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Authors

Margolis, Alyssa

Liu, Lin

Porwollik, Steffen

et al.

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BACTERIAL INFECTIONS

Arginine Metabolism Powers *Salmonella* Resistance to Oxidative Stress

Alyssa Margolis,[a] Lin Liu,[a] Steffen Porwollik,[b] James Karl A. Till,[a] Weiping Chu,[b] Michael McClelland,[b] Andrés Vázquez-Torres[a]

[a]Department of Immunology and Microbiology, University of Colorado Anschutz Medical Campus, Aurora, Colorado, USA
[b]Department of Microbiology and Molecular Genetics, University of California Irvine School of Medicine, Irvine, California, USA

ABSTRACT *Salmonella* invades host cells and replicates inside acidified, remodeled vacuoles that are exposed to reactive oxygen species (ROS) generated by the innate immune response. Oxidative products of the phagocyte NADPH oxidase mediate antimicrobial activity, in part, by collapsing the ΔpH of intracellular *Salmonella*. Given the role of arginine in bacterial resistance to acidic pH, we screened a library of 54 single-gene mutants in *Salmonella* that are each involved in, but do not entirely block, arginine metabolism. We identified several mutants that affected *Salmonella* virulence in mice. The triple mutant Δ*argCBH*, which is deficient in arginine biosynthesis, was attenuated in immunocompetent mice, but recovered virulence in phagocyte NADPH oxidase deficient *Cybb*$^{-/-}$ mice. Furthermore, Δ*argCBH Salmonella* was profoundly susceptible to the bacteriostatic and bactericidal effects of hydrogen peroxide. Peroxide stress led to a larger collapse of the ΔpH in Δ*argCBH* mutants than occurred in wild-type *Salmonella*. The addition of exogenous arginine rescued Δ*argCBH Salmonella* from peroxide-induced ΔpH collapse and killing. Combined, these observations suggest that arginine metabolism is a hitherto unknown determinant of virulence that contributes to the antioxidant defenses of *Salmonella* by preserving pH homeostasis. In the absence of phagocyte NADPH oxidase-produced ROS, host cell-derived L-arginine appears to satisfy the needs of intracellular *Salmonella*. However, under oxidative stress, *Salmonella* must additionally rely on *de novo* biosynthesis to maintain full virulence.

KEYWORDS Salmonella, arginine, innate immunity, metabolism, nox2, oxidative stress, pH, phagocyte NADPH oxidase

Despite significant progress by global health initiatives to ensure access to potable water, adequate sanitation, and food safety education, diarrheal disease caused by pathogens like *Salmonella* remains one of the top 10 leading causes of mortality worldwide (1). Non-typhoidal *Salmonella* are responsible for roughly 1.3 billion cases of intestinal disease per year (2). Given the broad host range, high prevalence in the food chain (3), and rising rates of antimicrobial resistance (4), *Salmonella* infections are a major public health concern.

After crossing the intestinal epithelium, *Salmonella* establishes an intracellular niche inside host epithelial cells and macrophages, replicating inside a modified endosome termed the *Salmonella*-containing vacuole (SCV). Within the SCV of professional phagocytes, *Salmonella* are exposed to reactive oxygen species (ROS) produced by the phagocyte NADPH oxidase (NOX2), one of the most potent arms of the innate host defense (5). NOX2-generated ROS can damage DNA, iron-sulfur cluster-containing proteins, and protein cysteine and methionine residues (6, 7). To survive the initial response to infection that is dominated by ROS produced by phagocyte NADPH oxidase, *Salmonella* employs a multitude of antioxidant enzymes (8, 9). For instance, superoxide dismutases degrade superoxide anions (O_2-) into oxygen (O_2) and hydrogen peroxide (H_2O_2) (10), catalases decompose H_2O_2 into O_2 and H_2O, peroxidases catalyze the reduction of H_2O_2 and other hydroperoxides into

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Address correspondence to Andrés Vázquez-Torres, Andres.Vazquez-Torres@cuanschutz.edu.

