

# The ABC-Type Efflux Pump MacAB Protects *Salmonella enterica* serovar Typhimurium from Oxidative Stress

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**ABSTRACT** Multidrug efflux pumps are integral membrane proteins known to actively excrete antibiotics. The macrolide-specific pump MacAB, the only ABC-type drug efflux pump in *Salmonella*, has previously been linked to virulence in mice. The molecular mechanism of this link between *macAB* and infection is unclear. We demonstrate that *macAB* plays a role in the detoxification of reactive oxygen species (ROS), compounds that salmonellae are exposed to at various stages of infection. *macAB* is induced upon exposure to H<sub>2</sub>O<sub>2</sub> and is critical for survival of *Salmonella enterica* serovar Typhimurium in the presence of peroxide. Furthermore, we determined that *macAB* is required for intracellular replication inside J774.A1 murine macrophages but is not required for survival in ROS-deficient J774.D9 macrophages. *macAB* mutants also had reduced survival in the intestine in the mouse colitis model, a model characterized by a strong neutrophilic intestinal infiltrate where bacteria may experience the cytotoxic actions of ROS. Using an Amplex red-coupled assay, *macAB* mutants appear to be unable to induce protection against exogenous H<sub>2</sub>O<sub>2</sub> *in vitro*, in contrast to the isogenic wild type. In mixed cultures, the presence of the wild-type organism, or media preconditioned by the growth of the wild-type organism, was sufficient to rescue the *macAB* mutant from peroxide-mediated killing. Our data indicate that the MacAB drug efflux pump has functions beyond resistance to antibiotics and plays a role in the protection of *Salmonella* against oxidative stress. Intriguingly, our data also suggest the presence of a soluble anti-H<sub>2</sub>O<sub>2</sub> compound secreted by *Salmonella* cells through a MacAB-dependent mechanism.

**IMPORTANCE** The ABC-type multidrug efflux pump MacAB is known to be required for *Salmonella enterica* serovar Typhimurium virulence after oral infection in mice, yet the function of this pump during infection is unknown. We show that this pump is necessary for colonization of niches in infected mice where salmonellae encounter oxidative stress during infection. MacAB is required for growth in cultured macrophages that produce reactive oxygen species (ROS) but is not needed in macrophages that do not generate ROS. In addition, we show that MacAB is required to resist peroxide-mediated killing *in vitro* and for the inactivation of peroxide in the media. Finally, wild-type organisms, or supernatant from wild-type organisms grown in the presence of peroxide, rescue the growth defect of *macAB* mutants in H<sub>2</sub>O<sub>2</sub>. MacAB appears to participate in the excretion of a compound that induces protection against ROS-mediated killing, revealing a new role for this multidrug efflux pump.

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Nontyphoidal salmonellae, including *Salmonella enterica* serovar Typhimurium, are the leading cause of bacterial foodborne enteritis. Infection with these organisms results in an estimated 1.4 million cases in the United States and hundreds of millions of cases worldwide annually. Symptoms of disease, including abdominal cramps, nausea, and diarrhea, appear 12 to 72 h after consumption of contaminated food or water and generally subside 5 to 7 days postinfection (1).

In humans, antibiotic therapy does not shorten the duration of gastrointestinal salmonellosis (2–6) and may in fact result in prolonged fecal shedding and increased frequency of relapses (3, 7). For example, *S. enterica* serovar Java isolated from patients was sensitive to ciprofloxacin before and after antibiotic therapy (3) yet was resistant to antibiotic treatment *in vivo*. Despite the fact

that ciprofloxacin is present at high doses in the intestinal lumen after oral administration and it is known to penetrate mammalian cells (8), bacteria can protect themselves from antibiotics in this environment.

Resistance to antibiotics can be caused by a large number of mechanisms. Drug modification, target modification, and inhibition of uptake are reported mechanisms of resistance to aminoglycosides (9, 10). Reduction in expression of porins protected bacteria against  $\beta$ -lactams (11), and mutations in *gyrA* and *gyrB* confer resistance of *Salmonella* to fluoroquinolones (12). Nevertheless, the first line of bacterial defense is to actively efflux antimicrobial compounds (13).

Drug efflux pumps are integral membrane proteins that are thought to actively pump antibiotics from the cell and are recog-

nized as an important mechanism of cellular protection against the toxic action of antibiotics and other drugs (14–16). Drug efflux pumps are usually chromosomally encoded and belong to ancient families of proteins (17, 18). Bacterial drug efflux pumps have been categorized into five families: ABC superfamily (19), the major facilitator superfamily (MFS) (20), the small multidrug resistance (MDR) (SMR) family (21), the resistance-nodulation-division (RND) superfamily (17), and the multidrug and toxic compound extrusion (MATE) family (22). The natural substrates extruded by some of these pumps are known, particularly the RND superfamily members, and include molecules relevant for survival in the natural setting of the bacterium expressing these pumps (17).

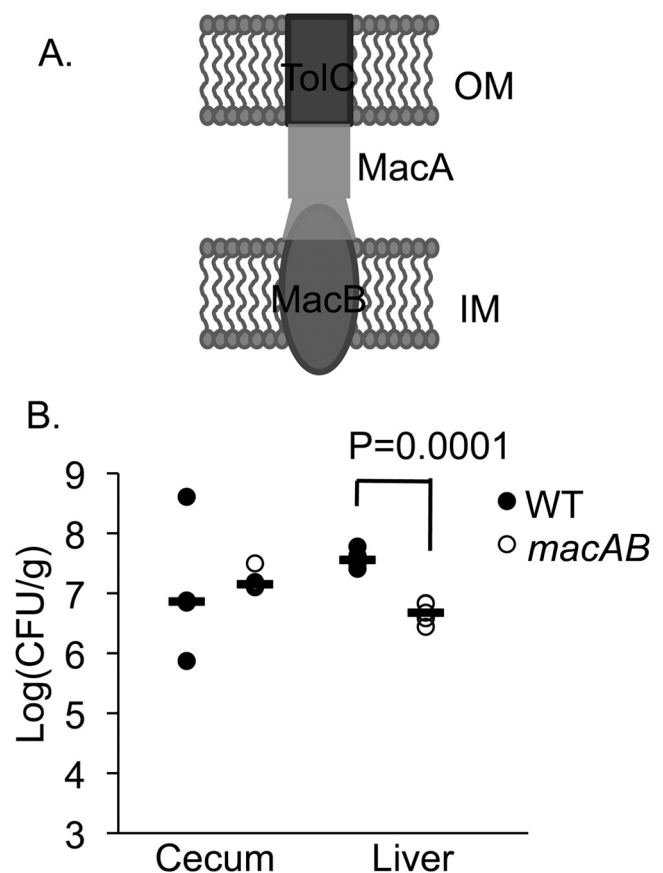
*Salmonella* encodes at least 11 multidrug efflux (MDR) pumps belonging to four different classes: ABC family, MATE, RND, and MFS (14, 23). Eight of these pumps are also found in *Escherichia coli* (14, 23). Only the AcrAB-TolC drug efflux pump is expressed in laboratory media (24), while other pumps require additional signals for expression. In *E. coli*, transcription of the *acrAB* pump is further activated under general stress conditions, including transition to a stationary phase in LB broth, high osmolarity (0.5 M NaCl), or exposure to 4% ethanol (25). We became interested in an ABC-type macrolide-specific efflux pump, MacAB-TolC (26), because the mutant lacking *macAB* appeared to be avirulent in mice (24), but the mechanism of this attenuation is unknown. Whether the *macAB* system transports other, perhaps host-derived molecules is unknown, as are the evolutionary pressures for *Salmonella* to express this system.

