 405 nm was obtained from BD FACSuite software and the 488/ 405 ratio was determined to estimate the level of acidification of the bacterial cytosol.

Measurement of extracellular H_2O_2 by phenol red assay

H_2O_2 produced by RAW264.7 cells infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* at MOI 10 was measured by modifying a protocol of phenol red assay as demonstrated before (Lahiri et al., 2008b). Briefly, two hours post-infection, infected RAW264.7 cells were supplemented with phenol red solution having potassium phosphate (0.01 M; pH 7.0), glucose (0.0055 M), NaCl (0.14 M), phenol red (0.2 g/ L), and HRPO (8.5 U/ mL; Sigma- Aldrich). 16 hours post-infection the culture supernatant was collected and subjected to the [OD] measurement at 610 nm wavelength in TECAN 96 well microplate reader. In presence of H_2O_2 produced by macrophage horseradish peroxidase (HRPO) converts phenol red into a compound that has enhanced absorbance at 610 nm. The concentration of H_2O_2 produced by macrophages was measured by using a standard curve of H_2O_2 in phenol red solution with known concentrations ranging from 0.5 to 5 µM.

Measurement of intracellular ROS

The level of intracellular ROS in infected macrophages was measured using membrane-permeable redox-sensitive probe 2',7'- dichlorodihydrofluorescein diacetate (H_2DCFDA) (Lahiri et al., 2008b). Upon its oxidation by intracellular esterases this non-fluorescent dye is converted into highly fluorescent 2',7'- Dichlorofluorescein (H_2DCF), which has emission at 492- 495 nm and excitation at 517 to 527 nm. Briefly, RAW264.7 cells were infected with

STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* at MOI 10 as described before. 16 hours post-infection the culture supernatants were replaced with DMEM media, supplemented with 10 µM concentration of H_2DCFDA, which was followed by further incubation of the infected cells at 37^0C temperature in presence of 5% CO_2 for 30 minutes. The cells were washed with sterile PBS and acquired immediately for analysis by flow cytometry (BD FACSVerse by BD Biosciences-US) using 492 nm excitation channel and 517 nm emission channel.

Sensitivity assay of bacteria against *in vitro* nitrosative and oxidative stress

The sensitivity of STM (WT) and *ΔompA* was tested against *in vitro* nitrosative and oxidative stress. H_2O_2 dissolved in PBS of pH 5.4 was used for creating *in vitro* oxidative stress. Acidified nitrite ($NaNO_2$ in PBS of pH 5.4) alone and a combination of acidified nitrite along with H_2O_2 were used to generate *in vitro* nitrosative stress (Heaselgrave et al., 2010). Sensitivity was checked in both concentration and time-dependent manner.

Concentration-dependent sensitivity

10^8 CFU of overnight grown stationary phase cultures of STM- (WT) and *ΔompA* were inoculated in varying concentrations of acidified nitrite and peroxide ranging from 200 µM to 5 mM and further incubated for 12 hours. At the end of the incubation period, supernatants from each concentration were collected, serially diluted, plated on *Salmonella- Shigella* agar and the $\log_{10}$[CFU/ mL] values were acquired to determine the inhibitory concentrations of nitrite, peroxide, and both together.

Time-dependent sensitivity

10^8 CFU of overnight grown stationary phase cultures of STM- (WT) and *ΔompA* were inoculated in 800µM concentration of acidified nitrite and further incubated for 12 hours. Aliquots were collected, serially diluted, and plated on *Salmonella- Shigella* agar at 0, 3, 6, 9, 12 hours post-inoculation to monitor the CFU.

Bacterial cell viability assay by resazurin

Bacterial cell viability under the treatment of acidified nitrite and peroxide was measured using resazurin assay. Resazurin, a blue-colored non-fluorescent redox indicator is reduced into resorufin, a pink-colored fluorescent compound (having excitation at 540 nm and emission at 590 nm) by aerobic respiration of metabolically active cells. Briefly, STM- (WT) and *ΔompA* were treated with varying concentrations of acidified nitrite and peroxide as mentioned above. At the end of the incubation period, supernatants were collected and incubated with resazurin (1 µg/ mL) in a 37^0C shaker incubator at 180 rpm for 2 hours in a 96 well plate. At the end of the incubation period, the fluorescence intensity was measured using TECAN 96 well microplate reader and percent viability was calculated.

Nitrite uptake assay

Nitrite uptake by different bacterial strains [STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*:pQE60, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, *ΔompC*, *ΔompD*, *ΔompF*, and PFA fixed dead bacteria] was determined by measuring the remaining concentration of nitrite in the uptake mixture using a protocol described earlier (Das et al., 2009). Briefly, 10^8 CFU of overnight grown stationary phase bacterial cultures were inoculated in an uptake mixture, which consists of 40 mM glucose, 80 mM MOPS-NaOH buffer (pH= 8.5), and nitrite (50/ 100/ 200 µM) in a final volume of 5 mL. The assay mixtures were kept in a 37^0C shaker incubator after the inoculation is done. At indicated time points, 150 µL of suspension from each assay mixture was collected and subjected to Griess assay to determine the level of remaining nitrite as mentioned earlier.

Examination of *in vitro* redox homeostasis of STM (WT) and *ΔompA* in response to acidified nitrite

Stationary phase cultures of STM- (WT) and *ΔompA* harboring pQE60-Grx1-roGFP2 plasmid were sub-cultured in freshly prepared 5 mL LB broth at 1: 33 ratios in presence of appropriate antibiotic in 37^0C shaker incubator at 175 rpm. Once the $[OD]_{600\ nm}$ has reached 0.3 to 0.4, 500 µM of IPTG (Sigma-Aldrich) was added and the cells were further grown in 30^0C temperature at 175 rpm for 10 to 12 hours. At the end of the incubation period, 4.5 X 10^7 CFU of bacteria were subjected to the treatment of acidified nitrite [varying concentrations (as mentioned in the figure legend) of $NaNO_2$ in PBS of pH= 5.4] for 15, 30, 45, and 60 minutes. At the end of every indicated time point, the cells were analyzed in flow cytometry (BD FACSVerse by BD Biosciences-US) using 405 nm and 488 nm excitation and 510 nm emission channel. The mean fluorescence intensity at 405 nm and 488 nm was obtained from the FITC positive (GFP expressing) population and the 405/ 488 ratio was determined.

Determination of outer membrane porosity of intracellular and extracellular bacteria by bisbenzimide

The outer membrane porosity of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, and *ΔompA*:pQE60 grown in low magnesium acidic N s medium (pH= 5.4) was measured using bisbenzimide (Sigma-Aldrich) by modifying a protocol as specified previously (Coldham et al., 2010). The bacterial strains were grown in low magnesium acidic N salt medium for 12 hours. At the end of the incubation period, the culture supernatants were collected and the $[OD]_{600\ nm}$ was adjusted to 0.1 with sterile PBS. 20 µL of bisbenzimide (10 µg/ mL) solution was added to 180 µL of culture supernatants (whose $[OD]_{600\ nm}$ has already been adjusted to 0.1) in 96 well a microplate and further incubated for 10 minutes in 37^0C shaker incubator. Because of enhanced outer membrane porosity, when bisbenzimide is taken up by the bacterial cells, it binds to the bacterial DNA and starts fluorescing. The fluorescence intensity of DNA bound bisbenzimide was measured in TECAN 96 well microplate reader using 346 nm excitation and 460 nm emission filter.

To check the outer membrane porosity of intracellular STM- (WT) and *ΔompA*, infected RAW264.7 macrophage cells were lysed with 0.1% Triton X-100. The lysate was collected and centrifuged at 300g for 5 minutes to settle down eukaryotic cell debris. The sup was collected and further centrifuged at 5000 rpm for 20 minutes to settle down the bacteria. This was followed by decanting the sup and resuspending the bacterial pellets with PBS. Finally, the suspension was subjected to bisbenzimide treatment to measure the fluorescence intensity as mentioned above.

Determination of bacterial membrane polarity using $DiBAC_4$

Outer membrane polarity of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, and *ΔompA*:pQE60 grown in low magnesium acidic N salt medium (pH= 5.4) for 12 hours was measured using a fluorescent membrane potential sensitive dye called bis-(1,3-dibutyl barbituric acid)-trimethylene oxonol (Invitrogen) (Marathe et al., 2010). Briefly, 4.5 X 10^7 CFU of each bacterial strain was incubated with 1 µg/ml of $DiBAC_4$ for 15 minutes in a 37^0C shaker incubator. The $DiBAC_4$ treated bacterial cells were further analyzed by flow cytometry (BD FACSVerse by BD Biosciences-US) to evaluate the change in membrane polarity upon knocking out *ompA*.

Comparison of *Salmonella* Containing Vacuolar pH between WT and *ΔompA*

The SCV pH of macrophages infected with STM- (WT) and *ΔompA* was compared using a protocol reported earlier (Gogoi et al., 2018). Briefly, overnight grown stationary phase cultures of STM- (WT) and *ΔompA* were labeled with 20 µM pH rhodo red succinimidyl ester at 37^0C incubator. The cells were further washed with 0.1 (M) sodium bicarbonate buffer (pH= 8.4) post- labeling. RAW264.7 cells were infected with these labeled bacteria at MOI 50. 12 hours post-infection the cells were washed with PBS and acquired for analysis by flow

cytometry (CytoFLEX by Beckman Coulter Life Sciences) using 560 nm excitation and 585 nm emission filter.

Expression profiling of *ompC, ompD, ompF* in STM- (WT), *ΔompA* and complement strains growing in LB broth, acidic N media, and macrophage

Overnight grown stationary phase cultures of STM- (WT), *ΔompA,* & *ΔompA*: pQE60-*ompA* were inoculated in freshly prepared LB broth, low magnesium acidic F media (pH= 5.4) at a ratio of 1: 100. The cells were further grown in a 37^0C shaker incubator at 180 rpm for 12 hours. RAW264.7 cells were infected with above mentioned bacterial strains at MOI 50 and incubated further for 12 hours as mentioned earlier. At the end of the specified incubation, period RNA was isolated, cDNA was synthesized and expression of *ompC, ompD, ompF* were checked as mentioned earlier.

Animal survival assay

4-6 weeks old BALB/c and C57BL/6 mice housed in the specific-pathogen-free condition of central animal facility of Indian Institute of Science, Bangalore was used for all the *in vivo* infection and survival studies. All the animal experiments were approved by the Institutional Animal Ethics Committee and the National Animal Care Guidelines were strictly followed. Two cohorts of twenty 4-6 weeks old BALB/c and C57BL/6 mice were infected with 10-12 hours old overnight grown stationary phase cultures of STM (WT) and *ΔompA* by oral gavaging at lethal dose 10^8 CFU/ animal respectively **(n= 10)**. The survival of infected mice was observed for the next few days until all the mice infected with STM (WT) died. The survival was recorded, and the data was represented as percent survival.