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H_2O using a variety of reductants (11), and the low-molecular-weight thiol glutathione can scavenge H_2O_2 (12). Yet, despite decades of research on the topic, the mechanisms by which *Salmonella* protects itself against the ROS produced during the innate host response are incompletely understood (13, 14).

During periods of high electron flow through the electron transport chain (ETC), damage caused by NOX2 to the NADH dehydrogenase encoded by the *nuo* operon results in a collapse in the bacterial ΔpH, disrupting membrane energetics, and precipitating increased protein misfolding events (15). In response, *Salmonella* undergoes overflow metabolism and utilizes fermentation-produced acetate to stabilize the alkaline pH of the bacterial cytoplasm (15, 16). Here, we investigated whether there are additional mechanisms *Salmonella* could employ to maintain ΔpH during oxidative stress.

Salmonella utilizes a number of different inducible and constitutive strategies to maintain pH homeostasis (17). Constitutive acid resistance systems, which enable survival at the typical gastric pH of less than 2.5, depend on the presence of L-arginine (18, 19), lysine (20), or ornithine (21). Inducible acid tolerance systems in *Salmonella* require a pre-adaption exposure to a mildly acidic pH prior to an acid challenge at pH $\geq$ 3 (22). The inducible acid tolerance systems include acid shock proteins that are controlled by RpoS, OmpR, Fur, and PhoPQ (23), regulation of F_0F_1-ATPase (24–26), and induction of amino acid decarboxylase (27) or deiminase (28) systems.

L-arginine is the only amino acid identified as one of the seven key nutrients that support full *Salmonella* virulence in mice (29). While both L-arginine catabolism (28) and L-arginine transport (30) are known to be critical for *Salmonella* virulence in mice, it is not yet known whether this necessity for L-arginine during host infection is related to resistance to the innate host response. Interestingly, research in other organisms implicates L-arginine catabolism in resistance to oxidative stress (31, 32), and *Salmonella* deficient in both cytoplasmic superoxide dismutases displays increased levels of L-arginine (33). Given these observations, the following experiments sought to explore how *Salmonella* utilizes L-arginine metabolism during host infection to promote pathogenesis during periods dominated by the antimicrobial activity of phagocyte NADPH oxidase.

RESULTS

L-arginine metabolism is a critical determinant of *Salmonella* pathogenesis. In the following investigations, we examined the role of L-arginine metabolism in *Salmonella* pathogenesis. Towards this end, a targeted, single-gene knockout library of 54 genes involved in L-arginine metabolism and several neutral control mutants was screened in a C57BL/6 murine model of acute *S. Typhimurium* infection. This screen identified several critical "pinch points" in L-arginine biosynthesis, transport, and catabolism that impact the ability of *Salmonella* to establish an infection in the liver tissue of C57BL/6 mice (Fig. 1; Table S1). Deletions in the *argA* or *argG* genes, which are both required for *de novo* L-arginine biosynthesis, substantially decreased *Salmonella* fitness *in vivo*. In addition, analysis of the combined effect of deletions in *argCBH* arginine biosynthesis genes significantly ($P = 0.0077$) attenuated *Salmonella* in C57BL/6 mice. These data suggest that *de novo* arginine biosynthesis from α-ketoglutarate is necessary for *Salmonella* to maintain full virulence in the host. Our screen also identified L-arginine transport as an important determinant of bacterial survival in the host. *Salmonella* harboring a deletion in either *artP* or *artQ*, subunits of the L-arginine uptake system encoded by the *artPIQM-artJ* gene cluster, or in *argT*, the periplasmic binding protein of the *argT-hisJQMP* gene cluster (34), suffered impaired growth during infection. Additionally, deletion of a putative L-arginine/ornithine antiporter, *ydgI*, decreased bacterial survival in the host. Combined, the decreased fitness of this facultative intracellular pathogen in the absence of either L-arginine transport or biosynthesis suggests that L-arginine is a critical nutrient that promotes *Salmonella* survival in the host. Not only do our results complement the results of a previous study that showed that L-arginine transport-deficient *Salmonella* is attenuated in BALB/c mice (30), but our data also suggests that *Salmonella*'s L-arginine requirements during infection