Here, we show that the *macAB* efflux pump is required for survival of *Salmonella* in the inflamed intestines, as well as in macrophages, where these organisms are exposed to highly toxic reactive oxygen species (ROS). We further show that *macAB* is induced upon exposure to H<sub>2</sub>O<sub>2</sub> and is critical for survival of *S. Typhimurium* in the presence of peroxide. Furthermore, we employed an Amplex red-coupled assay to demonstrate that *macAB* mutant cells are unable to detoxify exogenous H<sub>2</sub>O<sub>2</sub>, in contrast to the isogenic wild type. The presence of either intact wild-type cells or soluble secreted metabolites from cells pretreated with peroxide was sufficient to rescue the *macAB* mutant from H<sub>2</sub>O<sub>2</sub>-mediated killing. We hypothesize that the MacAB drug efflux pump has functions beyond conferring resistance to antibiotics, and at least one of these functions is to protect *Salmonella* against oxidative stress.

## RESULTS

***ΔmacAB* mutants colonize the liver poorly after intraperitoneal infection of *Salmonella*-susceptible mice.** The *macAB* locus in *Salmonella* Typhimurium encodes a homolog of the macrolide-specific ABC-type drug efflux pump in *E. coli* (Fig. 1A). Both MacA and MacB in *Salmonella* share 82 to 83% identity and over 90% similarity to corresponding proteins in *E. coli*. In *E. coli*, MacAB is involved in efflux of macrolide antibiotics with 14- and 15-membered rings (26).

In *Salmonella* Typhimurium, inactivation of *macAB* results in severe attenuation of this pathogen in BALB/c mice after oral infection (24). Because the degree of attenuation in work done by previous authors in this mouse model appeared to be severe (24) but was measured based on survival of the mouse and not on quantitative colonization by a *ΔmacAB* mutant, we chose to determine whether the *ΔmacAB* mutant was defective for coloniza-

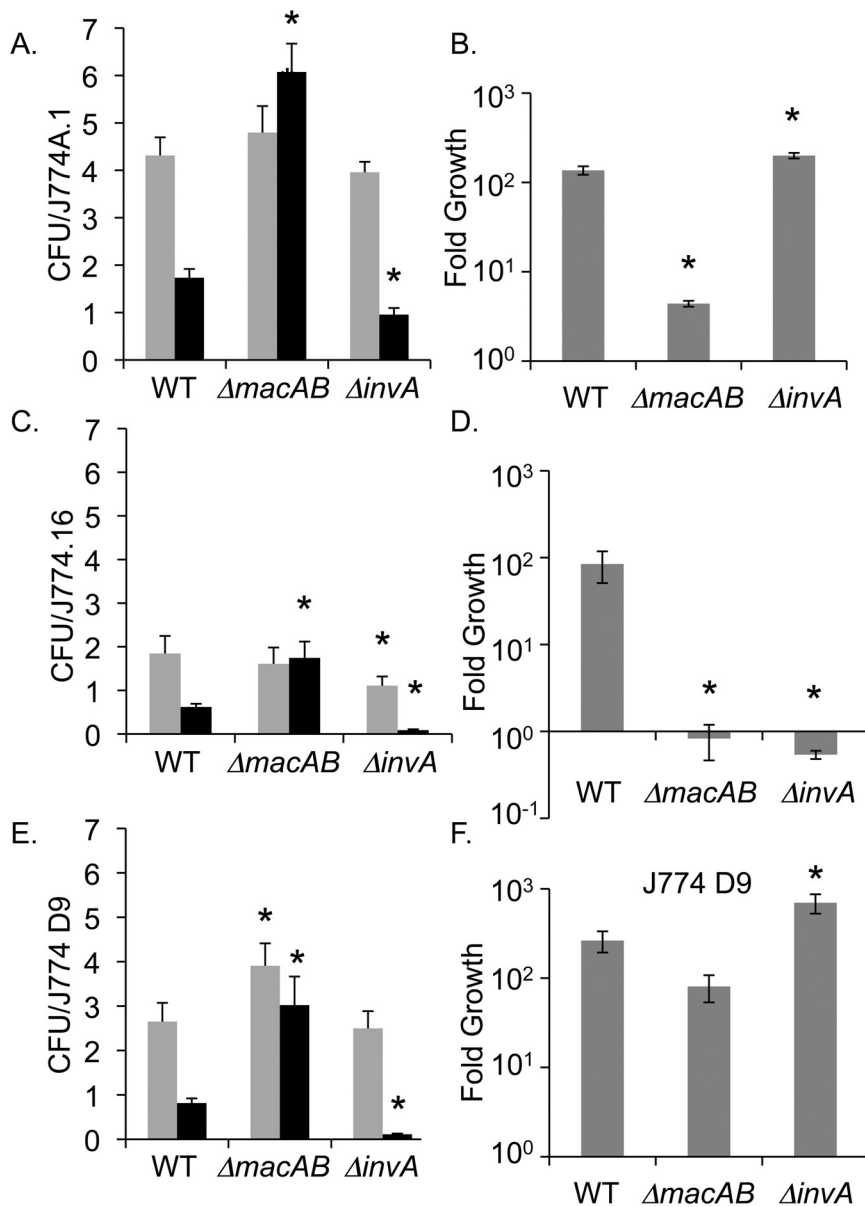


**FIG 1** The *macAB* mutant is defective in colonization of liver of intraperitoneally (i.p.) infected *Salmonella*-susceptible BALB/c mice. (A) Schematic model of the tripartite MacAB-TolC complex. (B) The *macAB* mutant is defective in colonization of liver but not cecum of BALB/c mice. Eight-week-old BALB/c mice were inoculated by intraperitoneal injections with approximately  $1.0 \times 10^6$  CFU of the wild type or the *macAB* mutant. Cecum and liver were collected at 48 h postinfection, and bacteria were recovered and plated for enumeration.

tion at systemic sites. We infected groups of 5 *Salmonella*-susceptible BALB/c mice intraperitoneally (i.p.) with either HA420 (wild type) or *ΔmacAB* mutants. The *ΔmacAB* mutant strains colonized the liver of BALB/c mice poorly after intraperitoneal infection (Fig. 1B). Thus, *macAB* mutants poorly colonize systemic organs during acute infection.

**MacAB is required for intracellular growth in macrophages.** The ability of *S. Typhimurium* to adhere, to invade, and to replicate inside macrophage cells is directly linked with virulence and systemic colonization of the host by this organism (27–29). We hypothesized that the defect of the *ΔmacAB* mutant in colonization of the liver could be linked to the inability of this mutant to successfully survive and grow within macrophages. In gentamicin protection assays, the *ΔmacAB* mutant was cell associated and internalized into J774.A1 murine macrophages as well as, or better than, the wild-type organism (Fig. 2A). Despite this, intracellular replication of the *ΔmacAB* mutant in J774.A1 macrophages was more than 30-fold reduced ( $4.39 \pm 0.33$ -fold replication) compared to that of the wild-type organism ( $136.7 \pm 14.8$ -fold) over a 24-h period (Fig. 2B).

In response to bacterial invasion, macrophages activate two



**FIG 2** MacAB is required for intracellular survival in macrophages capable of a respiratory burst. Cell association, internalization, and intracellular growth of the wild type, *macAB* mutant, and *invA* mutants were evaluated in J774.A1 macrophages (A and B), J774.16 macrophages (robust respiratory burst) (C and D), and J774.D9 macrophages (lack of a respiratory burst) (E and F). (A, C, and E) Cell association (attachment and internalization, gray bars) of *Salmonella* was assayed after 1 h of coinubation with macrophages, and internalization was evaluated at 2 h after gentamicin treatment (black bars). (B, D, and F) Intracellular growth was evaluated 24 h after gentamicin treatment. Data are expressed as fold growth and were calculated as CFU/J774 at 24 h after gentamicin treatment and CFU/J774 at 2 h after gentamicin treatment. Data are shown as the means from at least three experiments, each assay performed in triplicate, and error bars indicate standard errors. The asterisk indicates statistical significance ( $P < 0.05$ , Student's *t* test).

