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MOLECULAR PATHOGENESIS

Cyclopropane Fatty Acids Are Important for *Salmonella enterica* Serovar Typhimurium Virulence

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ABSTRACT A variety of eubacteria, plants, and protozoa can modify membrane lipids by cyclopropanation, which is reported to modulate membrane permeability and fluidity. The ability to cyclopropanate membrane lipids has been associated with resistance to oxidative stress in *Mycobacterium tuberculosis*, organic solvent stress in *Escherichia coli*, and acid stress in *E. coli* and *Salmonella*. In bacteria, the *cfa* gene encoding cyclopropane fatty acid (CFA) synthase is induced during the stationary phase of growth. In the present study, we constructed a *cfa* mutant of *Salmonella enterica* serovar Typhimurium 14028s (*S.* Typhimurium) and determined the contribution of CFA-modified lipids to stress resistance and virulence in mice. Cyclopropane fatty acid content was quantified in wild-type and *cfa* mutant *S.* Typhimurium. CFA levels in the *cfa* mutant were greatly reduced compared to CFA levels in the wild type, indicating that CFA synthase is the major enzyme responsible for cyclopropane modification of lipids in *Salmonella*. *S.* Typhimurium *cfa* mutants were more sensitive to extreme acid pH, the protonophore CCCP, and hydrogen peroxide compared to the wild type. In addition, *cfa* mutants exhibited reduced viability in murine macrophages and could be rescued by the addition of the NADPH phagocyte oxidase inhibitor diphenyleneiodonium (DPI) chloride. *S.* Typhimurium lacking *cfa* was also attenuated for virulence in mice. These observations indicate that CFA modification of lipids makes an important contribution to *Salmonella* virulence.

KEYWORDS cyclopropane fatty acid, *Salmonella*, virulence

Cyclopropanation of unsaturated fatty acids has been described in a variety of eubacteria, plants, and protozoa (reviewed in references 1 and 2). An *S*-adenosylmethionine-dependent methyltransferase called cyclopropane fatty acid (CFA) synthase (encoded by the *cfa* gene) mediates the formation of cyclopropane rings in lipid bilayers (2, 3). The reaction catalyzed by CFA synthase requires *S*-adenosylmethione (SAM or AdoMet) to donate a methylene group to the double bond of a membrane unsaturated fatty acid (UFA), creating a cyclopropane ring on the alkyl chain of the membrane phospholipid (Fig. 1) (2). Although cyclopropanated fatty acids in bacteria were discovered more than 50 years ago, their role in acid stress resistance and bacterial virulence has been established relatively recently (4–12).

Bacteria can alter the composition of their membrane lipids in response to environmental stress conditions (reviewed in references 13 and 14). Lipopolysaccharide modifications in the outer membranes of Gram-negative bacteria render them more resistant to antimicrobial peptides and to host innate immunity (reviewed in reference 15). Nutrient deprivation or changes in growth phase can also affect lipid composition and modification (16, 17). Bacteria can remodel phospholipid composition in membranes, as well as induce modifications in phospholipids, by desaturation, *cis-trans* isomerization, or cyclopropanation in response to stresses from heat, cold, osmolarity, pH, or organic solvent exposure (18–24).

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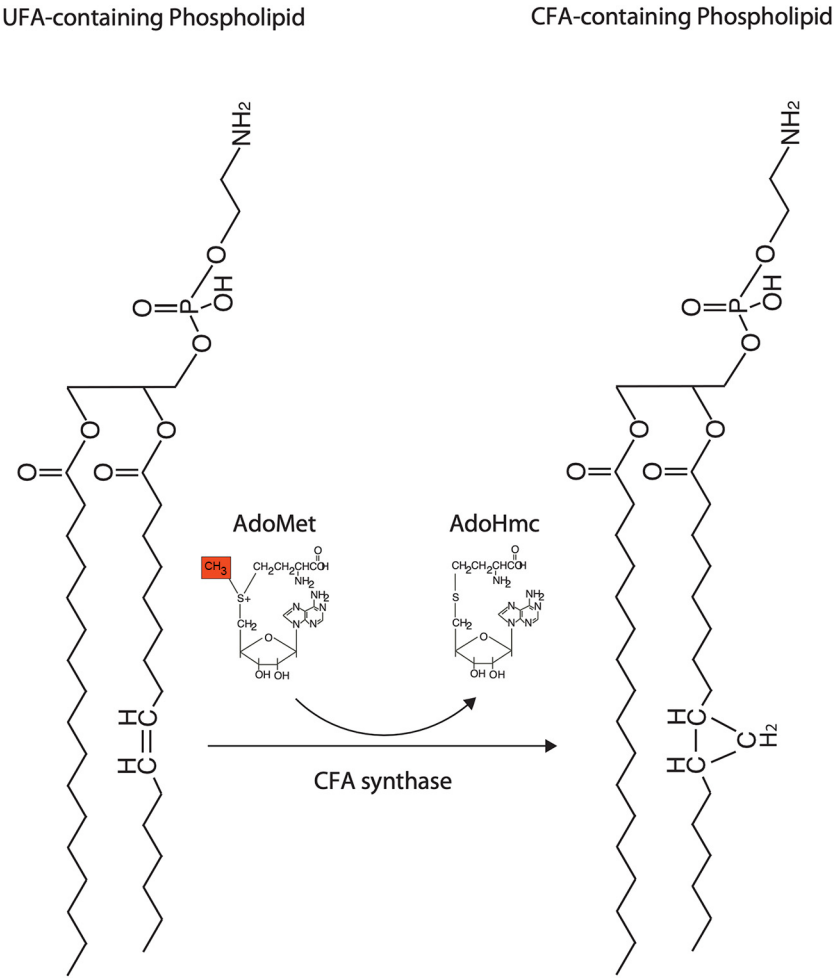

FIG 1 Biosynthesis of cyclopropane fatty acids. Phospholipids in the membrane serve as substrates for the reaction. A methylene group from *S*-adenosylmethione (AdoMet) is transferred to the double bond of an unsaturated fatty acid in the phospholipid to create a cyclopropane ring on the alkyl chain. A cellular enzyme, *S*-adenosylmethionine-dependent methyltransferase, known as CFA synthase, catalyzes the reaction.