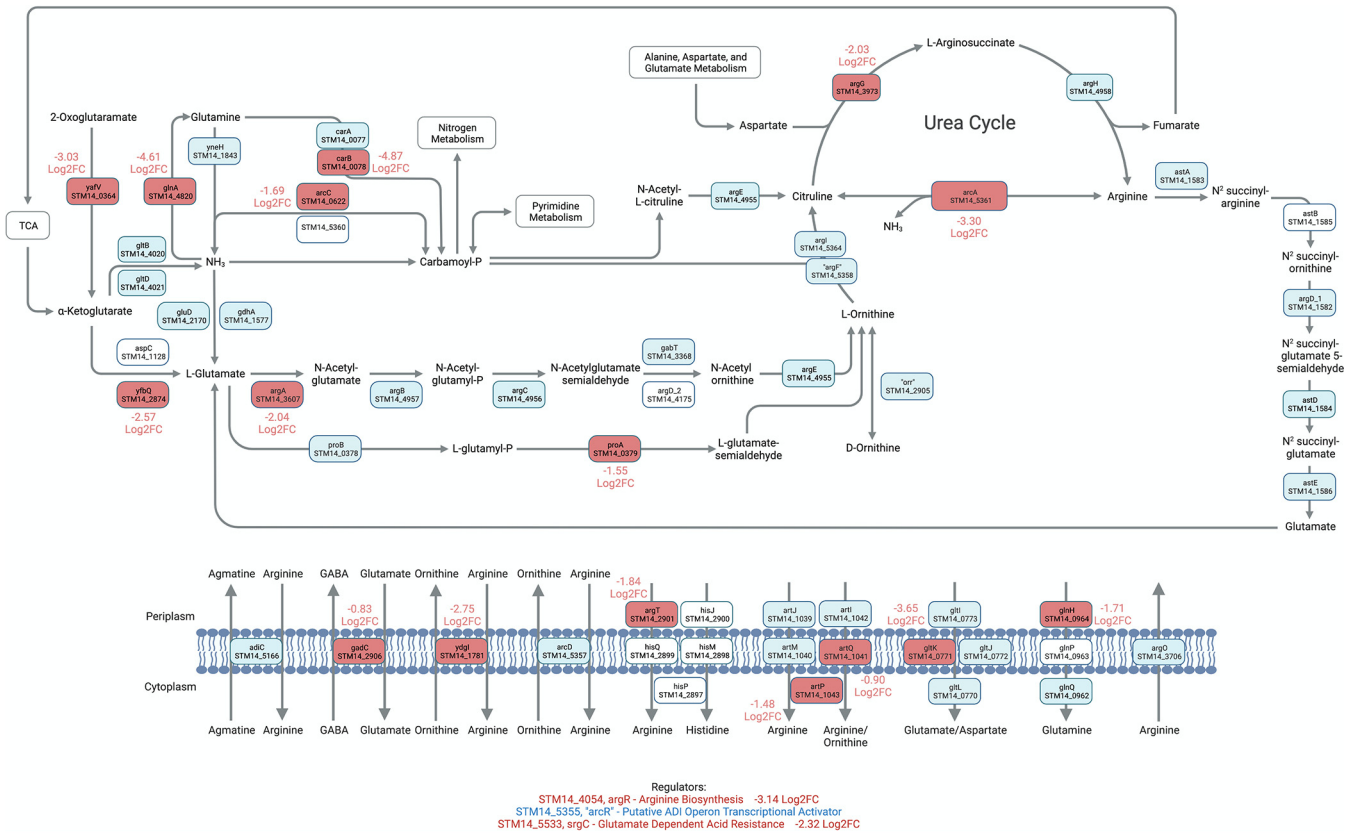

FIG 1 Significant hits from an arginine metabolism-specific single-gene KO library screen in mice. Five C57BL/6 mice were inoculated intraperitoneally with 3,000 CFU of a library containing barcoded deletion mutants of 54 arginine metabolism genes, and then livers and spleens were harvested 3 days after infection. Log_2 fold changes in barcode abundance were determined between the input and output libraries. Fitnesses with an adjusted *P*-value of less than 0.05 in both organs are marked. Color key: fitness defect (red), no difference (blue), not tested (no color). Figure created with BioRender.com.