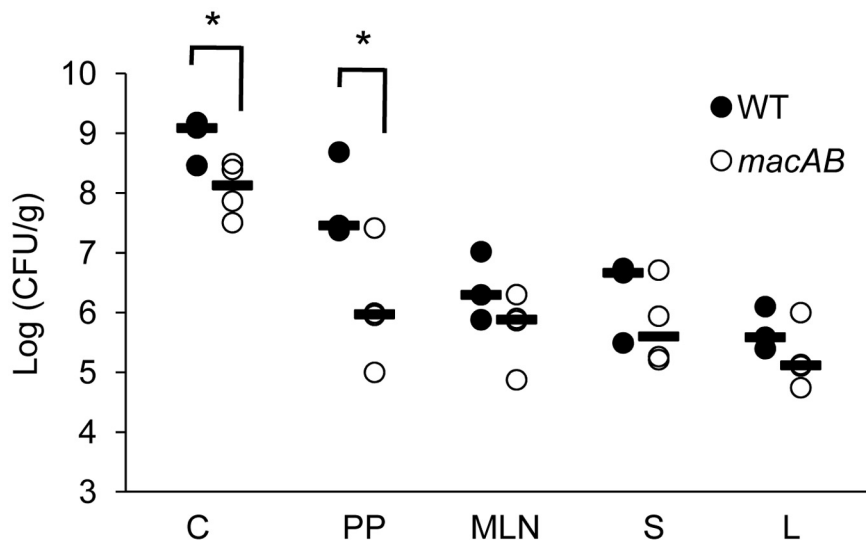
antimicrobial systems: NADPH phagocyte oxidase (*phox*) and inducible nitric oxide synthase (iNOS), responsible for generation of reactive oxygen and nitrogen species, respectively (30). To test whether reduced intracellular replication of the  $\Delta macAB$  mutant was linked to increased sensitivity to ROS production by cultured macrophages, we infected two additional murine macrophage cell lines, J774.16 and J774.D9, with the wild type or the  $\Delta macAB$  or

$\Delta invA$  mutants (Fig. 2C to F). J774.16 macrophages generate a strong oxidative burst (31), while J774.D9 macrophages do not produce ROS after stimulation with phorbol myristate acetate (32).  $\Delta macAB$  mutants are cell associated and internalized into J774.16 macrophage-like cells, like wild-type cells (Fig. 2C), but replicate poorly ( $0.83 \pm 0.36$ -fold) inside these cells, which produce a strong oxidative burst (Fig. 2D). In contrast, in the infection of ROS-deficient J774.D9 macrophages,  $\Delta macAB$  mutants associated with, were internalized by (Fig. 2E), and replicated intracellularly similar to wild-type organisms ( $80.64 \pm 27.16$ -fold) (Fig. 2F). These data support the hypothesis that MacAB is needed for intracellular survival and growth in macrophages, as well as for survival in oxidative stress.

**MacAB is required for survival in the inflamed intestine.** Intestinal infection with *S. Typhimurium* results in classic inflammatory diarrhea accompanied by a massive influx of neutrophils (33). To determine whether MacAB is required for survival in the inflamed intestine (34), a site of neutrophil-mediated ROS release, we performed oral infection of streptomycin-treated *Salmonella*-susceptible C57BL/6 mice with the wild type or the  $\Delta macAB$  mutants. Streptomycin treatment 24 h prior to infection disrupts normal microbiota and allows *Salmonella* to induce a neutrophilic inflammatory response in the murine intestines similar to that observed in cattle and humans (34). Neutrophils effect a massive release of ROS (35). In these assays, the  $\Delta macAB$  mutant was recovered in significantly lower numbers from the cecum and Peyer's patches of infected mice compared to the wild type (Fig. 3). These data support previous observations that MacAB is needed after oral infection (24) and for *Salmonella* survival in the presence of ROS.

**MacAB is required for resistance to hydrogen peroxide *in vitro*.** To further test the hypothesis that MacA and MacB are needed for resistance to oxidative stress, we assayed the ability of  $\Delta macAB$  mutants to grow in the presence of 1 mM

$H_2O_2$  (Fig. 4A). Hydrogen peroxide was selected for *in vitro* experiments because of its relative stability compared to other ROS ( $O_2^-$ ,  $HO^\cdot$ ) (36). For *E. coli*,  $H_2O_2$  concentrations greater than 0.5 mM are bactericidal for the wild type (37). When wild-type *Salmonella* were inoculated into 1 mM peroxide-containing medium at  $10^7$  CFU/ml, there was no increase in cell numbers for the first 2 h of incubation, followed by fast recovery (Fig. 4A, gray



**FIG 3** The *macAB* mutant survives poorly in the inflamed intestines of orally infected streptomycin-pretreated C57BL/6 mice. Eight-week-old C57BL/6 mice were treated with streptomycin 24 h before infection. Mice were inoculated by oral gavage with  $2 \times 10^8$  CFU of the wild type or the *macAB* mutant. Cecum (C), Payer's patches (PP), mesenteric lymph nodes (MLN), spleen (S), and liver (L) were collected 4 days postinfection, and bacteria were recovered and plated for enumeration. The asterisk indicates statistical significance ( $P < 0.05$ , Student's *t* test).

squares). In contrast,  $\Delta macAB$  mutants rapidly and dramatically lost viability (Fig. 4A, black circles), similar to known  $H_2O_2$ -sensitive mutants in *recA* (Fig. 4A, gray triangles) (38, 39). Returning the intact copy of *macAB* in *trans* reversed this  $H_2O_2$  sensitivity (Fig. 4A, open circles). The growth of *macAB* mutants is indistinguishable from the growth of the wild type in media that are not supplemented with hydrogen peroxide (see Fig. S1 in the supplemental material).

Bacterial cell membranes are semipermeable to hydrogen peroxide (40, 41). Given temporal sensitivity of wild-type cells to 1 mM  $H_2O_2$ , we wanted to determine the stability of hydrogen peroxide in the bacteriologic media. Concentrations of  $H_2O_2$  in the supernatants of both uninoculated and cultures inoculated with either wild-type, *macAB*-deficient, *macAB*-complemented, or *recA*-deficient cells were monitored by the Amplex red-horseradish peroxidase detection method (42). Using this assay, the  $H_2O_2$  concentration in this cell-free medium was unchanged over a 6-h incubation at 37°C (Fig. 4B, gray X). The hydrogen peroxide concentration also remained relatively stable in the medium inoculated with wild type or with  $H_2O_2$ -sensitive *recA* mutants for the first 2 h of incubation but rapidly dropped to negligible amounts by 5 h of incubation (Fig. 4B, gray square = wild type, gray triangle =  $\Delta recA$  mutant). Unexpectedly, the concentration of extracellular hydrogen peroxide remained stable in *macAB*-inoculated medium over the duration of the experiment (Fig. 4B, black circles =  $\Delta macAB$  mutant). The ability to decrease the concentration of  $H_2O_2$  in the medium was restored by providing the intact copy of *macAB* in *trans* (Fig. 4B, open circles =  $\Delta macAB$  mutant complemented in *trans*). Thus, MacAB appears to be involved in protection of *Salmonella* against extracellular hydrogen peroxide by decreasing  $H_2O_2$  concentration in the medium.