Determination of bacterial burden in different organs

Four cohorts of five 4-6 weeks old C57BL/6 mice were infected with STM (WT) and *ΔompA* by oral gavaging at sub-lethal dose 10^7 CFU/ animal respectively **(n= 5)**. Two of these cohorts

infected with STM- (WT) and *ΔompA* strains respectively were further intraperitoneally injected with iNOS inhibitor aminoguanidine hydrochloride (AGH- 10mg/ kg of body weight) regularly for 5 days post-infection. The other two cohorts were treated with a placebo. Two cohorts of *iNOS*$^{-/-}$ mice were orally infected with STM (WT) and *ΔompA* at 10^7 CFU/ animal **(n=5)**. Two cohorts of five *gp91phox*$^{-/-}$ mice, which are unable to generate ROS were orally gavaged with STM- (WT) and *ΔompA* at 10^7 CFU/ animal **(n=5)**. On the 5th day post-infection all the mice were sacrificed, which was followed by isolation, weighing, and homogenization of specific organs like- liver, spleen, and MLN. The organ lysates were plated on *Salmonella Shigella* agar to determine the bacterial burden in different organs. The CFU corresponds to an organ was further normalized with organ weight and the $\log_{10}$[CFU/ gm-wt.] value has been plotted.

Statistical analysis

Each assay has been independently repeated 2 to 5 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 and *p* values below 0.05 were considered significant. Results obtained from *in vitro* sensitivity assays were analyzed by 2way ANOVA. Data obtained from *in vivo* infection of mice were analyzed by Mann- Whitney *U* test from GraphPad Prism 8 software. Flow cytometry data were analyzed and plotted using BD FACSuite (by BD Biosciences-US) and CytoFLEX (by Beckman Coulter Life Sciences) software respectively. The results are expressed as mean ± SD or mean ± SEM. Differences between experimental groups were considered significant for $p < 0.05$.

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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.

Author Contributions

ARC and DC conceived the study and designed the experiments. ARC performed all the experiments, analyzed the data, and wrote the manuscript. DC supervised the study. All the authors have read and approved the manuscript.

Declarations

Ethics statement

All the animal experiments were approved by the Institutional Animal Ethics Committee and the Guidelines provided by National Animal Care were strictly followed. (Registration No: 48/1999/CPCSEA).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflict of interests.

Table 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	This study
ΔompA: pQE60-*ompA*	Kan^R, Amp^R	This study
ΔompA: pQE60	Kan^R, Amp^R	This study
S. Typhimurium *ΔompC*	Chl^R	This study
S. Typhimurium *ΔompD*	Chl^R	This study
S. Typhimurium *ΔompD*	Kan^R	This study
S. Typhimurium *ΔompF*	Chl^R	This study
S. Typhimurium *ΔompA ΔompC*	Kan^R, Chl^R	This study
S. Typhimurium *ΔompA ΔompD*	Kan^R, Chl^R	This study
S. Typhimurium *ΔompA ΔompF*	Kan^R, Chl^R	This study
pKD4	Plasmid with FRT-flanked Kanamycin resistance gene	KA Datsenko & BL Wanner, PNAS, 2000
pKD46	Plasmid expressing λ red recombinase, Amp^R	KA Datsenko & BL Wanner, PNAS, 2000
pQE60 vector	Low copy number plasmid, Amp^R	Gifted by Prof.

		Umesh Varshney, MCB, IISc
pFV- mCherry (RFP)	Amp^R	Laboratory stock
pFV: GFP	Amp^R	Laboratory stock
STM (WT): *LLO*	Amp^R	Laboratory stock
STM *ΔompA*: *LLO*	Amp^R	This study
S. Typhimurium wildtype: pHG86 *spiC-LacZ*	Amp^R	This study
S. Typhimurium *ΔompA*: pHG86 *spiC-LacZ*	Amp^R	This study
pHG86 *spiC-LacZ*	Amp^R	Laboratory stock
pQE60-Grx1-roGFP2	Amp^R	Gifted by Dr. Amit Singh, CIDR, IISc

Table 2. Primer sequences (5' to 3')

ompA knockout forward-

TCGTTGGAGATATTCATGGCGTATTTTGGATGATAACGAGCATATGAATATCCTC

CTTAG

ompA knockout reverse-

AAGAAGTAACGCTGAAAGGCGTTGTCATCCAGACCAGAGCGTGTAGGCTGGAGC

TGCTTC

ompC knockout forward-

ATAACTGTAACATCTTAAAAGTTTTAGTATCATATTCGTGGTGTAGGCTGGAGCTGCTTC

ompC knockout reverse-

TATCAAAACGTCGTATTTGTACGCCGGAATAAGGCATGATGGGAATTAGCCATGGTCC

ompD knockout forward-

TTATTAAAATGAAACTTAAGTTAGTGGCAGTGGCAGTGTTTAAATGGCGCGCCTTACG

ompD knockout reverse-

CAAAATTAGAACTGGTAGTTCAGACCAACAGCAACGATGTGGAAGATCACTTCGCAGAA

ompF knockout forward-

ATTGACGGAATTTATTGACGGCAGTGGCAGGTGTCATAGTGTAGGCTGGAGCTGCTTC

ompF knockout reverse-

TACAAAATGCCAACCGTTAGCGCTAAAAAGCCCGCCTGTTATGGGAATTAGCCATGGTCC

ompA cloning forward- CATGCCATGGATGAAAAAGACAGCTATCGC

ompA cloning reverse- CCCAAGCTTTTGTCATCCAGACCAGAG

ompA confirmatory forward- CGGTAGAGTAACTATTGAG

ompA confirmatory reverse- TTACAGGCGTTATTAGGC

ompA expression forward- ATCCAATCACTGACGATCTG

ompA expression reverse- GCATCACCGATGTTGTTAGT

ompC confirmatory forward- GGTAAACAGACATTCAGA

ompC confirmatory reverse- AGTCATTTTCATCGCTGTT

ompD confirmatory forward- GAACTTATGCCACTCCGTCATT

ompD confirmatory reverse- CAGCATTTCGACGTCAACGGTA

ompF confirmatory forward- GTCAGACACATAAAGACACC

ompF confirmatory reverse- CGAGGTTCCATTATAGTTACAG

Kanamycin^{R} internal forward- CGGTGCCCTGAATGAACTGC

Kanamycin^{R} internal reverse- CGGCCACAGTCGATGAATCC

Chloramphenicol^{R} internal forward- ACAAACGGCATGATGAACCT

Chloramphenicol^{R} internal reverse- GCTCTGGAGTGAATACCACG

spiC expression forward- ACCTAAGCCTTGTCTTGCCT

spiC expression reverse- CCATCCGCTGTGAGCTGTAT

sseC expression forward- TTTGGCGAGGAAGTGGTTGA

sseC expression reverse- AGCCATTTCACGTTCAAGCG

sseD expression forward- TGTTGTCGGGTGTACTGACG

sseD expression reverse- ACGGCTTGACCCGCTATAAG

16srRNA forward- GAGCGCAACCCTTATCCTTTG

16srRNA forward- CACTTTATGAGGTCCGCTTGCT

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Figure Legends

Figure 1.

OmpA promotes the evasion of phagocytosis and intracellular survival of *Salmonella* in macrophages.

(A) RAW 264.7 cells and PMA activated U937 cells were infected with STM wild type (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 respectively at MOI 10. 2 hours post-infection the cells were lysed and plated. The CFU at 2 hours was normalized with pre-

inoculum to calculate the percent phagocytosis, (n=3, N=3). (B) RAW 264.7 cells were infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 respectively at MOI 50. Cells were fixed at 15 minutes post-infection with PFA. Externally attached bacteria were probed with anti-*Salmonella* antibody without saponin. 20 microscopic fields were analyzed to calculate the no. of adherent bacteria/ total no. of cells per field. Scale bar = 20μm, (n=20, N=3). (C) RAW264.7 cells and PMA activated U937 cells were infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 respectively at MOI 10. 16- and 2-hours post-infection the cells were lysed and plated. The CFU at 16 hours were normalized with the CFU at 2 hours to calculate the fold proliferation of bacteria in macrophage, (n=3, N=3). (D) RAW264.7 cells were infected with STM- (WT): RFP, *ΔompA*: RFP, *ΔompA*: pQE60-*ompA* at MOI of 20. PFA fixed wild-type bacteria was used for infection at MOI 25. Cells were fixed at 16 hours post-infection & LAMP-1 was labeled with anti-mouse LAMP-1 antibody. To stain the complemented strain and PFA fixed dead bacteria anti-*Salmonella* antibody was used. The quantification of LAMP-1 recruitment on bacteria in RAW 264.7 cells has been represented in form of a graph. Percent colocalization was determined after analyzing 50 different microscopic fields from three independent experiments. Scale bar = 5μm, (n=50, N=3). (E) Chloroquine resistance assay of RAW 264.7 cells infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* strains respectively, (n=3, N=2).

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

Figure 2.

OmpA dependent protection of *Salmonella* against nitrosative stress inside RAW264.7 cells.

(A) Estimation of extracellular nitrite from the culture supernatant of RAW264.7 cells infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, & heat-killed bacteria respectively at MOI 10. 16 hours post-infection the culture supernatants were collected and subjected to Griess assay, (n=3, N=5). (B) Estimation of the level of intracellular nitric oxide in RAW 264.7 cells infected with STM- (WT), *ΔompA*, and *ΔompA*: pQE60-*ompA* at MOI 10, 16 hours post-infection using DAF-2 DA [5 µM] by flow cytometry. Unstained and uninfected RAW264.7 cells have also been used as controls. Both dot plots (SSC-A vs DAF-2 DA) and histograms (Count vs DAF-2 DA) have been represented. The percent population of DAF-2 DA positive RAW264.7 cells has been represented in form of a graph, (n=3, N=3). (C) Immunofluorescence image of RAW264.7 cells infected with STM- (WT): RFP, *ΔompA*: RFP, (WT): *LLO*, and *ΔompA*: *LLO* at MOI 20. 16 hours post-infection the cells were fixed with PFA and labeled with anti-*Salmonella* antibody and anti-mouse nitrotyrosine antibody followed by visualization under confocal microscopy. Percent colocalization of STM- (WT), *ΔompA*, (WT): *LLO*, and *ΔompA*: *LLO* with nitrotyrosine was determined by analyzing 50 different microscopic fields from three independent experiments, (n=50, N=3). Scale bar = 5µm. (D) RAW264.7 cells were infected with STM- (WT), *ΔompA* & (WT): *LLO* at MOI 10. 16- and 2-hours post-infection the cells were lysed and plated. The CFU at 16 hours were normalized with the CFU at 2 hours to calculate the fold proliferation of bacteria, (n=3, N=3). (E) The transcript-level expression profile of *spiC* from RAW264.7 cells infected with STM- (WT), *ΔompA* & (WT): *LLO* at MOI 50. STM (WT) infected RAW264.7 cells treated with bafilomycin A (50 nM) was used as a control. 12 hours post-infection the cells were lysed, and total RNA was extracted. After the synthesis of cDNA, the expression of *spiC* was measured by RT PCR, (n=3, N=3). (F) The measurement of the activity of *spiC* promoter in stationary phase culture of STM- (WT) and *ΔompA* growing overnight in LB media, (n=6, N=2). The measurement of the activity of *spiC* promoter in STM- (WT) and *ΔompA* proliferating

intracellularly in RAW264.7 cells (MOI= 50) 12 hours post-infection. Macrophage infected with STM- (WT) and further treated with bafilomycin A (50 nM) has been used as a negative control, (n=5, N=2). (G) Estimation of intracellular acidification of STM- (WT), *ΔompA*, and *ΔompA*: pQE60-*ompA* in phosphate buffer of pH 5.5, 6, 6.5, and 7 respectively using 20 µM of ratiometric pH indicator BCECF-AM by flow cytometry. The ratio of median fluorescence intensity of BCECF-AM labeled on STM- (WT), *ΔompA*, and *ΔompA*: pQE60-*ompA* at 488 and 405 nm respectively, (n=4, N=3).