Various growth and environmental conditions such as diverse carbon sources, pH, oxygenation, temperature, pressure, Mg^{2+} concentration, and exposure to acetate, organic solvents, terpenes, or fullerenes, can modulate CFA levels (21, 25–29). The expression of *cfa* is RpoS (σ^S)-dependent, which is consistent with the higher proportion of CFA-modified lipids in *E. coli* and *Salmonella* during stationary phase (8, 21, 30). The activity of CFA synthase during stationary phase is also controlled by RpoH (σ^H)-dependent protease activity (31). As a result of this additional level of regulation, the greatest quantities of CFA are observed during early stationary phase (31).

CFA membrane modification in bacteria has been associated with stress-related phenotypes. An *E. coli cfa* mutant exhibits reduced viability after exposure to ethanol, repeated freeze-thaw cycles, or extreme acidic conditions (5, 6, 11, 32). Some data suggest that *E. coli* survival in acidified minimal medium is partly due to the reduced membrane proton permeability which results from increased CFA levels (12). Both pathogenic and nonpathogenic strains of *E. coli* exhibit increased CFA in their cell membranes following acid adaptation, suggesting that CFA lipid modification is a conserved survival response (5).

The role of cyclopropanated lipids in other pathogenic bacteria has also been examined. At least five different *S*-adenosylmethionine-dependent methyltransferases able to cyclopropanate mycolic acids unique to mycobacteria have been identified (7, 33–36). Cyclopropanated mycolic acids are more widespread in pathogenic mycobacterial species (37). Cyclopropanation of mycolic acids by the *S*-adenosylmethionine-dependent methyltransferase Cma1 has been

TABLE 1 Fatty acids of stationary-phase *S.* Typhimurium ATCC14028s wild-type and *cfa* mutant strains

		Wild type		*cfa* mutant	
Fatty acid[a]	No. of carbons:no. of double bonds	Fatty acid level[d]	Conversion of CFA (%)[e]	Fatty acid level[d]	Conversion of CFA (%)[e]
SFA	12:0	1.3		0.88	
SFA	14:0	4.6		6.2	
SFA	15:0	4.4		6.0	
SFA	16:0	29.0		27.7	
UFA	16:1[b]	3.2		30.0	
SFA	17:0	3.2		4.4	
CFA	17:cyclopropane	27.4	89.5	1.1	3.5
SFA	18:0	0.36		1.4	
UFA	18:1[c]	9.3		23.0	
CFA	19:cyclopropane	17.1	64.8	0	0

[a]Saturated fatty acid (SFA), unsaturated fatty acid (UFA), cyclopropane fatty acid (CFA).
[b]6:1 UFA precursor of 17:cyclopropane.
[c]18:1, UFA precursor of 19:cyclopropane.
[d]Fatty acid levels in %weight = ([peak area/sum of all peaks] × 100).
[e]%Conversion of CFA = (%CFA/[%UFA + %CFA]/100).

found to play a role in resistance to oxidative stress (36). Moreover, the cyclopropanation of mycolic acids can affect the virulence of *M. tuberculosis* in infection models (7, 9, 10, 34, 38). Recently, cyclopropane fatty acid synthase has been found to be required for acid resistance, macrophage survival, and gastric colonization in *Helicobacter pylori* (39).

This study investigated whether *cfa*-dependent lipid cyclopropanation is important for stress resistance in *S.* Typhimurium. We found that *cfa* mutant *S.* Typhimurium is more sensitive to oxidative and extreme acid stress compared to the wild type. Enhanced susceptibility to the protonophore CCCP (carbonyl cyanide 3-chlorophenylhydrazone) suggests that CFA-modified lipids may help to maintain proton motive force (PMF) during stationary phase. In addition, a *cfa* mutant strain exhibited reduced survival in cultured macrophages and attenuated virulence in mice, compared to the wild-type strain.

RESULTS

An *S.* Typhimurium *cfa* mutant has an altered fatty acid profile and is defective for survival at extreme acid pH. To determine the effect of a *cfa* mutation on fatty acid composition, wild-type and isogenic *cfa* mutant *S.* Typhimurium ATCC14028s were grown in liquid culture to stationary phase, after which lipids were extracted and analyzed by gas chromatography/mass spectrometry (see Materials and Methods). The fatty acid composition of wild-type *S.* Typhimurium was comparable to previously published values (Table 1) (40). The total CFA levels in the *S.* Typhimurium *cfa* mutant were reduced to 1.1% of wild-type levels in stationary phase (Table 1). The CFA precursors 16:1 and 18:1 UFA were elevated 10- and 2.5-fold, respectively, in the *cfa* mutant, confirming the absence of CFA synthase activity (Table 1). *S.* Typhimurium ATCC14028s had elevated percent conversion values of 17:cyclopropane and 19:cyclopropane fatty acid (89.5% and 64.8%) compared to previously reported values for *S.* Typhimurium strain UK-1 (81.3 and 47.2) (Table 1) (8). The percent conversion of the sum of both types of CFA was approximately 15% higher in *S.* Typhimurium strain ATCC14028s compared to strain UK-1 (Table 1) (8).

A *cfa* mutant derivative of *S.* Typhimurium strain UK-1 was previously shown to be defective for survival at extreme acid pH (8). To confirm that a *cfa* mutation conferred a similar phenotype in strain 14028s, the mutant was tested for viability after acid stress. Strains grown in LB medium experienced lower survival compared to the wild type following exposure to pH 3, relative to cells grown in M9 minimal medium (compare Fig. 2A and B). In addition, the *cfa* mutants experienced higher mortality at pH 3 compared to the wild type (Fig. 2A and B). These observations confirm earlier findings that CFA contributes to the survival of *S.* Typhimurium 14028s during extreme acid stress.