cannot be maintained exclusively by transport from the host, but must also involve *de novo* synthesis.

Our screen revealed that L-arginine deiminase pathway genes, STM14_5361 (*arcA*) and *arcC*, were also important for *Salmonella* virulence in the C57BL/6 mouse model. The attenuation of the ΔSTM14_5361 and Δ*arcC* mutants in C57BL/6 mice is consistent with a prior study that showed that the L-arginine deiminase pathway contributes to the virulence of *Salmonella* in C3H/HeN mice (28). While the results of our *in vivo* screen indicate that both host-derived and *de novo*-synthesized L-arginine appear to be important for *Salmonella* fitness in the host, the fitness advantage conferred by the L-arginine deiminase pathway may reflect the eventual fate of these L-arginine pools. In light of these observations, the following investigations sought to characterize the mechanism by which L-arginine biosynthesis contributes to *Salmonella* virulence.

L-arginine metabolism enhances *Salmonella* oxidative stress resistance. To further analyze the role of L-arginine biosynthesis in *Salmonella* pathogenesis, we generated a deletion mutant deficient in the *argCBH* operon, which was identified in our screen as a critical component of *Salmonella* fitness *in vivo*. As expected from previous studies in related organisms (35), Δ*argCBH Salmonella* exhibited an auxotrophy for L-arginine as demonstrated by the inability of this mutant to grow in E salts minimal medium containing glucose (EG) as the only carbon source (Fig. 2a). The growth defect of Δ*argCBH Salmonella* in EG minimal medium was reversed upon complementation with the low copy plasmid pWSK29 expressing the *argCBH* operon from its native promoter or upon the addition of exogenous L-arginine (Fig. 2a).

To investigate whether L-arginine metabolism contributes to *Salmonella*'s resistance to oxidative stress, wild-type and Δ*argCBH Salmonella* were challenged with bacteriostatic concentrations (150 μM) of hydrogen peroxide in EG minimal media supplemented with