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

Figure 3.

Alteration in the activity of iNOS by using specific inhibitor or activator changes the fate of STM *ΔompA* in *in vitro* and *in vivo* infection model.

Intracellular survival of STM- (WT) and *ΔompA* (MOI-10) in RAW264.7 cells (16 hours post-infection) in presence and absence of iNOS (A) inhibitor- 1400W dihydrochloride [10 µM] and (B) activator- mouse IFN-ɣ [100U/ mL]. Fold proliferation of bacteria was calculated by normalizing the CFU at 16 hours to CFU at 2 hours, (n=3, N=3). (C) Immunofluorescence image of RAW264.7 cells infected with STM- (WT) and *ΔompA* (MOI=20) in presence and absence of iNOS inhibitor- 1400W dihydrochloride [10 µM] and activator- mouse IFN-ɣ [100U/ mL]. 16 hours post-infection the cells were fixed with PFA and probed with anti-*Salmonella* antibody and anti-mouse nitrotyrosine antibody. The quantification of the recruitment of nitrotyrosine on STM- (WT) and *ΔompA* (MOI-20) in RAW264.7 cells (16 hours post-infection) in presence and absence of iNOS (D) inhibitor- 1400W dihydrochloride [10 µM] and (E) activator- mouse IFN-ɣ [100U/ mL]. Percent colocalization of bacteria with nitrotyrosine was determined after analyzing more than 50 different microscopic fields from

two independent experiments, (n≥ 50, N=2). (F) The schematic representation of the strategy of animal experiment. (G) Four cohorts of 4 to 6 weeks old five C57BL/6 mice were orally gavaged with STM- (WT) and *ΔompA* at a sublethal dose of 10^7 CFU/ animal. Two of these four cohorts were intraperitoneally injected with iNOS inhibitor aminoguanidine hydrochloride (10mg/ kg of body weight) regularly for 5 days post-infection. Two cohorts of five *iNOS*$^{-/-}$ and *gp91phox*$^{-/-}$ mice were orally gavaged with STM- (WT) and *ΔompA* at a sublethal dose of 10^7 CFU/ animal separately. On the 5th-day post-infection, the mice were sacrificed, which was followed by isolation, homogenization of liver, spleen, and MLN. The organ lysates were plated on *Salmonella Shigella* agar. The colonies were counted 16 hours post-plating. The log_{10}[CFU/ gm-wt.] for each CFU has been plotted, (n=5, N=3). (H) Two cohorts- each consisting of 4 to 6 weeks old 20- BALB/c and C57BL/6 mice infected with a lethal dose (10^8 CFU/ animal) of STM- (WT) and *ΔompA*. After they were infected by oral gavaging, the survival of the mice was monitored till the death of all the wild type infected mice, (n=10).

(P)* *< 0.05, *(P)* **< 0.005, *(P)* ***< 0.0005, *(P)* *< 0.0001, ns= non-significant, (Student's *t* test, Mann-Whitney *U* test- for animal survival).**

Figure 4.

OmpA dependent regulation of outer membrane permeability in *Salmonella* controls cytoplasmic redox homeostasis in response to *in vitro* nitrosative stress.

(A) Time-dependent *in vitro* death kinetics of STM- (WT) and *ΔompA* in presence of acidified nitrite (Nitrite concentration 800 µM in PBS of pH 5.4). 10^8 CFU of overnight grown stationary phase culture of both the strains were inoculated in acidified nitrite. The survival of both the strains was determined by plating the supernatant with appropriate dilution in SS agar plates at 0, 3, 6, 9, 12 hours post-inoculation, (N=5). (B) *In vitro* nitrite uptake assay of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, & PFA fixed dead bacteria. 10^8 CFU of

overnight grown stationary phase culture of all the strains were inoculated in MOPS- NaOH buffer (pH= 8.5) with an initial nitrite concentration of 200 µM. The remaining nitrite concentration of the media was determined by Griess assay at 0, 3, 6, 9, 12 hours post-inoculation, (n=3, N=4). (C) Measurement of redox homeostasis of STM- (WT) and *ΔompA* harboring pQE60-Grx1-roGFP2 in response to varying concentrations of acidified nitrite in a time-dependent manner. 4.5X 10^7 CFU of overnight grown stationary phase culture of STM (WT) and *ΔompA* expressing pQE60-Grx1-roGFP2 were subjected to the treatment of acidified nitrite (concentration of 800µM, 1mM, 5mM) for 15, 30, 45, and 60 minutes. Median fluorescence intensities of Grx1-roGFP2 at 405nm and 488nm for FITC positive population were used to obtain the 405/ 488 ratio in 800 µM, 1mM, 5 mM concentration of acidified nitrite respectively, (n=3, N=3). (D) The transcript level expression profiling of larger porin genes namely- *OmpC*, *OmpD*, *OmpF* by real-time PCR in STM- (WT), *ΔompA*, & *ΔompA*: pQE60-*ompA* in nutritionally enriched LB media, low magnesium acidic F media (pH= 5.4) mimicking the internal environment of *Salmonella* containing vacuole, and RAW264.7 murine macrophage cells (MOI 50), [12 hours post inoculation], (n=3, N=2). (E) Measurement of membrane porosity of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 in acidic F media [12 hours post inoculation] using a slightly negatively charged dye named $DiBAC_4$ (final concentration- 1 µg/ mL) by flow cytometry. Unstained bacterial cells were used as control. Both dot plots (SSC-A vs $DiBAC_4$) and histograms (Count vs $DiBAC_4$) have been represented. The median fluorescence intensity of $DiBAC_4$ has been represented here, (n=3, N=2). (F) Measurement of outer membrane porosity of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* & *ΔompA*: pQE60 in acidic F media [12 hours post inoculation] using a nuclear binding fluorescent dye bisbenzimide [excitation- 346 nm and emission- 460 nm] (final concentration- 1 µg/ mL). (WT) treated with 0.1% saponin for 15 minutes has been used as a positive control, (n=8, N=3). (G) Measurement of outer membrane porosity of STM- (WT) and *ΔompA* isolated

from infected RAW264.7 cells 12 hours post-infection by bisbenzimide (Sigma) (final concentration- 1 µg/ mL), (n=6, N=3).

(P)* *< 0.05, *(P)* **< 0.005, *(P)* ***< 0.0005, *(P)* *< 0.0001, ns= non-significant, (2way ANOVA, Student's *t*-test).**

Figure 5.

The maintenance of the integrity of the SCV membrane inside RAW264.7 macrophage solely depends upon OmpA, not on other larger porins such as OmpC, OmpD, and OmpF.

(A) RAW264.7 cells were infected with STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, & WT: *LLO* at MOI 20. Cells were fixed at 16 hours post-infection, followed by labeled with anti-*Salmonella* antibody and anti-mouse LAMP-1 antibody. (B) Quantification of LAMP-1 recruitment on STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, (WT): *LLO*. Percent colocalization between bacteria and LAMP-1 was determined after analyzing more than 50 different microscopic fields from two independent experiments, (n≥50, N=2). Scale bar = 5µm. (C) RAW264.7 cells were infected with STM- (WT), *ΔompA*, *ΔompC*, *ΔompD*, and *ΔompF* at MOI 20. Cells were fixed at 16 hours post-infection, followed by labeled with anti-*Salmonella* antibody and anti-mouse LAMP-1 antibody respectively. (D) Quantification of LAMP-1 recruitment on STM- (WT), *ΔompA*, *ΔompC*, *ΔompD*, and *ΔompF*. Percent colocalization of bacteria with LAMP-1 has been determined after analyzing more than 60 different microscopic fields from two independent experiments, (n≥60, N=2). Scale bar = 5µm.

(P)* *< 0.0001, (Student's *t* test).**

Figure 6.

In absence of OmpA, porins OmpC and OmpF enhance the susceptibility of *Salmonella* against the nitrosative stress of RAW264.7 cells.

(A) *In vitro* nitrite uptake assay of STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, & PFA fixed dead bacteria. 10^8 CFU of overnight grown stationary phase culture of all the strains were inoculated in MOPS- NaOH buffer (pH= 8.5) with 100 µM initial concentration of nitrite. The remaining nitrite in the media was estimated by Griess assay at 0, 2, 4, 6, 8 hours post-inoculation, (n=3, N=6). (B) *In vitro* viability assay of STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, & *ΔompAΔompF* in presence of acidified nitrite [800 µM] 12 hours post-inoculation using resazurin solution, (n=3, N=3). (C) Immunofluorescence image of RAW264.7 cells infected with STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, & (WT): *LLO* at MOI 20. Cells were fixed at 16 hours post-infection, followed by labeled with anti-*Salmonella* antibody and anti-mouse nitrotyrosine antibody. Quantification of nitrotyrosine recruitment on STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, (WT): *LLO* has been represented in form of a graph. Percent colocalization between bacteria and nitrotyrosine was determined after analyzing more than 60 different microscopic fields from two independent experiments, (n≥60, N=3). Scale bar = 5µm. (D) Intracellular survival of STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, & *ΔompAΔompF*, & (WT): *LLO* respectively (MOI 10) in RAW264.7 cells. 16- and 2-hours post-infection the cells were lysed and plated. The CFU at 16 hours were normalized with the CFU at 2 hours to calculate the fold proliferation of bacteria in macrophage, (n=3, N=3). (E) Estimation of the intracellular nitrite level in RAW 264.7 cells infected with STM- (WT), *ΔompA*, *ΔompAΔompC*, *ΔompAΔompD*, *ΔompAΔompF*, and (WT): *LLO* respectively at MOI 10, 16 hours post-infection using DAF-2 DA [5 µM] by flow cytometry. Unstained cells have also been used as control. Both dot plots (SSC-A vs DAF-2 DA) and histograms (Count vs

DAF-2 DA) have been represented. Median fluorescence intensity of DAF-2 DA has been represented in form of a graph, (n=3, N=3).