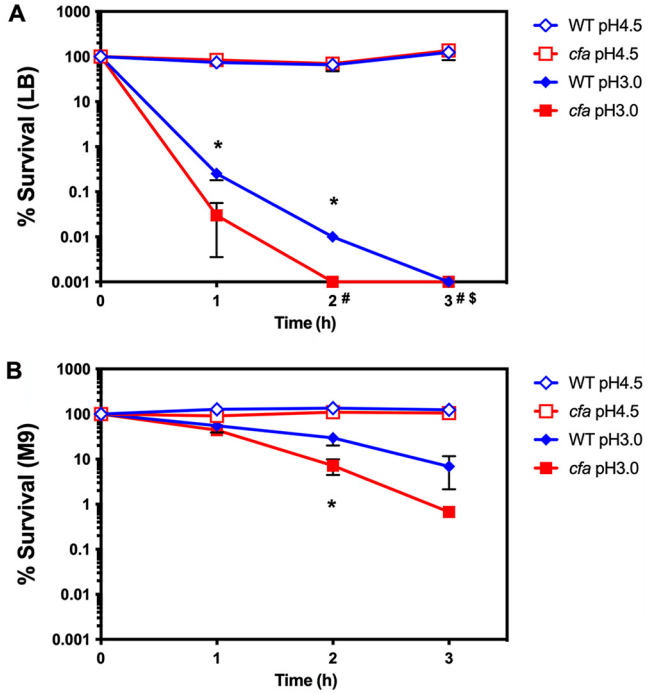

FIG 2 Cyclopropane fatty acids are required for survival at extreme acid pH. Stationary-phase *S.* Typhimurium strains were grown in either LB medium (A) or in M9 containing 0.4% glucose (B) and were diluted 1:1,000 into fresh media adjusted to pH 4.5 (wild-type, blue open diamonds; *cfa* mutant, red open squares) or pH 3.0 (wild-type, blue closed diamonds; *cfa* mutant, red closed squares). The CFU of surviving cells were determined at 0, 1, 2 and 3 h post-acid shock treatment. [$]The wild type and [#]*cfa* mutant were below the theoretical limit of detection at these time points. A Student's *t* test was performed between wild type and mutant. The asterisk (*) indicates $P < 0.05$.

Cyclopropane fatty acids are required for *Salmonella* resistance to the protonophore CCCP. Environmental stresses such as extreme acid pH and nutrient deprivation can affect membrane potential $\Delta\psi$ (41, 42). As bacteria enter stationary phase, nutrients are exhausted and energy utilization diminishes, resulting in decreased proton motive force (PMF) (43). Levels of the phage shock protein PspA increase during stationary phase and play a role in PMF maintenance (43, 44). Because CFA synthesis increases during stationary phase, we sought to determine whether CFA membrane modification contributes to the maintenance of PMF. *S.* Typhimurium strains were treated with the protonophore CCCP (carbonyl cyanide 3-chlorophenylhydrazone), which disrupts PMF (46). Stationary-phase cells were treated with 250 μM CCCP, and viability was determined by plating of serial dilutions. A *pspA* mutant previously shown to be CCCP-sensitive was included as a positive control (Fig. 3) (44). The *S.* Typhimurium *cfa* mutant was found to be more sensitive to CCCP than the wild type (Fig. 3). Moreover, a *cfa pspA* double mutant was found to have increased sensitivity to CCCP when compared to a strain carrying a single *cfa* mutation (Fig. 3). These observations suggest that cyclopropane fatty acids play a role in the maintenance of PMF in *Salmonella* that is independent of the phage shock response.

Cyclopropane fatty acids are important for *Salmonella* resistance to oxidative stress. The role of CFA in protection from oxidative stress has been previously demonstrated in *Pseudomonas* and *Mycobacterium* spp. Highly reactive singlet oxygen generated by fullerenes has been shown to increase CFA levels in *P. putida* (27). Overexpression of the *M. tuberculosis* CFA synthase homolog Cma1, an *S*-adenosylmethionine-dependent methyltransferase, confers increased resistance to hydrogen peroxide in *M. smegmatis* (36). However, whether CFA contributes to oxidative stress resistance in enteric bacteria has previously been unclear. An *E. coli* K-12 *cfa* mutant derivative exhibited wild-type resistance levels following exposure to singlet oxygen generated by illuminated eosin Y (32). To determine whether CFA contributes to oxidative stress resistance in *S.* Typhimurium, we monitored the growth of wild-type and isogenic *cfa* mutant strains in the presence of hydrogen peroxide

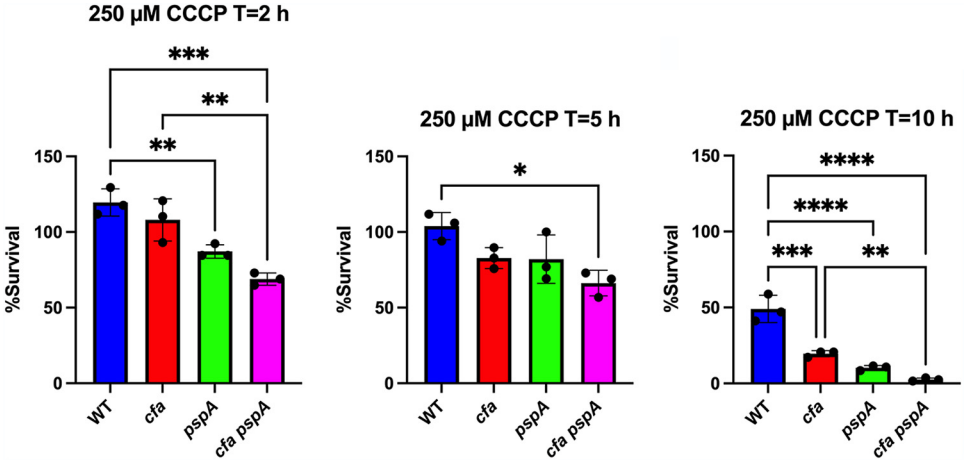

FIG 3 Cyclopropane fatty acids are needed for survival upon exposure to protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Stationary-phase *S.* Typhimurium wild type (WT), *cfa* mutant, *pspA* mutant, and *cfa pspA* double mutant strains were grown in LB, diluted to 10^5 CFU per ml in LB with 250 μM CCCP, and incubated for 10 h at 37°C. CFU of surviving cells were determined at $T = 2$ h, $T = 5$ h, and $T = 10$ h. Means and standard deviations from three independent experiments are shown. A one-way ANOVA with Tukey's multiple comparison test was used to compare WT and mutant strains at 2, 5, and 10 h. Asterisks indicate the P value as follows. (*) $P = 0.0332$, (**) $P = 0.0021$, (***) $P = 0.0002$, and (****) $P < 0.0001$.