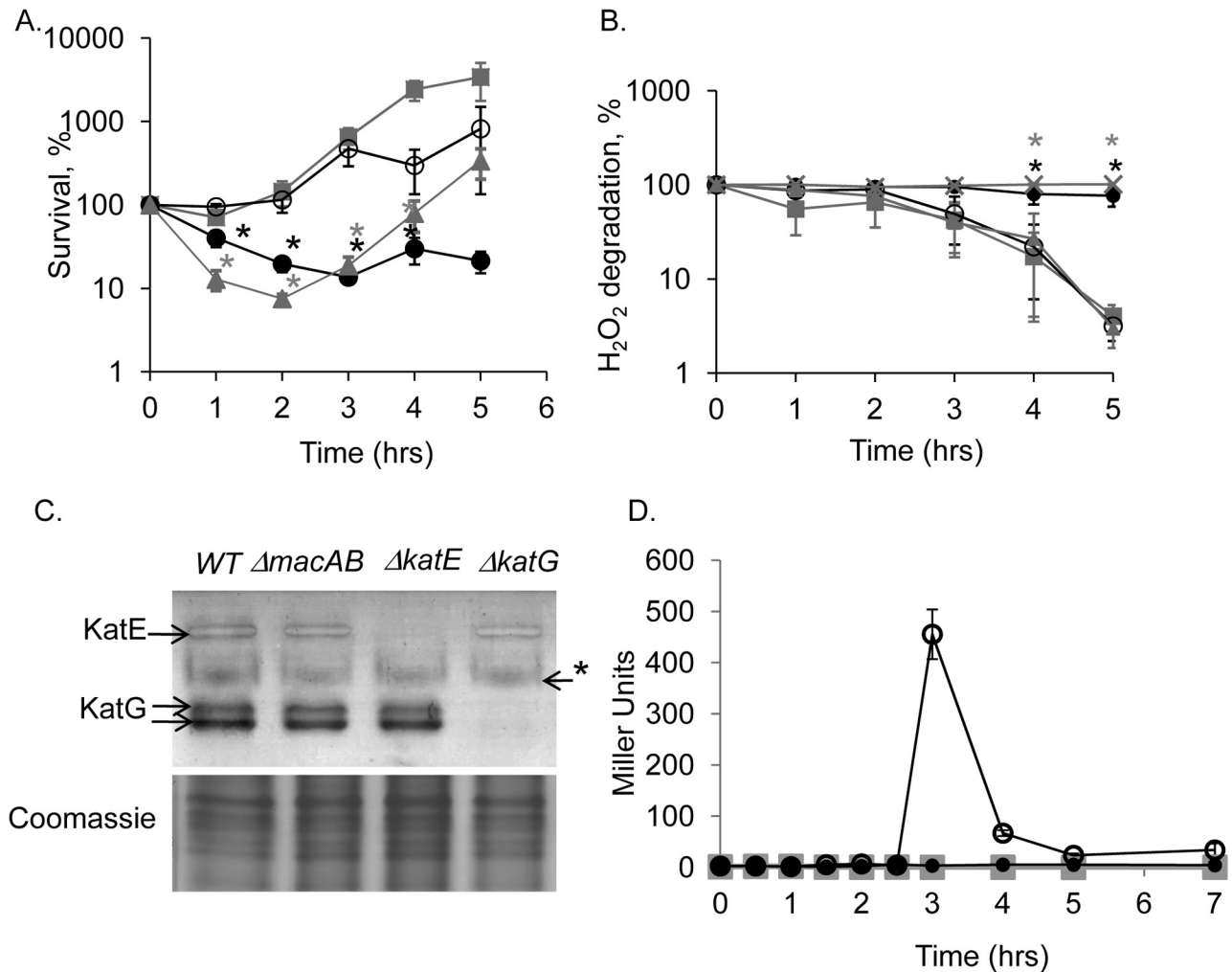
Bacteria are known to degrade  $H_2O_2$  using two classes of enzymes: catalases and peroxidases (43). *Salmonella* Typhimurium

encodes three catalases (KatE, KatN, KatG) and three peroxidases (AhpC, TsaA, Tpx) (43, 44). Examination of the primary sequence of *katG* also reveals a plant peroxidase-like domain, and thus the encoded protein may have some peroxidase activity, although this has not previously been described. We wanted to determine whether the sensitivity of our  $\Delta macAB$  mutant to peroxide was due to reduced expression of these enzymes using in-gel catalase and peroxidase staining techniques (45). As controls for this analysis, we generated deletion mutations in *katE* and *katG* and show that the appropriate activities are missing in the corresponding deletion mutants using in-gel staining. Furthermore, lysates of the wild type and the *macAB* deletion mutants showed identical patterns of catalase and peroxidase activities, indicating that gross changes in the repertoire and abundance of these enzymes does not occur when *macAB* is deleted (Fig. 4C; see also Fig. S3 in the supplemental material).

The MacAB drug efflux pump is not activated when *Salmonella* is grown on LB plates (24). Because we found that *macAB*-null cells are sensitive to  $H_2O_2$ , we next determined whether hydrogen peroxide exposure would induce expression of the *macAB* promoter. We found that in a strain bearing a chromosomal *macAB-lacZY* transcriptional fusion integrated on the chromosome replacing the *macAB* locus (24), *macAB* expression, as measured by  $\beta$ -galactosidase assays, spiked at 3 h postinoculation of media containing  $H_2O_2$  (Fig. 4D). Taken together, our results indicate that the MacAB drug efflux pump is expressed in the presence of hydrogen peroxide and is involved in protection of *Salmonella* against oxidative stress.

**Wild-type *Salmonella* transcomplements the hydrogen peroxide sensitivity of the *macAB* mutant in the mixed culture.** Since MacAB is thought to function as an efflux pump (26), we hypothesized that the concentration of hydrogen peroxide in media in the presence of the *macAB* mutant remained stable, because these mutants were unable to efflux a compound that induces protection against  $H_2O_2$  in the broth. If this is the case, wild-type *Salmonella* should protect  $\Delta macAB$  mutant cells from  $H_2O_2$  action when the two strains are cocultured. In order to test this hypothesis, we mixed the wild type and the  $\Delta macAB$  mutants from individual overnight cultures and subcultured this mixture into fresh medium containing 1 mM  $H_2O_2$ . In contrast to single-culture experiments (Fig. 4A), the  $\Delta macAB$  mutant survived the exposure to hydrogen peroxide in the presence of wild-type cells (Fig. 5A,  $\Delta macAB$  mutant, black circles). The  $\Delta recA$  mutant is hypersensitive to  $H_2O_2$ -mediated damage due to inability to repair DNA (39), one of the major targets of hydrogen peroxide (44). The presence of the wild-type organism in coculture with the  $\Delta recA$  mutant did not rescue the peroxide sensitivity of this mutant (Fig. 5B,  $\Delta recA$  mutant, black triangles).