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

Figure 7.

The hypothetical model that depicts the fate of (A) STM (WT), (B) STM *ΔompA*, and (C) STM (WT): *LLO* inside the murine macrophage. (A) STM (WT) staying inside the acidic SCV can proliferate efficiently by suppressing the activity of iNOS in SpiC dependent manner, (B) STM *ΔompA,* which is unable to remain in SCV, is heavily bombarded with RNI in the cytosol of macrophage, (C) STM (WT): *LLO* can survive in the cytosol by reducing its outer membrane permeability to RNI with the help of OmpA.

Figure S1.

OmpA plays a crucial role in the survival of *Salmonella* Typhimurium in murine macrophage.

The transcript level expression profile of (A) *ompA*, (C) *ompC*, (E) *ompD*, and (G) *ompF* in STM- (WT) at indicated time points (3, 6, 9, 12 hours) in LB broth (n=3, N=3), low magnesium acidic F media (pH=5.4) (n=3, N=3), and RAW264.7 murine macrophage cells (MOI= 50), (n=3, N=3). The predicted structures of (A) OmpA, (B) OmpC, (C) OmpD, and (D) OmpF using SWISS-MODEL protein structure homology-modeling server.

***(P)* *< 0.05, *(P)* **< 0.005, (Student's *t* test).**

Figure S2.

STM *ompA* knockout and complement strains generation, confirmation and analysis of growth kinetics in LB media

(A) Confirmation of *ompA* knockout in *Salmonella* using confirmatory primers, [Colonies C-2, 3, 5, 6, 7, 9, 10, 12, 13 are *ompA* knockout (1.7kb size), W- STM (WT) (1.3 kb size)] (B) Confirmation of knockout using kanamycin internal primers (Kan^R) [Colonies C-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 are *ompA* knock out (.47 kb size), W- STM WT (no band)], (C) Confirmation of complement strain generation by restriction digestion [DD- Double digested plasmid , SD1, SD2- Single digested plasmid, NC- Negative Control (No Enzyme), UC- Uncut plasmid, L- Ladder], (D) Confirmation of *ompA* knockout and complementation strain by RT PCR, (n=4, N=2), (E-F) Growth kinetics of STM (WT), STM *ΔompA,* STM *ΔompA*: pQE60-*ompA,* STM *ΔompA*: pQE60 in (E) $[OD]_{600nm}$ and (F) log_{10}[CFU/mL] respectively, (n=3, N=3). ***(P)* *< 0.05, *(P)* **< 0.005, *(P)* ****< 0.0001, ns= non-significant, (Student's *t* test).**

Figure S3.

OmpA dependent invasion and intracellular proliferation of *Salmonella* inside the epithelial cell.

(A) Caco-2 cells and HeLa cells were infected with log phase culture of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 respectively at MOI 10. 2 hours post-infection the cells were lysed and plated. The CFU at 2 hours was normalized with pre-inoculum to calculate the percent invasion in epithelial cells, (n=3, N=3). (B) HeLa cells were infected with log phase culture of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 respectively at MOI 50. Cells were fixed at 25 minutes post-infection with PFA. Externally attached bacteria were probed with anti-*Salmonella* antibody without saponin. 20 microscopic fields were analyzed to calculate the no. of adherent bacteria/ total no. of cells per field, (n=20, N=3). Scale bar = 20µm. (C) Caco-2 and HeLa cells were infected with log phase culture of STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, & *ΔompA*: pQE60 respectively at MOI 10. 16- and 2-hours post-infection the cells were lysed and plated. The CFU at 16 hours were normalized with the CFU

at 2 hours to calculate the fold proliferation of bacteria in epithelial cells, (n=3, N=3). (D) Caco-2 cells were infected with STM- (WT): RFP, *ΔompA*: RFP, and *ΔompA*: pQE60-*ompA* at MOI 20. PFA fixed wild-type bacteria was used for infection at MOI 25. Cells were fixed at 16 hours post-infection & LAMP-1 was labeled with anti-human LAMP-1 antibody. The complemented strain and PFA fixed dead bacteria were tracked with an anti-*Salmonella* antibody. The quantification of LAMP-1 recruitment on bacteria in Caco-2 cells has been represented in form of a graph. Percent colocalization was determined after analyzing 50 different microscopic fields from three independent experiments. Scale bar = 5μm, (n=50, N=3). (E) Chloroquine resistance assay of Caco-2 cells infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA* strains, (n=3, N=2).

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

Figure S4.

STM *ΔompA* comes out into the cytoplasm of the macrophage after quitting the vacuole.

(A) Verification of the sub-cellular niche of STM- (WT) and *ΔompA* in macrophage by flow cytometry. RAW264.7 cells were infected with STM- (WT) and *ΔompA* labeled with pH rhodo red dye at MOI 50. Uninfected RAW264.7 cells were used as unstained control. 12 hours post-infection the cells were harvested and subjected to analysis by flow cytometry. The dot plots (of SSC-A vs pH rhodo red) and histograms from one experiment out of three independently carried out experiments have been represented here. The median fluorescence intensity of pH rhodo red for both STM- (WT) and *ΔompA* strains has been represented in form of a graph. The representative image has been chosen from three independently carried out experiments. (n=5, N=3). (B-E) RAW264.7 cells were infected with STM (WT)-: RFP, *ΔompA*: RFP, at MOI 20. Cells were fixed at 16 hours post-infection, *Salmonella* SPI-2 encoded translocon proteins

SseC and SseD were labeled with anti- *Salmonella* SseC/ SseD antibody. (F), (H) The quantification of SseC and SseD recruitment on bacteria in RAW 264.7 cells respectively. Percent colocalization was determined after analyzing 50 different microscopic fields from three independent experiments. Scale bar = 5µm, (n=50, N=3). (G), (I) The transcript level expression of *sseC* and *sseD* in STM (WT) and *ΔompA* growing intracellularly in RAW264.7 cells 12 hours post-infection, (n=3, N=3).

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

Figure S5.

OmpA doesn't have a significant role in building up *in vitro* and *in vivo* protection of *Salmonella* against oxidative stress.

(A) Estimation of the level of intracellular reactive oxygen species (ROS) in RAW 264.7 cells infected with STM- (WT), *ΔompA*, and *ΔompA*: pQE60-*ompA* at MOI 10, 16 hours post-infection using DCFDA [10 µM] by flow cytometry. Unstained and uninfected RAW264.7 cells have also been used as controls. Both dot plots (SSC-A vs DCFDA) and histograms (Count vs DCFDA) have been represented. (B) The percent population of DACFDA positive RAW264.7 cells, (n=4, N=3). (C) Estimation of extracellular ROS from the culture supernatant of RAW264.7 cells infected with STM- (WT), *ΔompA*, *ΔompA*: pQE60-*ompA*, *ΔompA*: pQE60, & PFA fixed dead bacteria respectively at MOI 10. 2 hours post-infection the cells were supplemented with phenol red solution having horse radish peroxidase. 16 hours post-infection the culture supernatants were collected, and the OD was measured at 610 nm, (n=3, N=2). (D) Checking the *in vitro* sensitivity of STM- (WT) and *ΔompA* in presence of H_2O_2 by serial dilution, plating, and CFU calculation and resazurin test. 10^8 CFU of overnight grown stationary phase culture of STM (WT) and *ΔompA* were inoculated in PBS with varying concentrations of H_2O_2. 12 hours post-inoculation the supernatants were collected for plating

on SS agar to determine the $\log_{10}$[CFU/mL] (N=3), and resazurin test to determine percent viability. (n=3, N=3). (E) Checking the *in vitro* sensitivity of STM– (WT) and *ΔompA* in presence of acidified nitrite by serial dilution, plating, and CFU calculation and resazurin test. 10^8 CFU of overnight grown stationary phase culture of STM (WT) and *ΔompA* were inoculated in PBS (pH=5.4) with varying concentrations of $NaNO_2$. 12 hours post-inoculation the supernatants were collected for plating on SS agar to determine the $\log_{10}$[CFU/mL], (N=3) and resazurin test to determine percent viability. (n=3, N=3). (F) Checking the *in vitro* sensitivity of STM– (WT) and *ΔompA* in presence of $NaNO_2$ and H_2O_2 combined, by serial dilution, plating, and CFU calculation and resazurin test. 10^8 CFU of overnight grown stationary phase culture of STM (WT) and *ΔompA* were inoculated in PBS (pH=5.4) with varying concentrations of $NaNO_2$ and H_2O_2. 12 hours post-inoculation the supernatants were collected for plating on SS agar to determine the $\log_{10}$[CFU/mL] (N=3), and resazurin test to determine percent viability, (n=3, N=3).

(P)* *< 0.05, *(P)* **< 0.005, *(P)* ***< 0.0005, *(P)* *< 0.0001, ns= non-significant, (2way ANOVA), (Student's *t* test).**

Figure S6.

Generation and confirmation of *ompA ompC, ompA ompD*, *ompA ompF* double knockouts in STM *ΔompA* and *ompC*, *ompD*, *ompF* single knockouts in STM (WT).

(A-C) To obtain *ompA ompC, ompA ompD*, *ompA ompF* double knockout strains, chloramphenicol resistant gene cassettes (size- 1.1 kb) having a flanking sequence homologous to *ompC, ompD, ompF* respectively, were introduced into STM *ΔompA* harboring pKD46 plasmid by electroporation. Confirmation of (A) *ΔompA ΔompC*, (B) *ΔompA ΔompD,* (C) *ΔompA ΔompF* using respective confirmatory primers. (D-F) To obtain *ompC*, *ompD*, *ompF*

single knockout strains chloramphenicol resistant gene cassette (size- 1.1 kb, having a flanking sequence homologous to *ompC, ompD,* and *ompF*) and kanamycin-resistant gene cassette (size- 1.6 kb having a flanking sequence homologous to *ompD*) were introduced into STM (WT) harboring pKD46 plasmid. Confirmation of (D) *ompC*, (E) *ompD*, (F) *ompF* using respective confirmatory primers.