(H_2O_2) (see Materials and Methods). A *dps* mutant strain previously shown to exhibit enhanced H_2O_2-susceptibility was included as a positive control (47). Treatment with 1 mM H_2O_2 prolonged the lag phase of wild-type *S.* Typhimurium by 5.5 h, and by 8.5 h in the isogenic *cfa* mutant (Fig. 4A). The *dps* mutant was more profoundly inhibited by 1 mM H_2O_2, with a growth delay of 15.5 h (Fig. 4A).

Stationary-phase wild-type and *cfa* mutant *S.* Typhimurium strains were also tested for survival after treatment with H_2O_2. Cells grown in LB were more resistant to H_2O_2 compared to strains grown in M9 minimal medium (Fig. 4B). The *cfa* mutant was more sensitive to H_2O_2 regardless of the growth medium used (Fig. 4B). The enhanced H_2O_2-susceptibility of a *cfa* mutant was restored to wild-type levels using a plasmid containing a copy of the *cfa* gene (Fig. 4C). Taken together, these observations demonstrate that CFA contributes to oxidative stress resistance in *S.* Typhimurium.

Cyclopropane fatty acids are required for *Salmonella* survival in macrophages and virulence in mice. Previous studies have demonstrated the role of lipid cyclopropanation in *M. tuberculosis* virulence (7, 9, 10, 34, 38). Here, we determined if cyclopropane-modified fatty acids contribute to *Salmonella* pathogenesis. A *cfa* mutant *S.* Typhimurium strain was assayed for its ability to survive in murine macrophages. Murine sodium periodate-elicted peritoneal macrophages were isolated from C3H/HeN (*Ity*^{R}) mice and activated with IFN-γ prior to infection. The *S.* Typhimurium *cfa* mutant was more vulnerable to macrophages than the wild type at 2 and 4 h postinfection (Fig. 5). The macrophage defect of the *cfa* mutant could be repaired by treatment using DPI (diphenyleneiodinium) (Fig. 5) to inhibit both the Nox2 NADPH phagocyte oxidase and the Nos2 inducible nitric oxide synthase, which are required for the production of antimicrobial reactive oxygen and nitrogen species, respectively (48). *S.* Typhimurium strains were also tested for sensitivity to the cathelicidin antimicrobial peptide CRAMP, produced by murine macrophages (49). We observed no difference in resistance to CRAMP in *cfa* mutant *S.* Typhimurium compared to the wild type (Fig. S1). In addition, an *S.* Typhimurium *cfa* mutant survived as well as the wild type at a pH of 4.5, corresponding to the pH of an acidified macrophage vacuole (Fig. 2A and B). Collectively, these studies suggest that CFA promotes intra-macrophage survival by conferring resistance to reactive species generated by activated macrophages.

Wild-type *S.* Typhimurium, an isogenic *cfa* mutant, and a complemented *cfa* mutant carrying a low-copy-number plasmid with *cfa* and its native promoter (see Materials and Methods) were assayed for virulence in C3H/HeN (*Ity*^{R}) mice. Mice were infected intraperitoneally with 3,000 CFU and monitored daily, and moribund mice were euthanized.