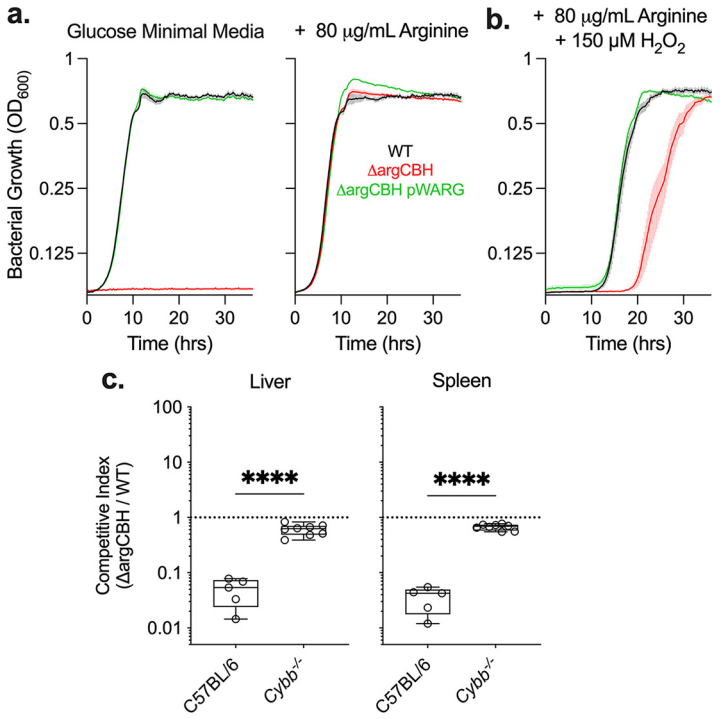

FIG 2 L-arginine biosynthesis is important during oxidative stress. (a) Aerobic growth of wild-type (WT) and Δ*argCBH* mutant *Salmonella* in EG minimal media in the presence or absence of 80 μg/mL supplemental L-arginine as measured by OD_{600} with a Biotek Synergy H1 plate reader. Data are shown as mean ± SEM (n = 2 to 6). (b) Aerobic growth of the indicated *Salmonella* mutants in the presence of 150 μM H_2O_2 in EG minimal media supplemented with 80 μg/mL L-arginine as measured by OD_{600} with a Biotek Synergy H1 plate reader. Data are shown as means ± SEM (n = 3 to 9). (c) Competitive index (CI) of the Δ*argCBH* mutant and WT *Salmonella* inoculated in competition into C57BL/6 and *Cybb*$^{-/-}$ mice. 250 CFU of each strain was inoculated intraperitoneally and then harvested 4.5 days after infection for C57BL/6 mice or 2.5 days after infection for *Cybb*$^{-/-}$ mice. Data were analyzed by unpaired, two-tailed t test with Welch's correction where ****, $P < 0.0001$. Box and whisker plots represent minimums to maximums, 25th and 75th percentiles, and medians. n = 5 for C57BL/6 and n = 8 for *Cybb*$^{-/-}$ mice.

80 μg/mL L-arginine (Fig. 2b). Despite sufficient L-arginine availability to fully restore the growth of the mutant to wild-type levels, Δ*argCBH Salmonella* took significantly longer than wild-type controls to recover from the H_2O_2 challenge. We also tested the virulence of Δ*argCBH Salmonella* by inoculating C57BL/6 mice with equal numbers of mutant and wild-type *Salmonella*. Δ*argCBH Salmonella* were at a competitive disadvantage for growth against wild-type controls in C57BL/6 mice. However, the L-arginine auxotroph was able to recover virulence in *Cybb*$^{-/-}$ mice lacking the membrane-bound gp91*phox* subunit of the phagocyte NADPH oxidase (Fig. 2c). Together, these data demonstrate that L-arginine biosynthesis plays a previously unappreciated role in *Salmonella's* defense against the ROS generated by phagocyte NADPH oxidase.

L-arginine metabolism mediates peroxide resistance in the presence of oxygen. To understand how L-arginine levels in *Salmonella* could impact resistance to ROS, an L-arginine re-feed protocol was utilized to probe the consequence of a low L-arginine supply in Δ*argCBH Salmonella* (Fig. S1). Briefly, mutant and wild-type *Salmonella* grown overnight in a limiting amount of exogenous L-arginine (20 μg/mL) were resuspended in fresh EG minimal medium that contained 80 μg/mL L-arginine, a nonlimiting concentration that promotes growth of the auxotroph Δ*argCBH Salmonella* (Fig. S2). Following a 30-min incubation period in L-arginine-containing media, bacterial cultures were then treated for an additional 30 min with 400 μM H_2O_2 in a shaking incubator. Using this approach, we uncovered that the L-arginine auxotroph is remarkably susceptible to the bactericidal effects of H_2O_2. Without functional L-arginine biosynthesis or sufficient intracellular L-arginine stores, the susceptibility of *Salmonella* to peroxide stress was so severe that within 30 min of peroxide exposure there was a 3-log reduction in the number of surviving Δ*argCBH Salmonella*