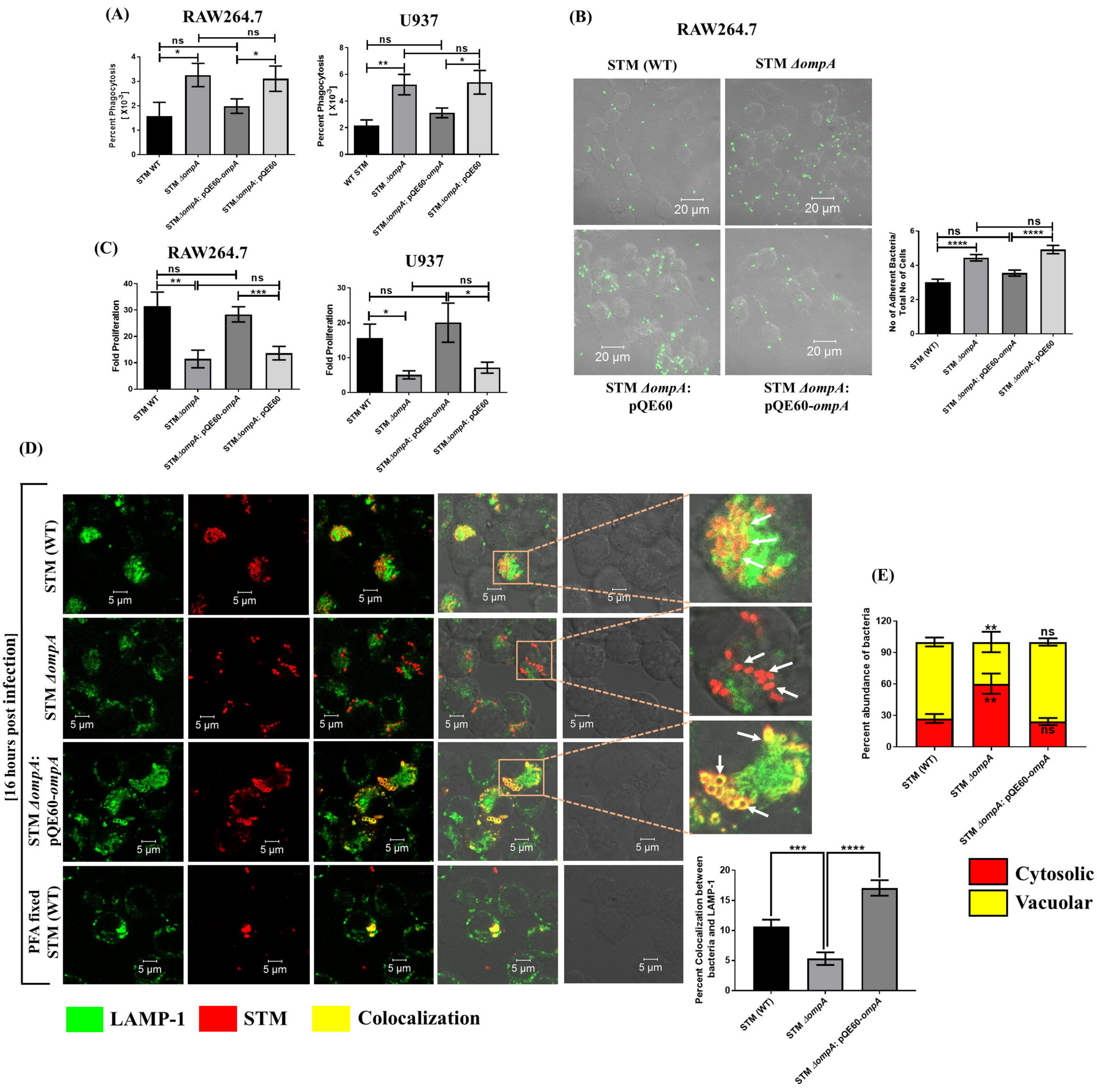
Figure S7.

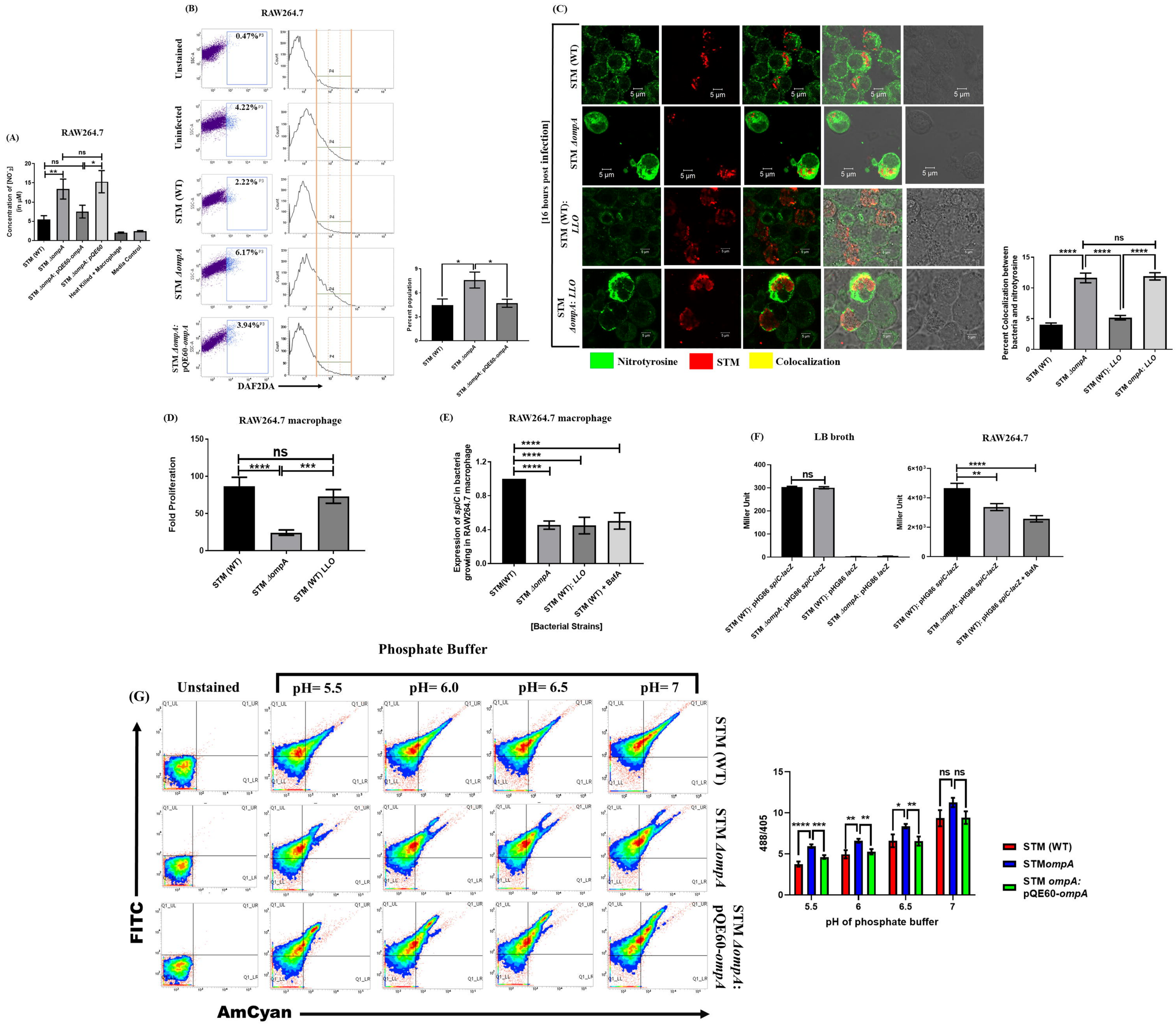
Unlike *ompA*, other outer membrane porins (*ompC, ompD, ompF*) don't have any significant role in the protection of *Salmonella* against intracellular and extracellular nitrosative stress.

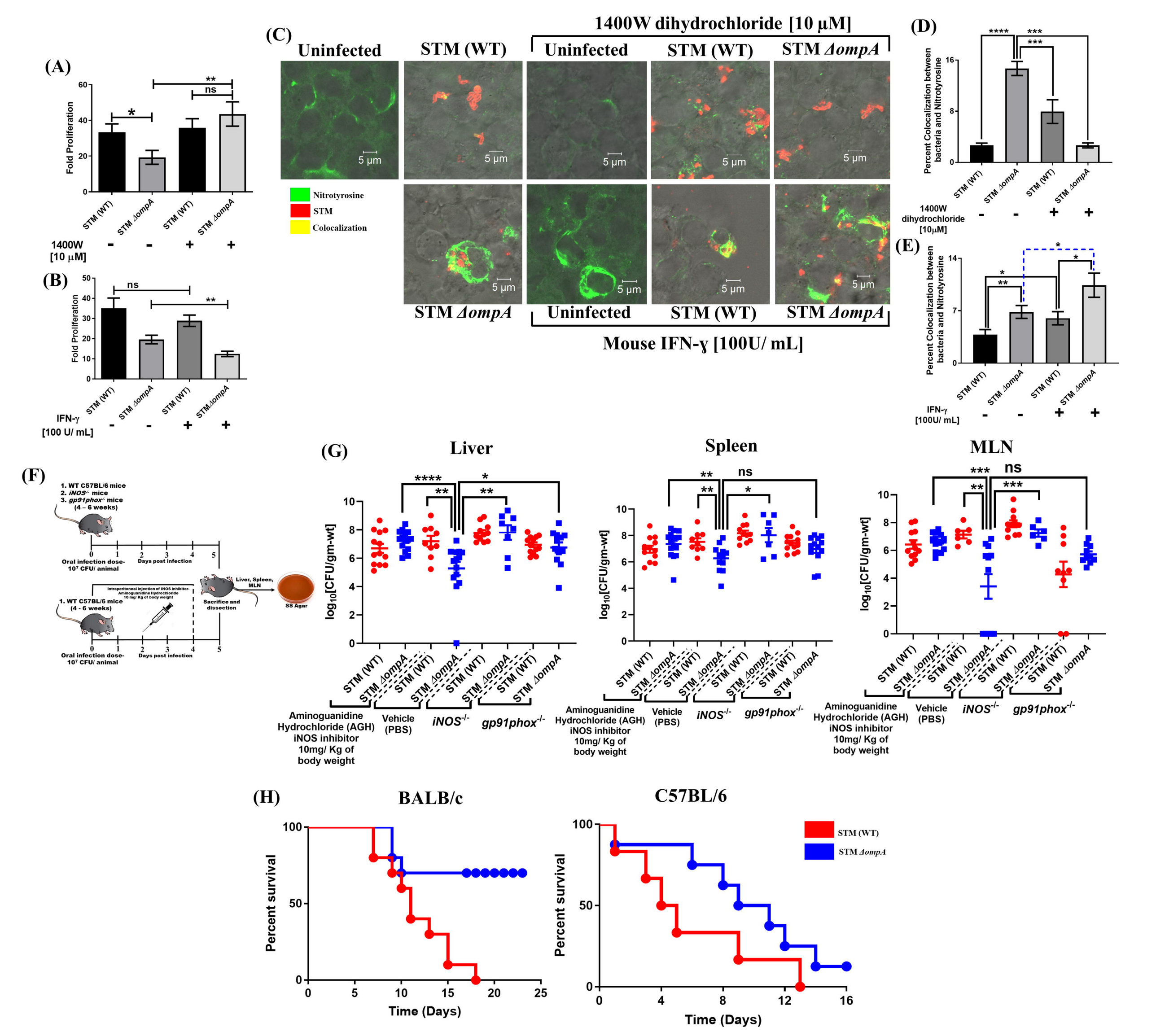
(A) RAW264.7 cells were infected with STM- (WT), *ΔompA*, *ΔompC*, *ΔompD*, and *ΔompF* at MOI 20. Cells were fixed at 16 hours post-infection, followed by labeled with anti-*Salmonella* antibody and anti-mouse nitrotyrosine antibody respectively. Quantification of nitrotyrosine recruitment on STM- (WT), *ΔompA*, *ΔompC*, *ΔompD*, and *ΔompF* has been represented in form of a graph. Percent colocalization of bacteria with nitrotyrosine has been determined after analyzing more than 60 different microscopic fields from two independent experiments, ($n \geq 60$, N=3). Scale bar = 5μm. (B) Checking the *in vitro* sensitivity of STM– (WT), *ΔompA*, *ΔompC*, *ΔompD*, and *ΔompF* in presence of acidified nitrite by serial dilution, plating, and CFU calculation. 10^8 CFU of overnight grown stationary phase culture of STM (WT), *ΔompA, ΔompC, ΔompD,* and *ΔompF* were inoculated in PBS (pH=5.4) with varying concentration of $NaNO_2$. 12 hours post-inoculation the supernatants were collected for plating on SS agar to determine the $\log_{10}$[CFU/mL], (N=3). (C) *In vitro* nitrite uptake assay of STM- (WT), *ΔompA*, *ΔompC, ΔompD, ΔompF* & PFA fixed dead bacteria. 10^8 CFU of overnight grown stationary phase culture of all the strains were inoculated in MOPS- NaOH buffer (pH= 8.5) with an initial