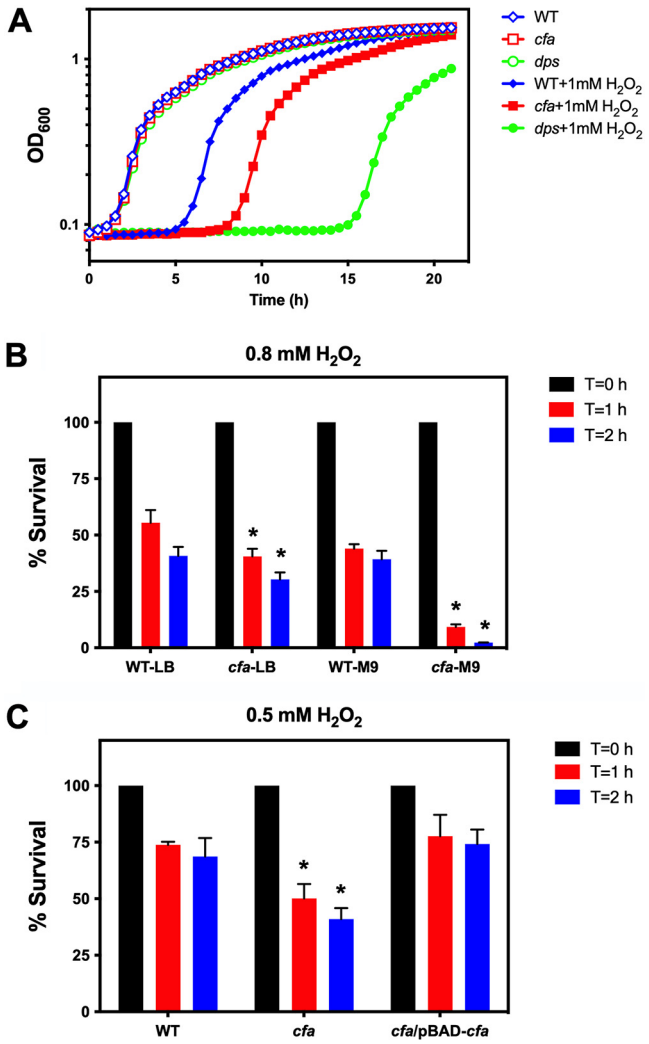

FIG 4 Cyclopropane fatty acids are important for are important for protection against hydrogen peroxide exposure. (A) *S.* Typhimurium strains that lack cyclopropane fatty acids are susceptible to growth inhibition by hydrogen peroxide. Stationary-phase *S.* Typhimurium strains were diluted to a final optical density (OD_{600}) of 0.002 in LB medium (LB), and cell growth was monitored by OD_{600} at 37°C for 21 h (wild type, open blue diamonds; *cfa* mutant, open red squares; *dps* mutant, open green circles). Strains were also monitored for cell growth in LB medium with the addition of 1 mM hydrogen peroxide (H_2O_2) (wild-type, blue closed diamonds; *cfa* mutant, red closed squares; *dps* mutant, green closed circles). (B) Cyclopropane fatty acids are important for survival in hydrogen peroxide. Stationary-phase *S.* Typhimurium strains (wild-type and *cfa* mutant) were grown in LB medium or in M9 medium (M9) with 0.4% glucose and diluted in PBS, containing 0.8 mM H_2O_2, for 2 h at 37°C. CFU of surviving cells were determined post-H_2O_2 challenge at $T = 0$ (black bars), $T = 1$ h (red bars), and $T = 2$ h (blue bars). A Student's *t* test was performed between the wild type and the *cfa* mutant at each time point. An asterisk (*) indicates $P < 0.05$. (C) The plasmid pBAD-*cfa* could complement a *cfa* mutant for survival in hydrogen peroxide. Stationary-phase *S.* Typhimurium strains (wild-type, *cfa* mutant, and *cfa*/pBAD-*cfa*) were grown in LB medium with 0.2% arabinose and incubated in PBS, containing 0.5 mM H_2O_2, for 2 h at 37°C. CFU of surviving cells were determined post-H_2O_2 challenge at $T = 0$ (black bars), $T = 1$ h (red bars) and $T = 2$ h (blue bars). A Student's *t* test was performed between wild-type and *cfa* mutant or *cfa*/pBAD-*cfa* strains at each time point. An asterisk (*) indicates $P < 0.05$.

Comparisons of survival between the wild type, the *cfa* mutant, and the complemented *cfa* mutant revealed that the *cfa* mutant strain is detective for virulence in mice (Fig. 6A). Survival analysis by a Gehan-Breslow-Wilcoxon test showed significant differences between the *cfa* mutant and the complemented *cfa* mutant ($P = 0.0463$) while a trend toward reduced survival of mice infected with wild-type *Salmonella* compared to those infected with *cfa* mutant strains did not achieve statistical significance ($P = 0.1085$). A competitive

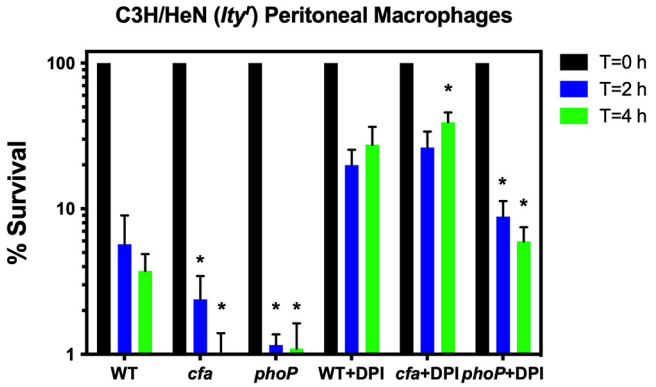

FIG 5 Cyclopropane fatty acids are important for survival in murine peritoneal macrophages. Peritoneal macrophages isolated from mice C3H/HeN (*Ity*^{R}) were infected with an MOI of 10:1 with *S.* Typhimurium wild-type, *cfa* mutant, and *phoP* mutant strains, and the CFU were determined postinfection at $T = 0$ (black bars), $T = 2$ h (blue bars), and $T = 4$ h (green bars). In addition, peritoneal macrophages were treated with 25 μM diphenyleneiodonium (DPI) chloride to inhibit both NADPH phagocyte oxidase and inducible nitric oxide synthase prior to infection. A Student's *t* test was performed between the wild-type, *cfa* mutant, and *phoP* mutant strains at $T = 2$ and $T = 4$ h within each group (with and without DPI). An asterisk (*) indicates $P < 0.05$.

survival assay was performed by intraperitoneally infecting mice with a 1:1 mixture of *cfa* mutant and wild-type strains, euthanizing mice at time intervals ranging from 4 to 17 days postinfection. Livers and spleens were harvested and homogenized prior to plating and enumeration of CFU, and competitive indices of wild-type and *cfa* mutant strains were determined (see Materials and Methods). An *S.* Typhimurium strain lacking *cfa* exhibited reduced competitive fitness beginning on day 7, with a median CI of 0.6 and 0.83, respectively (Fig. 6B). By day 17, the median CI of *cfa* mutant versus wild type in livers and spleens was 0.48 and 0.41, respectively (Fig. 6B). CI data from livers and spleens from days 4 to 17 were combined, and a Wilcoxon Signed Rank test showed a survival defect of the *cfa* mutant compared to the wild type ($P < 0.0001$) (Fig. 6C). These observations demonstrate that CFA contributes to *Salmonella* survival in macrophages and virulence in mice.

DISCUSSION

S. Typhimurium experiences diverse stress conditions in both external and host environments and has developed strategies to withstand these stresses (reviewed in references 50 and 51). Previous studies have shown that the cyclopropanation of bacterial membrane unsaturated fatty acids occurs in response to a diverse range of environmental stresses (19, 21). In the present study, we have demonstrated the importance of cyclopropane modification of membrane lipids in *S.* Typhimurium in resistance to CCCP treatment and oxidative stress, as well as its importance for virulence in mice.