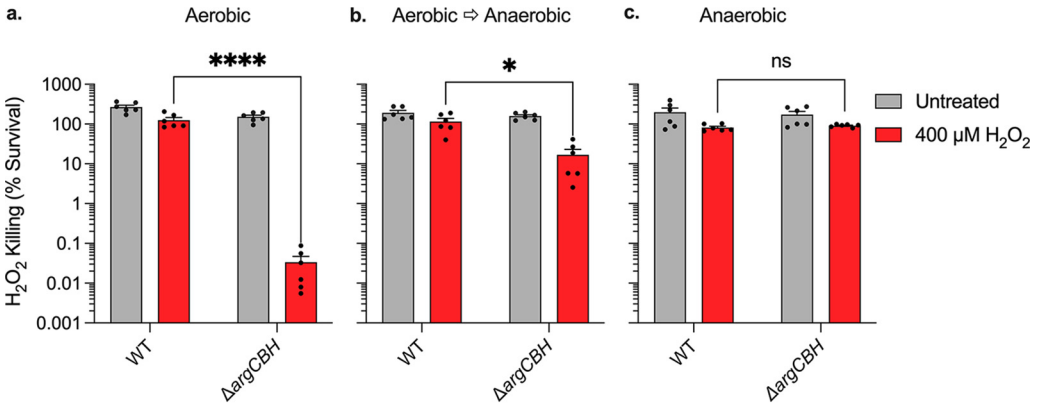

FIG 3 L-arginine biosynthesis-dependent peroxide killing is exacerbated in the presence of oxygen. Survival of wild-type (WT) and Δ*argCBH* mutant *Salmonella* grown overnight in EG minimal media supplemented with 20 μg/mL L-arginine after 30 min of treatment with 400 μM H_2O_2 in EG supplemented with 80 μg/mL L-arginine. (a) *Salmonella* in aerobic experiments were grown overnight and treated with H_2O_2 in the presence of oxygen. (b) *Salmonella* in aerobic to anaerobic shift experiments were grown overnight in the presence of oxygen and then treated with H_2O_2 under anaerobic conditions. (c) *Salmonella* in anaerobic experiments were grown overnight and treated with H_2O_2 under anaerobic conditions. Data are shown as means ± SEM (n = 6) and was analyzed by two-way ANOVA with Tukey's correction where *, $P < 0.05$; ****, $P < 0.0001$.

(Fig. 3a). In comparison, wild-type *Salmonella* completely resisted H_2O_2 killing under the experimental conditions used in these investigations. Building off prior research in *Salmonella* that showed that electron flux through the electron transport chain can dictate susceptibility to H_2O_2 (15), we employed the re-feeding assay to microaerobic and anaerobic cultures. When grown overnight in fully aerated conditions and then incubated under anaerobic conditions in fresh EG minimal medium for 30 min prior to treatment with H_2O_2, Δ*argCBH Salmonella* was partially resistant to the drastic killing seen in fully oxygenated media (Fig. 3b). In contrast, when both grown overnight and treated with H_2O_2 under anaerobic conditions, the hypersusceptibility of Δ*argCBH Salmonella* to peroxide stress was completely abolished (Fig. 3c). Combined, these observations suggest that L-arginine metabolism protects *Salmonella* against the toxic effect of ROS during periods of high electron flow through the electron transport chain.

L-arginine metabolism buffers *Salmonella*'s cytoplasm during oxidative stress. Expanding on these observations, we began to further dissect the molecular mechanisms that drive *Salmonella*'s reliance on L-arginine metabolism to resist oxidative stress. We uncovered that Δ*argCBH Salmonella* grown overnight in a limiting amount of L-arginine was hypersusceptible to the bactericidal effects of H_2O_2, regardless of the presence of L-arginine in the resuspension media (Fig. 4). In contrast, Δ*argCBH Salmonella* grown overnight in EG