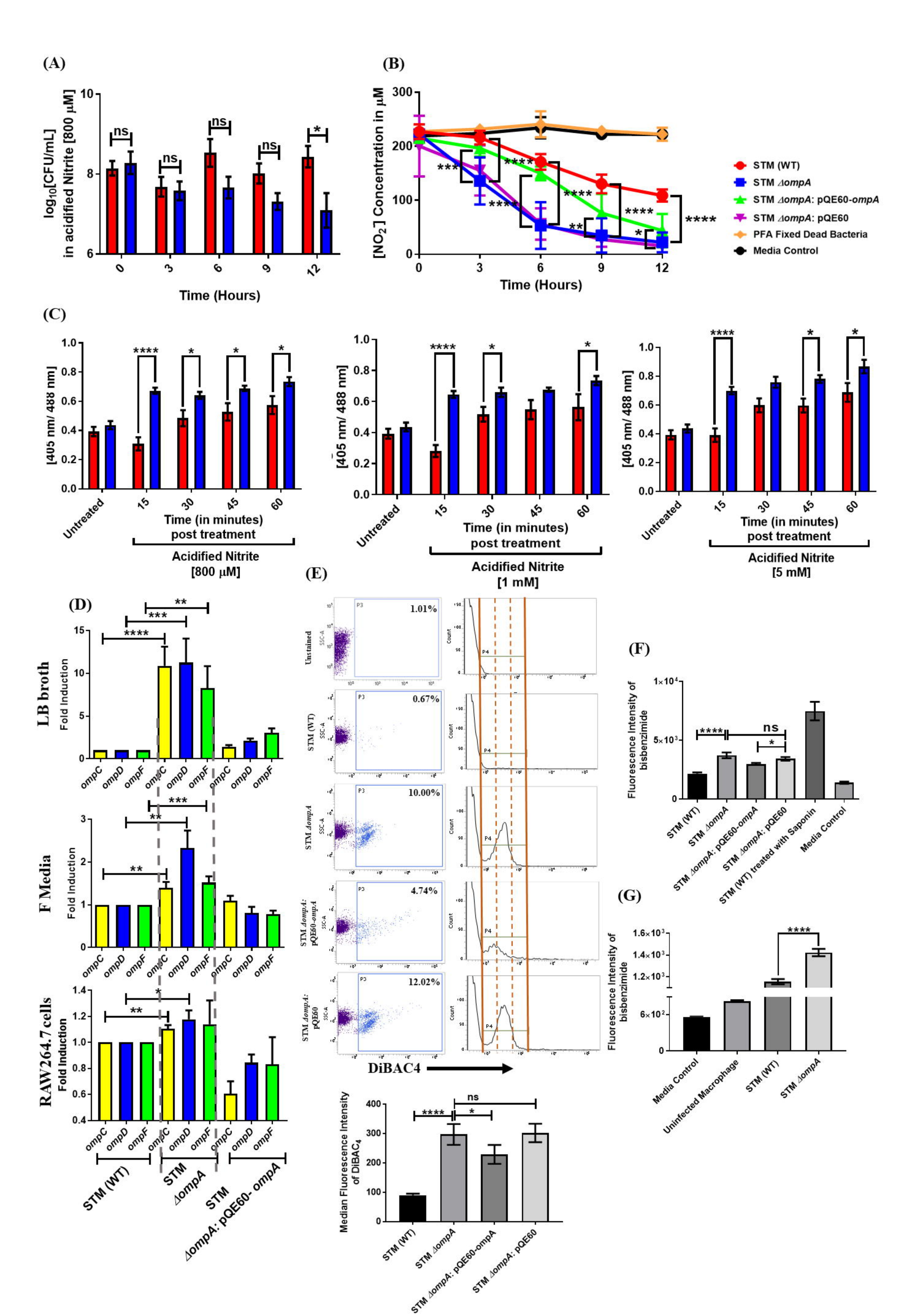
nitrite concentration of 50 µM. The remaining nitrite concentration of the media was determined by Griess assay at 0, 1, 2, 3, 4, 5, and 6 hours post-inoculation, (n=3, N=3).

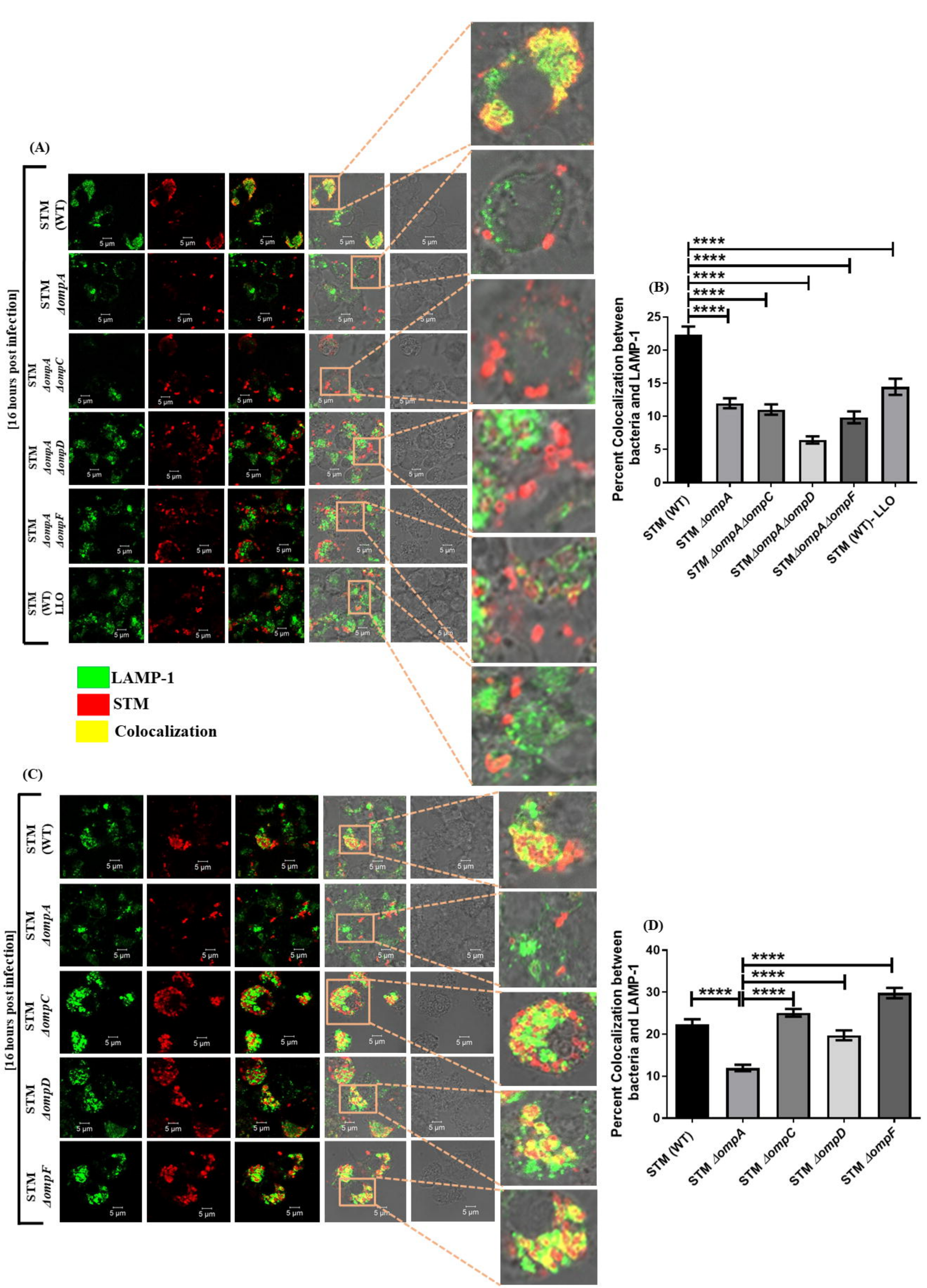
(P)* *< 0.05, *(P)* **< 0.005, *(P)* ***< 0.0005, *(P)* *< 0.0001, ns= non-significant, (2way ANOVA), (Student's *t* test).**