**The presence of a soluble anti- $H_2O_2$  compound in preconditioned medium is sufficient for protection of the  $\Delta macAB$  mutant from hydrogen peroxide.** One hypothesis for the ability of

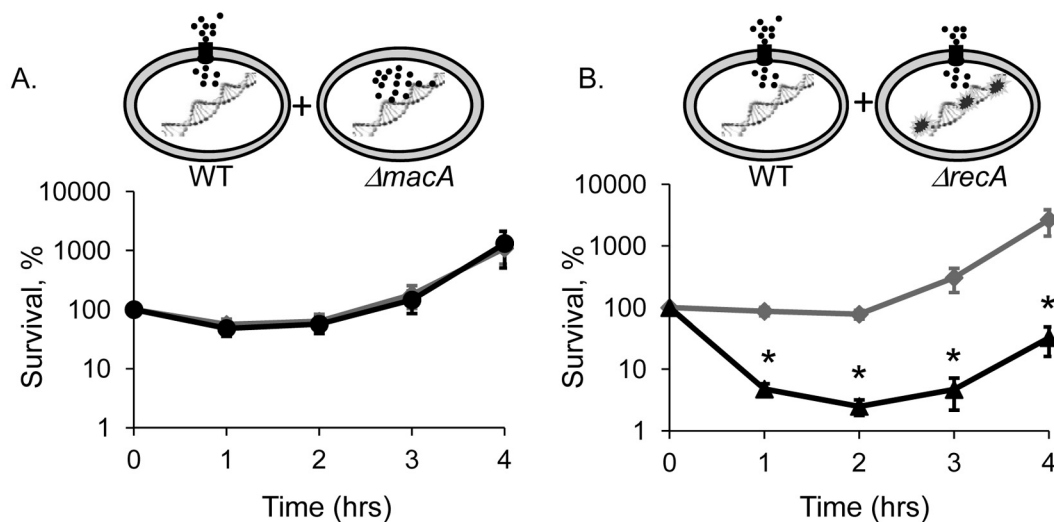


**FIG 4** *macAB* is required for resistance to 1 mM hydrogen peroxide. (A) Overnight cultures of the wild type (gray squares), the  $\Delta macAB$  mutant (black circles), the  $\Delta macAB$  mutant complemented with *macAB* in *trans* (*pmacAB*, open circles), and the  $\Delta recA$  mutant (gray triangles) were diluted 1/100 in LB broth, supplemented with 1 mM H<sub>2</sub>O<sub>2</sub>, and incubated at 37°C with aeration. Aliquots were collected hourly, serially diluted, and plated, and culture supernatants were retained for analysis in panel B. Data are shown as the survival means from at least three experiments and a standard error. The asterisk indicates statistical significance ( $P < 0.05$  by Student's *t* test). (B) Overnight cultures of the wild type (gray squares),  $\Delta macAB$  mutant (black circles),  $\Delta macAB$  p $macAB$  mutant (open circles), and  $\Delta recA$  mutant (gray triangles) were diluted 1/100 in LB broth containing 1 mM H<sub>2</sub>O<sub>2</sub> and incubated at 37°C with aeration. A sterile control (1 mM H<sub>2</sub>O<sub>2</sub> LB broth, gray X symbols) was included in all experiments. Hydrogen peroxide concentration in the collected supernatants was detected with the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen) according to the manufacturer's protocol. Data are shown as the means from at least three experiments and a standard error. The asterisk indicates statistical significance ( $P < 0.05$ ) in Student's *t* test. (C) Whole-cell lysates of the wild type,  $\Delta macAB$  mutant,  $\Delta katE$  mutant, and  $\Delta katG$  mutant grown in LB broth overnight at 37°C with aeration were separated on a 10% nondenaturing PAGE gel and stained to assess peroxidase and catalase activity. Bands representing KatE and KatG activities are noted. \*, a band representing additional peroxidase activity was also noted. (D) Hydrogen peroxide induces expression of *macAB*. Overnight cultures of wild type (gray squares) and a chromosomal *macAB-lacZY* fusion strain (black circles) were diluted 1/100 in LB broth (filled markers) or in LB broth supplemented with 0.5 mM H<sub>2</sub>O<sub>2</sub> (*macAB-lacZY*, open circles) and incubated at 37°C with aeration. Aliquots were collected and used for  $\beta$ -galactosidase activity assay. Data are shown as means of at least three experiments and a standard error.

the wild type to transcomplement the peroxide sensitivity of *macAB* mutants is that the wild type may secrete a compound that induces protection against H<sub>2</sub>O<sub>2</sub> into the medium. To test this hypothesis, we generated cell-free conditioned medium using wild-type *Salmonella* (supplemented with hydrogen peroxide) and tested the growth of  $\Delta macAB$  mutants in this conditioned medium supplemented with peroxide. First, the growth of  $\Delta macAB$  mutants in conditioned medium (Fig. 6A, filled black circles) and fresh LB broth was very similar (Fig. 6A, filled gray circles), while *macAB* mutants incubated in 1 mM H<sub>2</sub>O<sub>2</sub> lost viability (Fig. 6A, gray line, open gray circles). Second, the  $\Delta macAB$

mutant growth in medium preconditioned by growth of the wild-type organism partially rescued the sensitivity of these mutants to H<sub>2</sub>O<sub>2</sub> exposure (Fig. 6A, solid black line, open circles). Third, we found that heat treatment of conditioned medium at 100°C for 10 min did not destroy the protective ability of conditioned medium for the  $\Delta macAB$  mutants to hydrogen peroxide exposure (Fig. 6A, dashed line, open circles). Finally, we did not observe any protection of *macAB* cells against H<sub>2</sub>O<sub>2</sub>-mediated killing when *macAB*-conditioned medium was used in the identical experiment (Fig. 6B).

From this experiment, we conclude that *Salmonella* excretes, in



**FIG 5** Wild-type *Salmonella* transcomplements hydrogen peroxide sensitivity of the *macAB* mutant. (A) Individually grown overnight cultures of wild type (gray diamonds) and  $\Delta macAB$  mutants (black circles) were mixed in equal numbers. The resulting mixed culture was diluted 1/100 in LB broth, supplemented with 1 mM  $H_2O_2$ , and incubated at 37°C with aeration. Aliquots were collected hourly, serially diluted, and plated. The numbers of wild-type organisms and *macAB* mutants were enumerated from each aliquot. Data are shown as the means from at least three experiments and a standard error. The asterisk indicates statistical significance ( $P < 0.05$ , Student's *t* test). (B) Individually grown overnight cultures of the wild type (gray diamonds) and the  $\Delta recA$  mutant (black triangles) were mixed in equal ratios, grown, and analyzed as described for panel A.

a MacAB-dependent manner, a compound that increases resistance to  $H_2O_2$ . The presence of this soluble compound is sufficient for protection of *macAB* mutant cells against hydrogen peroxide.

## DISCUSSION

The ABC-type drug efflux pump MacAB is highly conserved and present in many bacterial species (24, 26, 46–49). In *E. coli*, MacAB is involved in active efflux of macrolides, such as erythromycin (26). In *Salmonella* Typhimurium, however, the role of MacAB in efflux of macrolides is much smaller (14, 24), leaving us with few clues regarding the natural substrate of this system. MacAB is required for the full virulence of *Salmonella* in mice (24). In our hands, a *macAB* deletion mutant had a defect in colonization of liver after intraperitoneal infection of *Salmonella*-susceptible BALB/c mice. In the liver, salmonellae grow within macrophages (28, 29, 50). *macAB* mutants survive and grow poorly in cultured J774 macrophages.