The proton motive force (PMF) represents the sum of electrical and chemical gradients across the cell membrane, which can be profoundly influenced by environmental stresses and by the metabolic status of the cell. When *E. coli* enters the stationary phase of growth, PMF begins to decrease as a consequence of diminishing nutrient availability and energy utilization (43). Recent studies have shown that *E. coli* exhibits increased membrane proton permeability when subjected to organic solvents, such as ethanol (52). Furthermore, *E. coli cfa* mutants are less viable than the wild type following exposure to ethanol (32), suggesting a role for CFA in PMF maintenance. Extreme acid conditions can enhance the transmembrane proton gradient, affecting intracellular pH homeostasis and membrane potential (53, 54). In *E. coli*, CFAs reduce membrane proton permeability and enhance bacterial survival in acidified minimal media (12). We found that *S.* Typhimurium that lack CFAs are defective for survival following exposure to extreme acid stress or treatment with the protonophore CCCP (Fig. 2 and 3). CCCP collapses the PMF by uncoupling the transport of protons across the membrane (46). The phage shock protein PspA has been proposed to play a role in PMF maintenance,

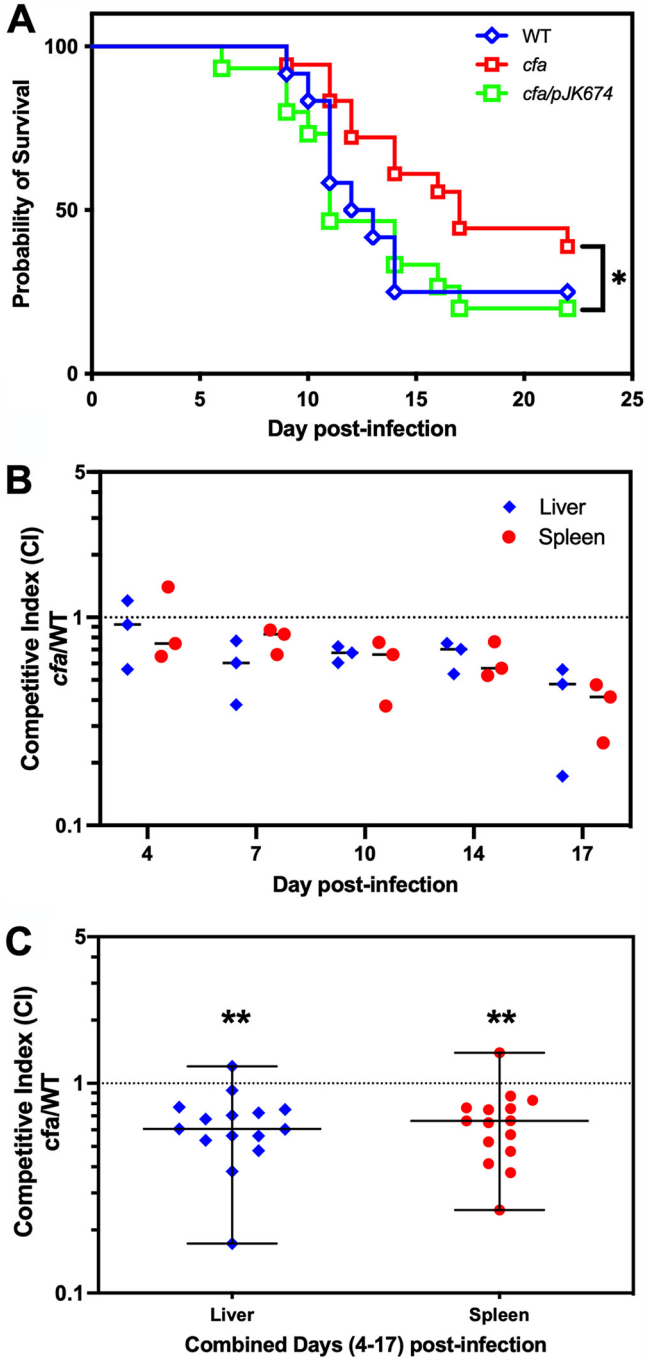

FIG 6 Cyclopropane fatty acids are important for virulence in mice. (A) C3H/HeN (*lty*^{R}) mice were infected intraperitoneally with *S.* Typhimurium strains wild-type (blue open diamonds), *cfa* mutant (red open squares), and *cfa*/pJK674 (green open squares) with 3000 CFU. Mice were monitored daily, and moribund mice were euthanized. *, $P = 0.0463$ between *cfa* mutant and *cfa* mutant with complementing plasmid pJK674 using Gehan-Breslow-Wilcoxon test. (B) Competitive survival experiment of *cfa* mutant versus wild type in C3H/HeN (*lty*^{R}). Mice were inoculated intraperitoneally with a mixture of *cfa* mutant and wild-type *S.* Typhimurium strains. Three independent mice were assayed for each time period postinfection. The competitive index (CI) of the *cfa* mutant versus the wild type was determined for both the liver (blue closed diamonds) and the spleen (red closed circles). A CI of 1 indicates that both strains grew equally well *in vivo*. (C) The competitive index data from panel B were combined from days 4 to 17 postinfection and a Wilcoxon signed rank test was performed. A double asterisk (**) indicates $P < 0.0001$.

and suppresses membrane proton permeability (44, 52, 55). *S.* Typhimurium *pspA* and *cfa* mutants are both defective for survival when treated with CCCP, compared to the wild type (Fig. 3). Furthermore, a *pspA cfa* double mutant is more sensitive to CCCP than a strain carrying a *cfa* mutation alone (Fig. 3). Taken together, these results suggest that the CFA modification of membranes contributes to the maintenance of PMF under stress conditions by reducing proton permeability.

The role of CFA-modified membranes in oxidative stress resistance has not been well characterized. Previous observations found no difference in survival between wild-type and *cfa* mutant *E. coli* following exposure to singlet oxygen (32). In contrast, we have found that *S.* Typhimurium *cfa* mutants are more susceptible to hydrogen peroxide (Fig. 4), indicating that fatty acid cyclopropanation confers resistance to oxidative stress. The difference between earlier results with singlet oxygen and the present study with hydrogen peroxide may suggest that the membrane poses a greater diffusion barrier for hydrogen peroxide than for singlet oxygen, and that this barrier function is sensitive to fatty acid cyclopropanation, which can influence packing within lipid bilayers (56). Alternatively, CFA-modified membranes may have greater integrity or enhance membrane protein function during oxidative stress. For example, changes in lipid composition have been shown to modulate the activity of osmoregulated uptake systems in *Corynebacterium* (57).