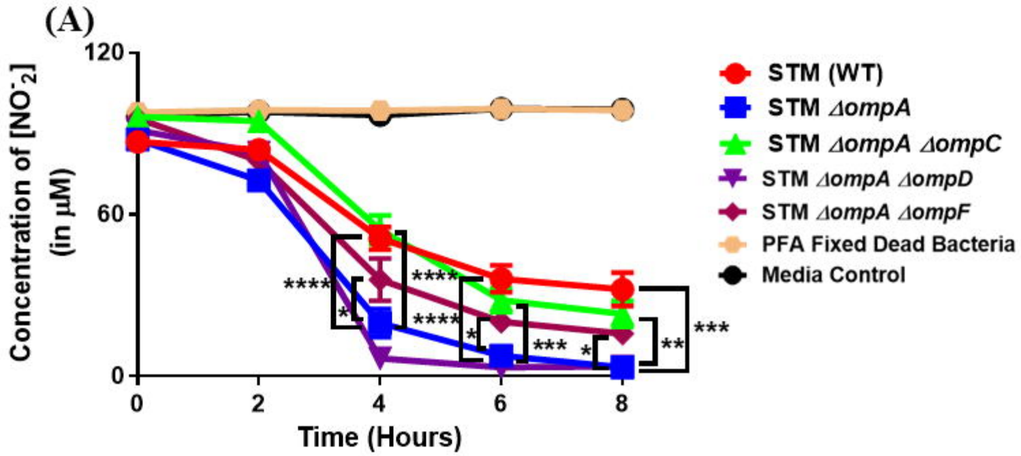

(A)
RAW264.7
U937
Percent Phagocytosis [X10^-3]
STM WT
STM ΔompA
STMΔompA: pQE60-ompA
STMΔompA: pQE60
WT STM
(B)
RAW264.7
STM (WT)
STM ΔompA
STM ΔompA: pQE60
STM ΔompA: pQE60-ompA
20 µm
No of Adherent Bacteria/ Total No of Cells
(C)
RAW264.7
U937
Fold Proliferation
(D)
[16 hours post infection]
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
PFA fixed STM (WT)
5 µm
LAMP-1
STM
Colocalization
Percent Colocalization between bacteria and LAMP-1
(E)
Percent abundance of bacteria
Cytosolic
Vacuolar

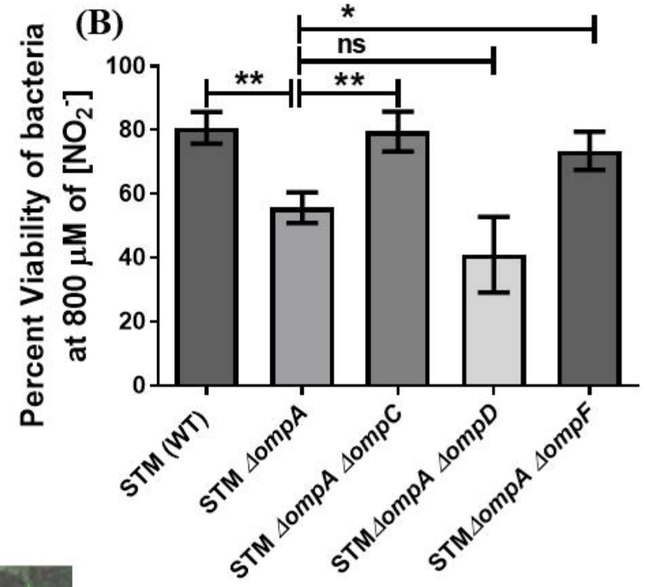

(A)
RAW264.7
Concentration of [NO-2] (in µM)
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60
Heat Killed + Macrophage
Media Control
(B)
RAW264.7
Unstained
Uninfected
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
0.47%
4.22%
2.22%
6.17%
3.94%
DAF2DA
Percent population
(C)
[16 hours post infection]
STM (WT)
STM ΔompA
STM (WT): LLO
STM ΔompA: LLO
Nitrotyrosine
STM
Colocalization
Percent Colocalization between bacteria and nitrotyrosine
(D)
RAW264.7 macrophage
Fold Proliferation
STM (WT)
STM ΔompA
STM (WT) LLO
(E)
RAW264.7 macrophage
Expression of spiC in bacteria growing in RAW264.7 macrophage
STM(WT)
STM ΔompA
STM (WT): LLO
STM (WT) + BafA
[Bacterial Strains]
(F)
LB broth
RAW264.7
Miller Unit
STM (WT): pHG86 spiC-lacZ
STM ΔompA: pHG86 spiC-lacZ
STM (WT): pHG86 lacZ
STM ΔompA: pHG86 lacZ
STM (WT): pHG86 spiC-lacZ + BafA
Phosphate Buffer
(G)
Unstained
pH= 5.5
pH= 6.0
pH= 6.5
pH= 7
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
FITC
AmCyan
488/405
pH of phosphate buffer
STMompA
STM ompA: pQE60-ompA

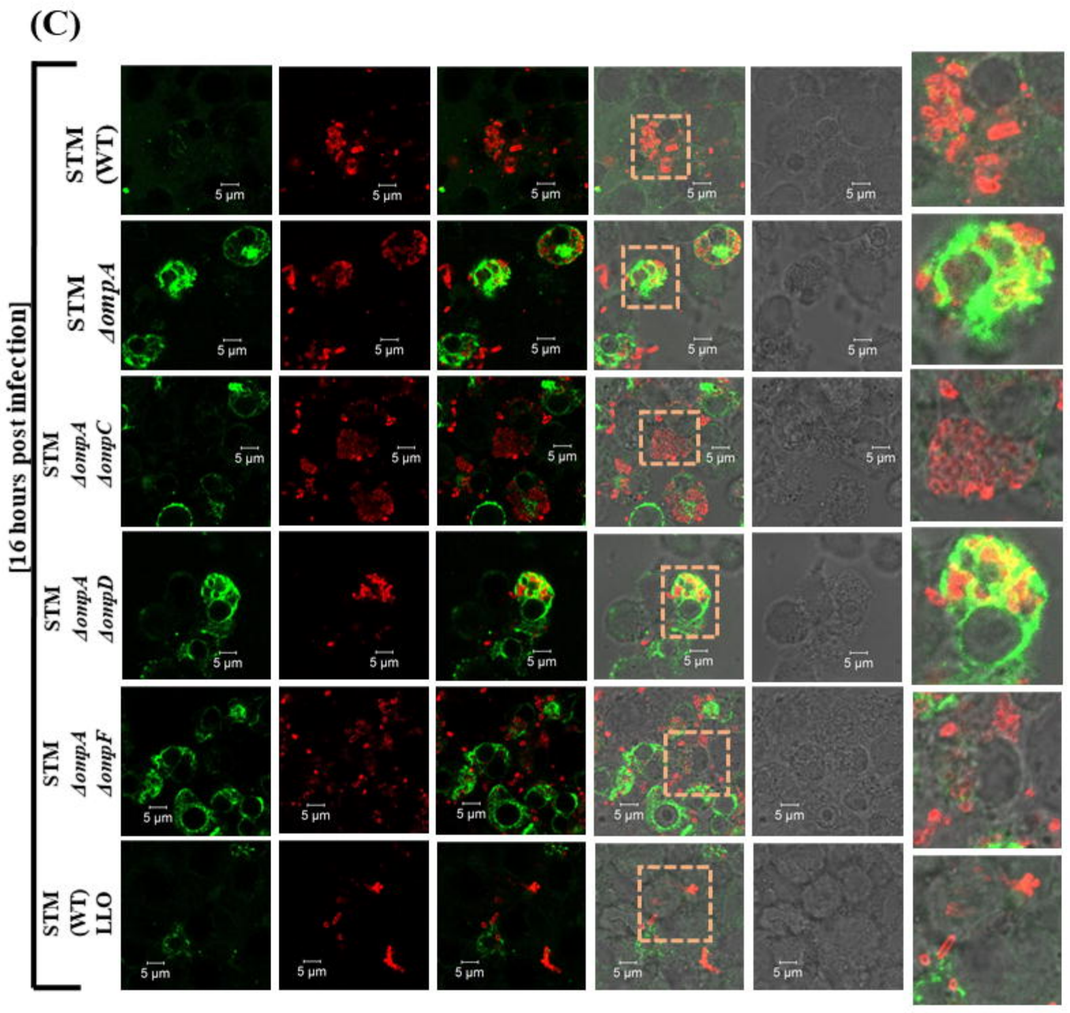

(A)
Fold Proliferation
STM (WT)
STM ΔompA
1400W [10 μM]
(B)
Fold Proliferation
IFN-γ [100 U/ mL]
(C)
Uninfected
STM (WT)
STM ΔompA
1400W dihydrochloride [10 μM]
Nitrotyrosine
STM
Colocalization
5 μm
Mouse IFN-γ [100U/ mL]
(D)
Percent Colocalization between bacteria and Nitrotyrosine
1400W dihydrochloride [10μM]
(E)
IFN-γ [100U/ mL]
(F)
1. WT C57BL/6 mice
2. iNOS-/- mice
3. gp91phox-/- mice
(4 – 6 weeks)
Oral infection dose- 10⁷ CFU/ animal
Days post infection
Intraperitoneal injection of iNOS inhibitor- Aminoguanidine Hydrochloride 10 mg/ Kg of body weight
Sacrifice and dissection
Liver, Spleen, MLN
SS Agar
(G)
Liver
Spleen
MLN
log10[CFU/gm-wt]
Aminoguanidine Hydrochloride (AGH) iNOS inhibitor 10mg/ Kg of body weight
Vehicle (PBS)
iNOS-/-
gp91phox-/-
(H)
BALB/c
C57BL/6
Percent survival
Time (Days)
STM (WT)
STM ΔompA

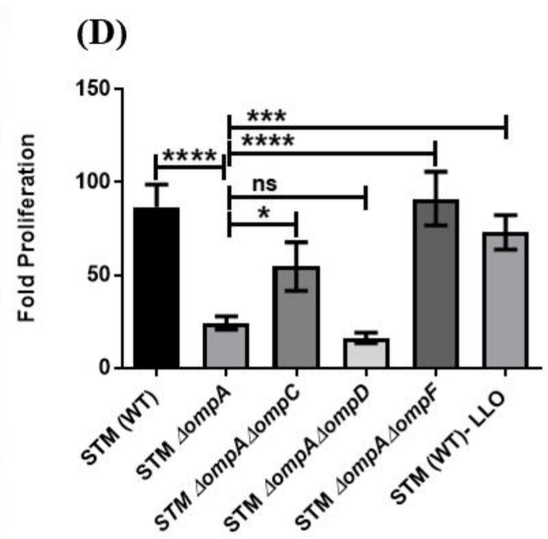

(A)
log10[CFU/mL] in acidified Nitrite [800 μM]
Time (Hours)
(B)
[NO2] Concentration in μM
Time (Hours)
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60
PFA Fixed Dead Bacteria
Media Control
(C)
[405 nm/ 488 nm]
Untreated
Time (in minutes) post treatment
Acidified Nitrite [800 μM]
Acidified Nitrite [1 mM]
Acidified Nitrite [5 mM]
(D)
LB broth
F Media
RAW264.7 cells
Fold Induction
ompC
ompD
ompF
STM (WT)
STM ΔompA
STM ΔompA: pQE60- ompA
(E)
Unstained
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60
1.01%
0.67%
10.00%
4.74%
12.02%
DiBAC4
Median Fluorescence Intensity of DiBAC4
(F)
Fluorescence Intensity of bisbenzimide
STM (WT)
STM ΔompA
STM ΔompA: pQE60-ompA
STM ΔompA: pQE60
STM (WT) treated with Saponin
Media Control
(G)
Fluorescence Intensity of bisbenzimide
Media Control
Uninfected Macrophage
STM (WT)
STM ΔompA