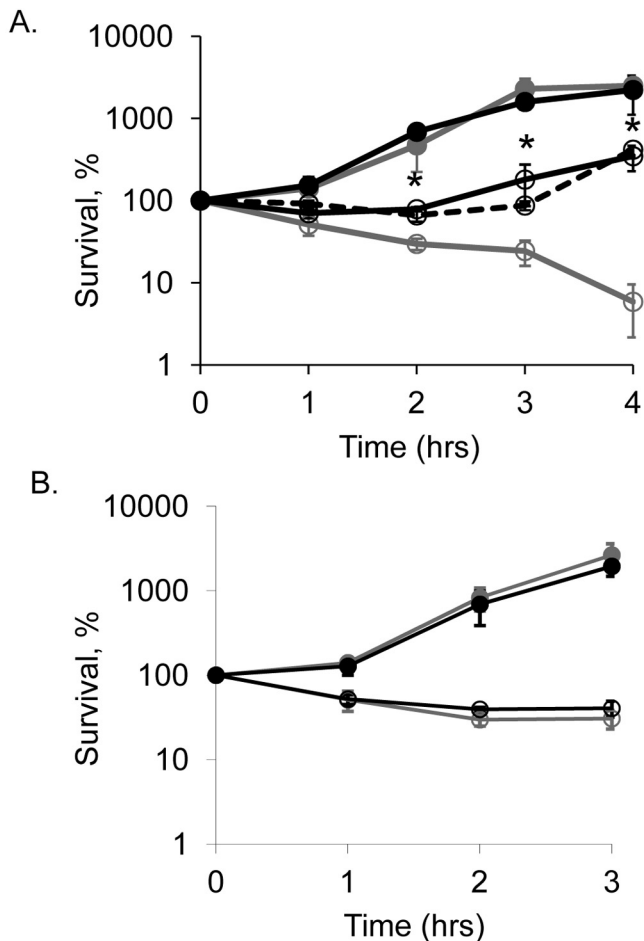
Macrophages employ various strategies to kill many bacterial species, including production of reactive oxygen and nitrogen species (ROS and RNS) via NADPH phagocyte oxidase (*phox*) and inducible nitric oxide synthase (iNOS) (30). In turn, salmonellae have evolved a unique mechanism to evade ROS-mediated damage. Once inside the macrophage, salmonellae are enclosed inside a *Salmonella*-containing vacuole (SCV). Within this compartment, *Salmonella* expresses the type three secretion system 2 (TTSS-2), encoded on *Salmonella* pathogenicity island 2 (SPI-2), that allows the organism to secrete effector proteins that interfere with vesicular trafficking of the NADPH phagocyte oxidase (44, 51).

We linked the *macAB* mutant's defects in intracellular survival and proliferation with the production of ROS by macrophages. We show that the ability of *macAB*-null mutants to survive inside macrophages correlates inversely with the ability of phagocytes to generate ROS. Notably, secretion of TTSS-2 effectors is not af-

fected in the *macAB* mutant (data not shown). We did not investigate a possibility that the *macAB* mutant might be also sensitive to RNS, but this seems unlikely, as growth of the *macAB* mutant in J774.D9 macrophages, which produce reactive nitrogen species, is not different from that of the wild type. MacAB is also required for survival in the mouse colitis model. Acute intestinal infection in the *Salmonella* Typhimurium-infected host is characterized by substantial neutrophilic inflammation accompanied by ROS production in the gut (1, 33, 34, 52). In support of our previous observations, we found that the *macAB* mutant was defective in colonization of the gut-associated sites: cecum and Peyer's patches of orally infected streptomycin-treated mice.

*macAB* is not expressed during growth in LB (24). Expression of some RND pumps in enteric bacteria is modulated by bile salts and fatty acids—compounds present in the intestinal tract. For example, the expression of the *acrAB* system in *E. coli* and *Salmonella* is induced by bile salts (25, 53, 54). Similarly, bile salt-mediated induction occurs for RND pumps in *Campylobacter jejuni*, *Vibrio cholerae*, and *Bacteroides fragilis* (55–57). However, our *macAB*-null mutant does not appear to be sensitive to bile salts (data not shown).

MacAB is also required for survival in the presence of hydrogen peroxide *in vitro*, and expression of the *macAB* promoter is activated upon exposure to hydrogen peroxide. Expression of a number of drug efflux pumps is positively regulated by ROS, suggesting that efflux systems in turn are playing a role in protection of bacteria against oxidative stress. The AcrAB efflux pump is controlled by SoxRS (58–60), a two-component system that responds to oxidative stress and redox cycling compounds (43, 61). In many clinical isolates of *E. coli* and *Salmonella*, the *soxS* gene is constitutively expressed, resulting in upregulation of the AcrAB efflux pump (62–64). The SoxSR system also regulates expression of AcrAB in *Klebsiella pneumoniae* and *Enterobacter cloacae* (65, 66). An association between oxidative stress and expression of drug



**FIG 6** Media preconditioned by the growth of wild-type *Salmonella* protects the *macAB* mutant against hydrogen peroxide-mediated killing. (A) We evaluated the growth of  $\Delta macAB$  mutants ( $10^7$  CFU) in either sterile LB broth or preconditioned LB broth (preconditioning by the growth of the wild-type organism in the presence of 1 mM  $H_2O_2$  for 3 h), either in the presence or absence of 1 mM  $H_2O_2$ . All cultures were incubated at 37°C with aeration. Aliquots were collected hourly, serially diluted, and plated. Data are shown as the survival means from at least three experiments and a standard error. The asterisk indicates statistical significance ( $P < 0.05$ , Student's  $t$  test).  $\Delta macAB$  mutants grown in sterile LB broth (solid gray line, solid gray circles) or in preconditioned medium without peroxide had identical growth (solid black line, solid black circles).  $\Delta macAB$  mutants incubated in LB broth containing 1 mM  $H_2O_2$  lost viability (solid gray line, open gray circles).  $\Delta macAB$  mutants incubated in the presence of 1 mM  $H_2O_2$  in medium preconditioned by the growth of the wild-type organism were partially rescued for growth, regardless of whether or not the preconditioned media were heat treated (untreated, solid black line, open circles; heat treated, dotted black line, open circles). (B) We evaluated the growth of  $\Delta macAB$  mutants ( $10^7$  CFU) in either sterile LB broth or preconditioned LB broth (preconditioning by the growth of the  $\Delta macAB$  mutant in the presence of 1 mM  $H_2O_2$  for 3 h), either in the presence or absence of 1 mM  $H_2O_2$ .  $\Delta macAB$  mutants grown in LB broth (gray line, closed gray circles) and LB broth preconditioned by growth of  $\Delta macAB$  mutants (black line, closed black circles) grew very similarly.  $\Delta macAB$  mutants incubated in either LB supplemented with 1 mM  $H_2O_2$  (solid gray line, open gray circles) or in  $\Delta macAB$  preconditioned media supplemented with 1 mM  $H_2O_2$  (black line, open black circles) lost viability.

efflux pumps has also been described for *Pseudomonas aeruginosa*, a bacterium frequently isolated from patients with cystic fibrosis (CF). CF lungs are known to be enriched in ROS (67). Expression

of MexXY, linked to aminoglycoside resistance, and MexAB-OprM pumps is induced by oxidative stress (68, 69).

Previous work indicates that *macAB* is repressed by PhoP (24), yet we show that *macAB* is needed inside macrophages. Both inactivation and constitutive expression of *phoP* result in severe attenuation of *Salmonella* in mice (70–72), suggesting that the levels or activity of PhoP/PhoQ must be tightly controlled during infection. When activated, PhoP undergoes rapid phosphorylation, binds to target promoters, and controls PhoP-dependent genes. However, this is a short-term response, as within 60 min, the phosphorylated PhoP level returns to the steady-state level, and the expression of PhoP-dependent genes returns to preactivation levels (72). *macA* and *macB* transcript levels are elevated in mRNA isolated from *Salmonella*-infected J774 macrophage-like cells at later time points, including 4, 8, and 12 h postinfection (73, 74), consistent with potential rapid inactivation of PhoP and with our data.

We show that under the conditions tested, the sensitivity of the *macAB* mutant to hydrogen peroxide is comparable to that of the *recA* mutant. However, this sensitivity is not due to the lack of  $H_2O_2$ -degrading enzymes—catalases and peroxidases. Using the Amplex red assay, we show that the concentration of hydrogen peroxide in the medium of the *macAB* mutant remains stable over time, in contrast to the wild-type organism and the peroxide-sensitive *recA* mutant. This unexpected stability of hydrogen peroxide in the *macAB* mutant-inoculated medium was comparable to that in medium alone and was reversed by complementation. This observation implies that the MacAB pump is involved in protection of *Salmonella* against extracellular hydrogen peroxide. The MacAB pump appears to be involved in excretion of a compound that increases resistance to  $H_2O_2$ . The nature of this thermostable molecule has not yet been determined, but we show that it is present in the supernatants of wild-type cells exposed to hydrogen peroxide and is sufficient for the protection of the otherwise  $H_2O_2$ -sensitive *macAB*-null mutant.