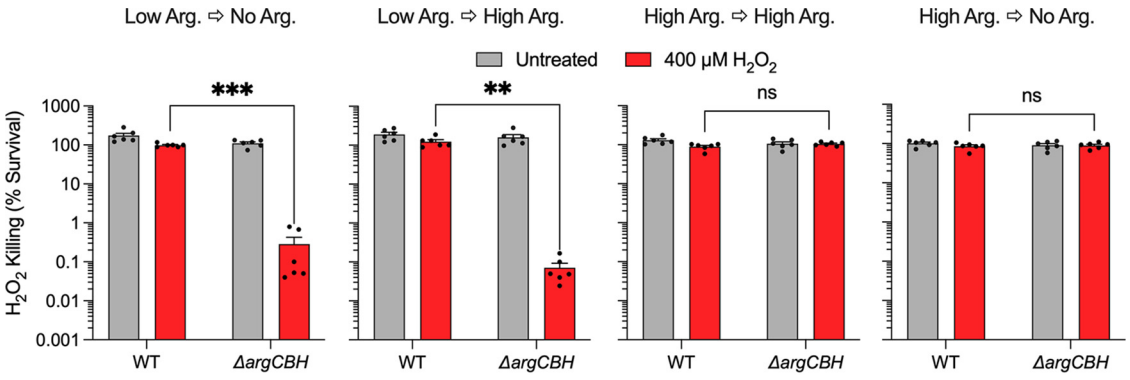

FIG 4 L-arginine levels help to maintain resistance to oxidative stress. *Salmonella* grown overnight in EG minimal media supplemented with either 20 μg/mL L-arginine (Low Arg.) or 80 μg/mL L-arginine (High Arg.) was resuspended in either EG media (No Arg.) or EG media with 80 μg/mL L-arginine (High Arg.) for 30 min prior to treatment. Susceptibility of wild-type (WT) and Δ*argCBH* mutant *Salmonella* to 30 min treatment of 400 μM H_2O_2 in EG media +/− L-arginine. Data are shown as means ± SEM (n = 6) and was analyzed by two-way ANOVA with Tukey's correction where **, $P < 0.01$; ***, $P < 0.001$.