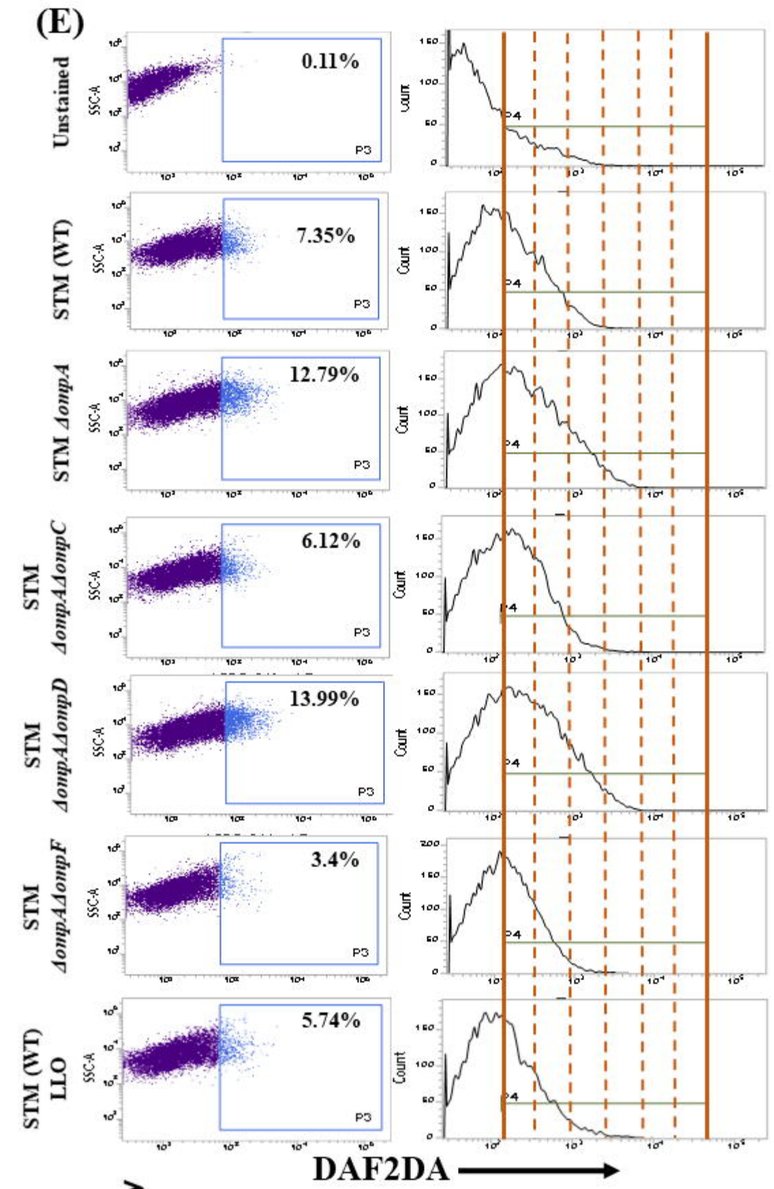

(A)
[16 hours post infection]
STM (WT)
STM ΔompA
STM ΔompA ΔompC
STM ΔompA ΔompD
STM ΔompA ΔompF
STM (WT) LLO
5 µm
LAMP-1
STM
Colocalization
(B)
Percent Colocalization between bacteria and LAMP-1
0
5
10
15
20
25
****
STM (WT)
STM ΔompA
STM ΔompA ΔompC
STM ΔompA ΔompD
STM ΔompA ΔompF
STM (WT)- LLO
(C)
[16 hours post infection]
STM (WT)
STM ΔompA
STM ΔompC
STM ΔompD
STM ΔompF
5 µm
(D)
Percent Colocalization between bacteria and LAMP-1
0
10
20
30
40
****
STM (WT)
STM ΔompA
STM ΔompC
STM ΔompD
STM ΔompF

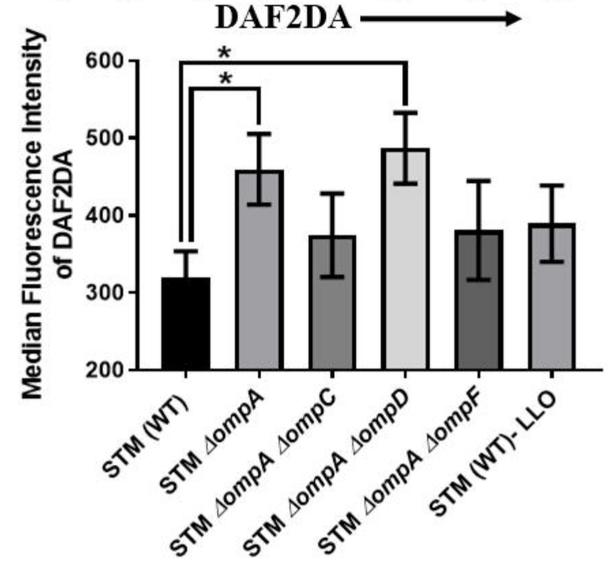




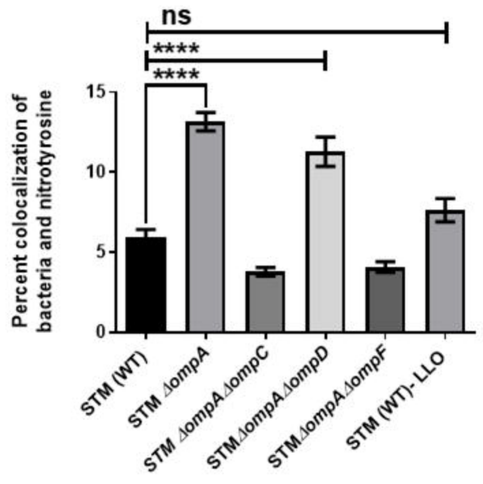


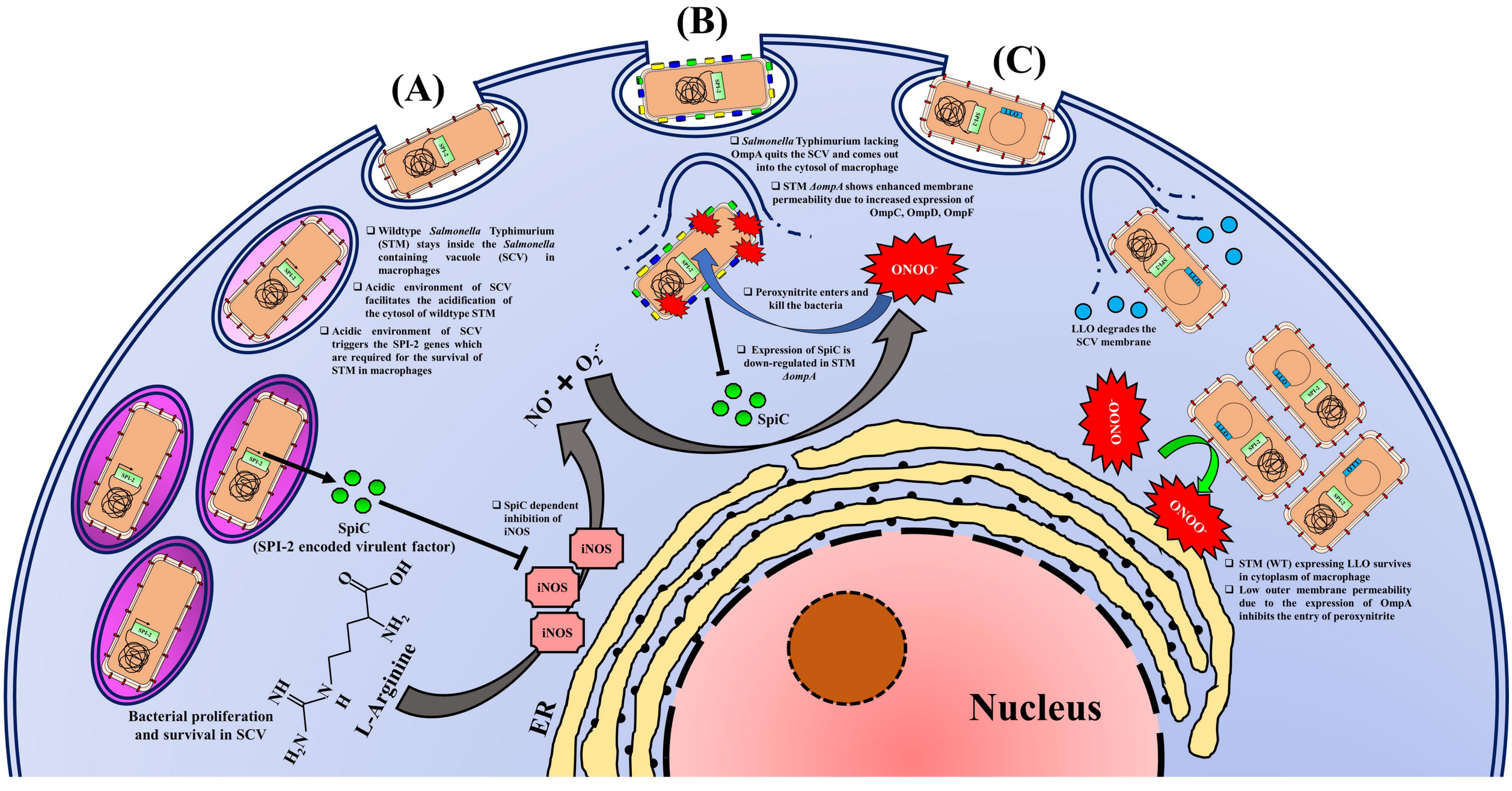






Nitrotyrosine
STM
Colocalization




(A)
(B)
(C)
SPI-2
LLO
❑ Wildtype *Salmonella* Typhimurium (STM) stays inside the *Salmonella* containing vacuole (SCV) in macrophages
❑ Acidic environment of SCV facilitates the acidification of the cytosol of wildtype STM
❑ Acidic environment of SCV triggers the SPI-2 genes which are required for the survival of STM in macrophages
❑ *Salmonella* Typhimurium lacking OmpA quits the SCV and comes out into the cytosol of macrophage
❑ STM *ΔompA* shows enhanced membrane permeability due to increased expression of OmpC, OmpD, OmpF
ONOO⁻
❑ Peroxynitrite enters and kill the bacteria
❑ Expression of SpiC is down-regulated in STM *ΔompA*
SpiC
LLO degrades the SCV membrane
NO· + O₂·⁻
SpiC
(SPI-2 encoded virulent factor)
❑ SpiC dependent inhibition of iNOS
iNOS
iNOS
iNOS
L-Arginine
Bacterial proliferation and survival in SCV
ER
Nucleus
ONOO⁻
ONOO⁻
❑ STM (WT) expressing LLO survives in cytoplasm of macrophage
❑ Low outer membrane permeability due to the expression of OmpA inhibits the entry of peroxynitrite