In summary, we show that the *Salmonella* Typhimurium MacAB efflux pump is required for infection in mice. The *macAB*-null mutant fails to grow in cultured J774.A1 macrophages, and the ability of the mutant to grow intracellularly is inversely dependent on the level of ROS generated by macrophages. The  $\Delta macAB$  mutant is also defective in colonization of intestinal sites of streptomycin-treated mice. While the level of ROS concentrations is known to be 100  $\mu M$  in macrophages (75), it may be much higher during neutrophilic inflammation in the intestine, where lactobacilli, intestinal epithelial cells, and neutrophils produce and release ROS (75–78). We show that our *macAB* mutant has poor survival in the presence of hydrogen peroxide *in vitro*. Sensitivity of the *macAB*-null mutant to hydrogen peroxide is suppressed by coculturing mutant with wild-type cells or by growing  $\Delta macAB$  in the medium containing soluble secreted metabolites from wild-type cells pretreated with peroxide. Identification of this antiperoxide molecule and the potential for the identification of other “natural” substrates of multidrug efflux pumps are fascinating areas of future work.

## MATERIALS AND METHODS

**Bacterial strains and media.** All *Salmonella enterica* serovar Typhimurium strains used for this study are listed in Table 1 and were derived from ATCC 14028. Mutants were generated by lambda red recombination-mediated homologous recombination (79, 80). HA420 is a fully virulent,

TABLE 1 Strain and plasmid list

Strain or plasmid name	Description	Reference or source
HA420	ATCC 14028, spontaneously Nal <sup>r</sup>	81
HA458	HA420 $\Delta invA::Kan^r$	81
HA531	HA420 $\Delta phoN::Cm^r$	This study
HA995	ATCC 14028 $\Delta macAB::Cm^r$	24
HA996	ATCC 14028 s $\Delta macAB::lacZY Kan^r$	24
LB250	HA420 $\Delta recA-hydN::Kan^r$	This study
LB272	HA995 pUC118- <i>macAB</i> , Amp <sup>r</sup>	This study
LB281	HA420 $\Delta phoN::Strep^r$	This study
LB282	HA995 $\Delta phoN::Strep^r$	This study
LB303	HA420 $\Delta spi-1::Kan$	This study
LB374	ATCC 14028 $\Delta katE::Kan^r$	This study
LB375	ATCC 14028 $\Delta katG::Kan^r$	This study
pMacAB	pUC118- <i>macAB</i> , Amp <sup>r</sup>	24

spontaneous nalidixic acid-resistant derivative of ATCC 14028 (81). Deletion mutants used in this study were moved into a clean genetic background using P22 transduction (82).

Strains were routinely cultured in LB broth and plates, supplemented with antibiotics when needed at the following concentrations: 50 mg/liter nalidixic acid, 100 mg/liter carbenicillin, 50 mg/liter kanamycin, or 20 mg/liter chloramphenicol. For the detection of *phoN* expression, 20 mg/liter of XP (5-bromo-4-chloro-3-indolyl- $\beta$ -D-phosphate) (Sigma) was added to LB agar plates.

Strains were grown aerobically at 37°C to stationary phase in LB broth for infection of *Salmonella*-susceptible BALB/c or C57BL/6 mice. For invasion assays, strains were grown statically for 16 h at 37°C in LB broth containing 0.3 M NaCl to promote SPI-1 expression (83, 84).

**Cell association, invasion, and intracellular replication assays.** J774.A1, J774.D9, and J774.16 murine macrophages were propagated in Dulbecco's modified Eagle's medium (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (Invitrogen) and were seeded at  $3.5 \times 10^5$  cells per well in 24-well tissue culture-treated dishes for all infections. Cultured macrophages were infected with *Salmonella* at a multiplicity of infection (MOI) of 50:1 (bacteria:eukaryotic cell) and incubated for 1 h at 37°C with 5% CO<sub>2</sub> in a humidified tissue culture incubator. Actual titer of the inoculum in each experiment was determined by serial dilution and plating on appropriate bacteriologic media. Infected monolayers were washed three times with 1 ml sterile phosphate-buffered saline (PBS) prior to lysis, to enumerate cell-associated bacteria, or treated with 100  $\mu$ g ml<sup>-1</sup> gentamicin sulfate (Invitrogen) for 2 h at 37°C with 5% CO<sub>2</sub> in a humidified tissue culture incubator to assess the number of intracellular bacteria. After macrophages were washed with sterile PBS three times, infected monolayers were lysed in 1% Triton X-100 (Sigma). CFU were determined by serial dilution and plating. For assessment of intracellular growth in J774 macrophages, infected, gentamicin-treated monolayers were washed with sterile PBS, and fresh DMEM supplemented with 10% fetal bovine serum (FBS) and 10  $\mu$ g ml<sup>-1</sup> gentamicin was added. Infected monolayers were incubated for 24 h, washed three times with sterile PBS, and lysed, and intracellular CFU were enumerated. At each stage when infected cells were lysed, the number of viable J774.A1 cells in duplicate monolayers infected with each strain was assessed by 0.4% trypan blue (Cellgro) exclusion and counting viable cells. No difference in viability was noted between J774 cells infected with the wild type and the *macAB* mutant. Each experiment was performed on three separate occasions, evaluating samples in triplicate.

**Intraperitoneal infections in mice.** All experiments involving animals described in this work were carried out in accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals of the National Institutes of Health*. The protocols used here were approved by the Institutional Animal Care and Use Committee at Texas A&M University.

A *macAB* mutant and virulent *Salmonella enterica* serotype Typhimurium ATCC 14028 derivative HA451 ( $\Delta phoN::Cm^r$ ) were tested for colonization of 8- to 10-week-old female BALB/c mice (Jackson Labs) in single infections by using the following protocol. Strains used as inocula were grown to stationary phase at 37°C with aeration and were diluted to  $1 \times 10^7$  CFU/ml in PBS. Resulting bacterial cultures were serially diluted and plated for bacterial CFU to determine the exact titer of both strains used for infections.

Groups of five mice were inoculated intraperitoneally with approximately  $0.5 \times 10^6$  to  $1 \times 10^6$  bacteria in 100  $\mu$ l of PBS. Two days postinfection, mice were humanely euthanized and livers and ceca of infected mice were excised and homogenized in preweighed tubes containing 5 ml ice-cold PBS. Organ homogenates were weighed, serially diluted, and plated to determine bacterial burden in the infected tissues. Data are expressed as CFU/g of tissue, converted logarithmically, and displayed graphically. Statistical significance was determined using a Student *t* test and *P* values as described in the figure legends.

**Oral infections in the murine colitis model.** Female 8- to 10-week-old C57BL/6 mice (Jackson lab) were treated with 20 mg of streptomycin by gavage 24 h prior to infection. Streptomycin-resistant *macAB* mutant (LB282) and virulent ATCC 14028 derivative LB281 were grown to stationary phase at 37°C with aeration, serially diluted, and plated for bacterial CFU to determine the exact titer of each strain used as inocula.