We observed no difference between *S.* Typhimurium wild-type and *cfa* mutant strains in their susceptibility to growth inhibition by the nitric oxide donor SperNO (Fig. S2). However, it is intriguing to note that *E. coli* flavohemoglobin (HMP), an important nitric oxide scavenger, preferentially binds to unsaturated (UFA) and cyclopropanated fatty acids (CFA), but not to saturated fatty acids (58). The binding of UFA or CFA to HMP augments HMP heme iron-binding properties *in vitro* (58). It is not presently known whether UFA or CFA influences HMP activity *in vitro* or *in vivo*.

The *S.* Typhimurium *cfa* mutant was found to be defective for survival in peritoneal murine macrophages (Fig. 5). The survival defect of the *cfa* mutant could be repaired by treating macrophages with DPI, which inhibits both the phagocyte NADPH oxidase and inducible nitric oxide synthase. These results suggest that lipid CFAs confer resistance to antimicrobial reactive species produced by activated macrophages, and support the *in vitro* finding that CFAs confer resistance to hydrogen peroxide. Finally, *S.* Typhimurium *cfa* mutants were also found to be deficient for virulence in mice, with a *cfa* mutant strain exhibiting a competitive defect compared to the wild type (Fig. 6B and C). A role for cyclopropanated lipids in pathogenic bacteria has recently been described in *M. tuberculosis* and *Helicobacter pylori*. *M. tuberculosis* has at least five putative cyclopropane synthase homologs that make at least three major types of cyclopropanated mycolic acids (7, 33–36). Some of these cyclopropanated mycolic acid derivatives have been found to be involved in hydrogen peroxide resistance, virulence, and long-term survival in mice, as well as influencing host inflammatory responses (7, 9, 10, 34, 38). Future studies will determine how CFA modification promotes *Salmonella* virulence and whether this includes modulation of the host response.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. Bacterial strains, plasmids, and primers used in this study are listed in Table 2.

Reagents and standard genetic and molecular techniques. Bacteria were grown in LB medium, minimal M9 medium supplemented with 0.4% glucose, or brain heart infusion (Difco Laboratories, Sparks, MD), at 37°C with aeration, unless otherwise indicated. Restriction and modifying enzymes were purchased from New England Biolabs (Ipswich, MA). Chemicals were purchased from Sigma (St. Louis, MO). *Taq* DNA polymerase was purchased from Promega (Madison, WI). Phage P22 transduction methods were performed as described (59).

Construction of *cfa* mutant. A chromosomal mutation in *cfa* was constructed by λ-Red-mediated recombination (60). The *cfa* gene was deleted from codons 16 to 47, which were replaced with the chloramphenicol resistance (*cat*) gene from pKD3 (60) as follows: the chloramphenicol resistance gene with flanking sequences from *cfa* was PCR-amplified using primers 5′cfa, 3′cfa, and plasmid DNA pKD3. The 1,100-bp PCR-amplified fragment was purified using a Qiagen PCR purification kit (Valencia, CA) and electroporated into *S.* Typhimurium containing the λ-Red-expressing plasmid pTP223 (61). Cells were plated onto LB agar containing 20 μg ml^{-1} chloramphenicol and incubated at 37°C. Chloramphenicol-

TABLE 2 Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or sequence (5′–3′)	Source or reference
Strains		
14028s	14028s wild type	ATCC
AF441	14028s *cfa*::*frt-cat-frt*	This study
LB133	14028s *pspA*::*kan*	(44)
AF453	14028s *cfa*::*frt-cat-frt pspA*::*kan*	This study
JV104	14028s *dps*::*cat*	(67)
CS015	14028s phoP102::Tn*10d*Cm	S. Miller
JK811	14028s *cfa*::*frt-cat-frt*/pJK674	This study
Plasmids		
pTP223		(61)
pKD3	*bla frt-cat-frt* PS1PS2 ori R6K	(60)
pBAD30	*bla araC araBAD* promoter ori p15A	(62)
pRB3-273C	*bla par* RK2 stable low-copy-no. cloning vector	(63)
pBR322	*bla tet* ori colE1	(64)
pBAD::*cfa*	*bla araC araBAD* promoter::*cfa* ori p15A	This study
pJK650	pBR322-(-389bp from ATG and coding region of *cfa*)	This study
pJK674	pRB3-(-389bp from ATG and coding region of *cfa*)	This study
Primers		
5′cfa	ATGGAGAAACTATGAGTTCATCGTGTATAGAAGAAGTCAGCGTA CCGGATGATAACTGGTGTGTAGGCTGGAGCTGCTTC	
3′cfa	CACCAGCCATCCATGTAACTCTCACCTAACCCCAACGATCCTT CCTGAAGGACGCGTTTACATATGAATATCCTCCTTAG	
cfa1	AGCCAAAAAAGCGTCTACGC	
cfa2	TCAGGCCGACATTGGCTAAC	
cfa3	GAATTCCAGTGATGGAGAAACTAT	
cfa4	CTAACGGAAAATAAGATTCCCCCGC	
JKP149	GGGGGAATTCGTTGTTCTGGCGGCGTTAGG	
JKP152	GGGGCTGCAGTAGAACGCGTACTGGCTGCG	

resistant colonies were screened by PCR, with primers cfa1 and cfa2, to confirm that the chloramphenicol-resistance cassette was inserted in *cfa*. The *cfa*: *frt-cat-frt* allele was reintroduced into a new *S.* Typhimurium background by P22 transduction. Quantitative RT-PCR was performed on the *cfa*::*frt-cat-frt S.* Typhimurium strain to verify the lack of *cfa* expression in log and stationary-phase cells.

Construction of *cfa* plasmid constructs. The *cfa* gene was cloned under the control of an arabinose-inducible promoter in the low-copy plasmid pBAD30 (62) as follows: the *cfa* gene was PCR-amplified with primers cfa3 and cfa4 using the genomic DNA template isolated from *S.* Typhimurium ATCC14028s. The 1,148-bp PCR-amplified fragment was digested with EcoRI, then gel-purified with a Qiagen gel purification kit (Valencia, CA). The *cfa*-containing PCR fragment was subsequently ligated, using T4 DNA polymerase, into pBAD30 digested with EcoRI and SmaI. The pBAD::*cfa* construct was confirmed by DNA sequencing. The promoter and coding region of *cfa* were cloned into the stable low-copy plasmid pRB3-273C (63), designated pJK674, as follows: primers JKP149 and JKP152 were used to PCR-amplify a 1637-bp fragment from *S.* Typhimurium 14028s which included both the promoter and the coding region of *cfa*. The fragment was digested with EcoRI and PstI, then ligated into plasmid pBR322 (64), digested with EcoRI and PstI, to create pJK650. The plasmid pJK650 was subsequently digested with EcoRI and PstI, and the 1,637-bp fragment containing the promoter and coding region of *cfa* was blunt ended with T4 DNA polymerase, then ligated into pRB3-273C digested with SmaI to create pJK674.

Isolation and analysis of fatty acid composition in *S.* Typhimurium. Wild-type *S.* Typhimurium and the isogenic *cfa* mutant derivative were grown overnight in brain heart infusion broth at 37°C with agitation. Cells were harvested by centrifugation at 3000 × *g* for 15 min. The resulting cell pellets were washed once with ice-cold 20 mM morpholinepropanesulfonic acid (pH 7.2) and stored frozen at −20°C. Lipids were extracted by the Kates modification of the method of Bligh and Dyer (65). The lower chloroform layer was washed once with 0.1 M KCl and filtered through a phase-separating filter (Whatman).

Alkaline methanolysis was performed as follows: solvent was evaporated under a stream of N_2 and the lipids suspended in 0.2 ml chloroform and 0.3 ml methanol, to which 0.2 M NaOH in methanol was added. The sample was mixed by vortexing and allowed to stand for 20 min at room temperature. Chloroform (0.8 ml), methanol (0.2 ml), and water (0.9 ml) were then added before the sample was mixed and centrifuged at 1,200 rpm for 4 min. The lower layer was washed once with methanol/water at a ratio of 10:9 (vol/vol) and twice filtered through a phase-separating filter (65).

The resulting fatty acid methyl esters were subjected to gas chromatography/mass spectrometry by standard methods. The mass spectra were compared to those of pure standards of saturated and unsaturated fatty acids. Cyclopropane fatty acids were confirmed to have the expected mass.

Hydrogen peroxide bioscreen and killing assays. A bioscreen assay to test strains for susceptibility to hydrogen peroxide (H_2O_2) was performed. Stationary-phase cells grown in LB were diluted to OD_{600} 0.002 with or without the addition of 1 mM H_2O_2. Cells were subsequently grown aerobically at 37°C with agitation and cell density monitored at OD_{600} with a Labsystems Bioscreen C microplate reader (Helsinki, Finland). Hydrogen peroxide killing assays were performed as follows: stationary-phase cells grown in LB or M9 containing 0.4% glucose were washed once in phosphate-buffered saline (PBS), diluted to 2×10^6 in PBS with or without 0.8 mM H_2O_2, and then incubated at 37°C for up to 2 h. Viable cells were determined by plating diluted aliquots onto LB agar. Percent survival was determined by calculating the CFU after H_2O_2 treatment divided by the CFU of untreated cells at each time interval.

Acid stress survival assays. Stationary-phase cells grown in LB or M9 broth containing 0.4% glucose were diluted 1:1,000 in fresh medium, with pH adjusted to 3 or 4.5 using HCl. Cells were subsequently grown aerobically at 37°C for up to 3 h. Viable cells were determined by plating diluted aliquots onto LB agar. Percent survival was determined by calculating the CFU of acid-stressed cells divided by the CFU of untreated cells.

CCCP survival assays. Stationary-phase cells grown in LB were diluted to 1×10^5 CFU in LB containing 250 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and incubated at 37°C for 10 h. Viable cells were determined by plating diluted aliquots onto LB agar. Percent survival was determined by calculating the CFU of CCCP-treated cells divided by the CFU of untreated cells.

Macrophage survival and mouse experiments. The animal experiments in this study were approved by the University of Washington Institutional Animal Care and Use Committee and performed as described in protocol 3373-01. Macrophage survival assays were performed using periodate-elicited macrophages isolated from 7-week-old female C3H/HeN (*Ity*^{s}) mice (Charles River Laboratories, Wilmington, MA) as previously described (66). To inhibit the NADPH phagocyte oxidase (Nox2), 25 μM diphenyleneiodonium (DPI) chloride (Sigma, Saint Louis, MO) was added 24 h prior to infection as indicated.

Virulence assays were performed as follows: 7-week-old female C3H/HeN (*Ity*^{s}) mice were infected intraperitoneally with 3000 CFU of wild-type, *cfa::frt-cat-frt* mutant, or complemented *cfa::frt-cat-frt*/pJK674 *S.* Typhimurium. The actual inocula were measured by plating serial dilutions onto LB agar. Mice were monitored daily for the duration of the experiment, and moribund mice were euthanized. Statistical analysis of the survival curves was determined using a Gehan-Breslow-Wilcoxon test with Prism 6.0c (GraphPad Software, Inc.).

Competitive index assays were performed as follows: 7-week-old female C3H/HeN (*Ity*^{s}) mice were infected as described above, but with an equal mixture of wild-type and *cfa::frt-cat-frt* mutant strains. At designated time intervals, the mice were euthanized, livers and spleens were harvested, organs homogenized in PBS, and viable counts determined by plating serial dilutions onto LB agar, with or without the addition of 20 μg ml^{-1} chloramphenicol. The competitive index of the *cfa::frt-cat-frt* mutant versus the wild type was determined as the ratio of $(\text{mutant/wild-type})_{\text{output}}$ to $(\text{mutant/wild-type})_{\text{input}}$. Statistical significance was determined using the Wilcoxon Signed Rank test with Prism 6.0c (GraphPad Software, Inc.).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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