Groups of five mice were inoculated by gavage with approximately  $1 \times 10^8$  bacteria in 100  $\mu$ l. After 4 days postinfection, mice were humanely euthanized, and livers, spleens, Peyer's patches, mesenteric lymph nodes, and ceca of infected mice were excised and homogenized in preweighed tubes containing 5 ml of ice-cold PBS. Organs were collected, homogenized, and serially diluted, bacteria were enumerated, and the results were displayed as described for intraperitoneal infections. Because the number of organisms that reach systemic sites (liver, spleen) in this model is near the limit of detection, and there can be substantial variation between animals, this assay cannot always reliably distinguish differences in colonization of these sites.

**Sensitivity of individual isolates to hydrogen peroxide.** Overnight cultures were subcultured at 1/100 in LB with appropriate antibiotics containing either no or 1 mM H<sub>2</sub>O<sub>2</sub> (VWR). Cultures were grown at 37°C with aeration. Aliquots for optical density at 600 nm (OD<sub>600</sub>) measurements and for CFU determination were taken every hour. Results were expressed as percentage of survival over time:  $[CFU(t_n)/CFU(t_0)] \times 100$ , where  $t_0$  corresponds to 0 hours and  $t_n$  corresponds to each subsequent time point. Experiments were performed on at least three separate occasions. Statistical significance was determined using a Student *t* test.

**$\beta$ -Galactosidase activity assay.** Cultures of the wild type or the chromosomal *macAB-lacZY* fusion strain (HA996) grown overnight were subcultured 1/100 in fresh LB with appropriate antibiotics containing no or 1 mM H<sub>2</sub>O<sub>2</sub> (VWR). Cultures were grown at 37°C with shaking. Aliquots



for OD<sub>600</sub> measurements as well as for  $\beta$ -galactosidase assay were taken every 30 min.  $\beta$ -Galactosidase was assayed by a modified Miller protocol (85). In brief, 20  $\mu$ l of the bacterial culture at each given time point was mixed with 80  $\mu$ l of permeabilization solution (100 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM KCl, 2 mM MgSO<sub>4</sub>, 0.8 mg/ml cetyltrimethylammonium bromide [CTAB], 0.4 mg/ml sodium deoxycholate, 5.4  $\mu$ l/ml  $\beta$ -mercaptoethanol) and incubated for 30 min at 30°C. A total of 600  $\mu$ l of substrate solution (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mg/ml *o*-nitrophenyl- $\beta$ -D-galactoside [ONPG; Sigma], 2.7  $\mu$ l/ml  $\beta$ -mercaptoethanol) was added to each tube and incubated at 30°C until color development, followed by the addition of 700  $\mu$ l of stop solution (1 M Na<sub>2</sub>CO<sub>3</sub>). Samples were centrifuged for 5 min at 13,000 rpm (Eppendorf 5415R) followed by OD<sub>420</sub> measurements.  $\beta$ -Galactosidase activity was expressed in Miller units. Miller units were calculated as OD<sub>420</sub>/OD<sub>600</sub>  $\times$   $t \times v \times 1,000$ , where  $t$  is reaction time in minutes and  $v$  is volume of culture assayed in milliliters ( $v = 0.02$ ).

Results were expressed as a function of time. The experiment was done in triplicate on three separate occasions.

**Hydrogen peroxide sensitivity of mixed cultures.** Overnight cultures of the wild type and the *macAB* mutant or the wild type and the *recA* mutant were mixed in a 1:1 ratio. The resulting cultures were diluted 1/100 in LB broth, supplemented with 1 mM H<sub>2</sub>O<sub>2</sub>, and incubated at 37°C with aeration. Aliquots were collected hourly, serially diluted, and plated on LB agar supplemented with the corresponding antibiotics. Results were expressed as a percentage of survival over time: [CFU( $t_n$ )/CFU( $t_0$ )]  $\times$  100. Each experiment was performed at least on three separate occasions.

**Hydrogen peroxide sensitivity of the *macAB* mutant grown in conditioned medium.** Overnight cultures of the wild type or the *macAB* mutant were subcultured at 1/100 in LB broth containing 1 mM H<sub>2</sub>O<sub>2</sub> and incubated at 37°C for 3 h with aeration. Bacterial pellets and cell-free supernatant from the resulting cultures were collected by centrifugation at 4,000 rpm for 15 min, and supernatants were filter sterilized (Steriflip; Millipore) to obtain bacterium-free conditioned medium. The resulting conditioned medium for each strain was divided into three tubes. One tube containing conditioned medium was boiled for 10 min and cooled on ice, while the other two were stored on ice. Each tube was inoculated with a 1/100 dilution of overnight culture of the *macAB* mutant. Two tubes containing conditioned media, one heat treated and one untreated, were supplemented with 1 mM H<sub>2</sub>O<sub>2</sub>, while the remaining tube containing conditioned medium that was not heat treated had no peroxide added. Aliquots were collected hourly, serially diluted, and plated on LB agar supplemented with the corresponding antibiotics. Results were expressed as percentage of survival over time: [CFU( $t_n$ )/CFU( $t_0$ )]  $\times$  100. Each experiment was performed on at least three separate occasions.

**In-gel peroxidase and catalase staining.** Overnight cultures of the wild type and the  $\Delta$ *macAB*,  $\Delta$ *katE*, and  $\Delta$ *katG* mutants were grown at 37°C with aeration. Equal numbers of cells were collected by centrifugation at maximum speed (Eppendorf 5415D) for 2 min, and cell pellets were resuspended in 200  $\mu$ l of PBS containing protease inhibitor cocktail (Sigma) and were disrupted by sonication on ice (Misonix Sonicator 3000). The resulting soluble fractions were collected by centrifugation at maximum speed (Eppendorf 5415D) for 2 min and separated on a 10% native PAGE gel. For visualization of catalases and peroxidases, the gel was incubated in 50 mM potassium phosphate buffer, pH 7.0, containing 0.2 U of horseradish peroxidase (Molecular Probes) for 45 min followed by incubation in 5 mM hydrogen peroxide-50 mM potassium phosphate buffer, pH 7.0, for 10 min and developed with 0.5 mg/ml diaminobenzidine in 50 mM potassium phosphate buffer, pH 7.0 (45). We also performed these assays with cultures grown in the presence of 1 mM H<sub>2</sub>O<sub>2</sub> for 3 hours as described in "Sensitivity of individual isolates to hydrogen peroxide" above (see Fig. S3 in the supplemental material). However, because the *macAB* mutant loses viability when peroxide is present, we were unable to collect sufficient bacteria to assay catalase activity using this in-gel staining technique.

**Hydrogen peroxide detection with Amplex red.** Overnight cultures were subcultured at 1/100 in LB broth with the appropriate antibiotics containing 1 mM H<sub>2</sub>O<sub>2</sub> (VWR). Uninoculated LB broth containing 1 mM H<sub>2</sub>O<sub>2</sub> was used as the no-cell control and was treated the same as the other samples. Cultures were grown at 37°C with aeration. Aliquots for hydrogen peroxide detection as well as for CFU determination were collected every hour. Samples to be used for H<sub>2</sub>O<sub>2</sub> detection were cleared by centrifugation at maximum speed (Eppendorf 5415D) for 3 min to remove bacteria. Supernatants were used for hydrogen peroxide detection using the Amplex red hydrogen peroxide/peroxidase kit according to the manufacturer's protocol (Invitrogen). Hydrogen peroxide concentration correlated with production of resorufin, and fluorescence was measured at 530/585 nm. Results were expressed as percent hydrogen peroxide degradation calculated as [Fluorescence<sub>530/585</sub>( $t_n$ )/Fluorescence<sub>530/585</sub>( $t_0$ )]  $\times$  100 over time. Each experiment was performed on at least three separate occasions. Statistical significance was determined by using a Student  $t$  test.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00630-13/-/DCSupplemental>.

Figure S1, PPT file, 0.1 MB.

Figure S2, PPT file, 0.1 MB.

Figure S3, PPT file, 3.4 MB.

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