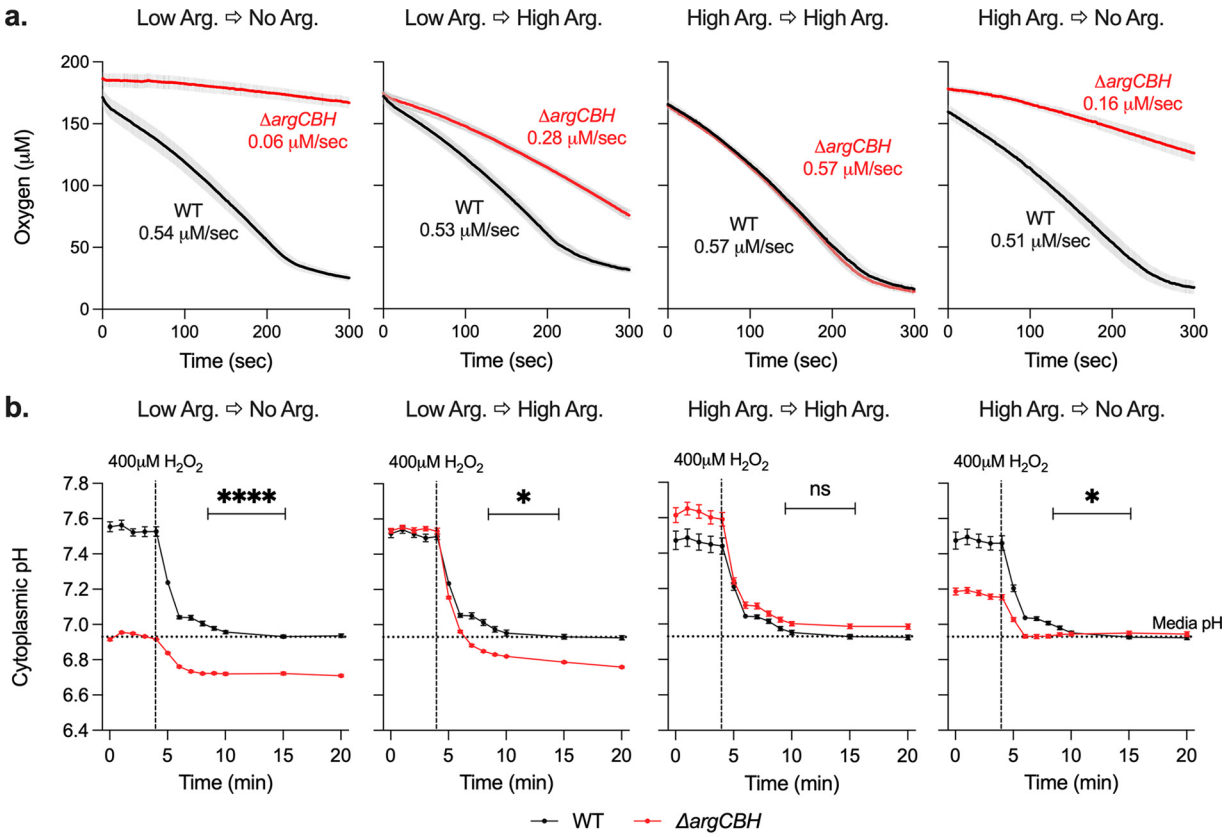

FIG 5 L-arginine levels help to maintain pH homeostasis during oxidative stress. All assays were performed using *Salmonella* grown overnight in EG minimal media supplemented with either 20 μg/mL L-arginine (Low Arg.) or 80 μg/mL L-arginine (High Arg.) and then resuspended in either EG media (No Arg.) or EG media with 80 μg/mL L-arginine (High Arg.) for 30 min prior to measurements. (a) Effect of supplemental L-arginine on respiration of wild-type (WT) and Δ*argCBH* mutant *Salmonella* in EG media +/− L-arginine. Aerobic respiration was measured polarographically. Data are shown as means ± SEM (n = 5). (b) Intracellular pH of wild-type (WT) and Δ*argCBH* mutant *Salmonella* measured ratiometrically with pHluorin before and after treatment with 400 μM H_2O_2 in EG media +/− L-arginine. Data are shown as means ± SEM (n = 6) and was analyzed by unpaired, two-tailed t test where *, $P < 0.05$; ****, $P < 0.0001$.

minimal medium that contained 80 μg/mL L-arginine was resistant to the bactericidal effects of H_2O_2, regardless of whether there was L-arginine in the resuspension media or not.

Increased respiratory activity has previously been associated with hypersusceptibility to oxidative stress (15). To understand if the concentration of L-arginine available to the auxotroph affected respiration levels, thus contributing to the increased susceptibility of Δ*argCBH Salmonella* to H_2O_2, we measured the oxygen consumption rate under each individual condition (Fig. 5a). Compared to wild-type controls, subculturing of Δ*argCBH Salmonella* grown in EG minimal medium containing 20 μg/mL or 80 μg/mL L-arginine into fresh EG minimal medium without L-arginine stopped respiratory activity. In contrast, moving Δ*argCBH Salmonella* from either EG minimal media containing 20 μg/mL or 80 μg/mL L-arginine into fresh EG minimal medium containing L-arginine did not cause any measurable arrests in aerobic respiration. Neither decreased nor increased respiratory capacity appeared to be predictive of susceptibility to peroxide stress. These results were surprising given that the susceptibility of Δ*argCBH Salmonella* to peroxide killing was greatly diminished under anaerobic conditions and that the sensitivity of *Salmonella* to oxidative stress is positively correlated with aerobic respiratory activity (15).

Given recent research that the killing activity of phagocyte NADPH oxidase collapses the cytoplasmic ΔpH of *Salmonella* (15) and the known role of L-arginine catabolism in maintaining pH homeostasis during acid stress (18, 19), we sought to measure the changes in intracellular pH during peroxide challenge in *Salmonella* grown in varying L-arginine concentrations (Fig. 5b). Compared to wild-type controls, the intracellular pH of Δ*argCBH Salmonella* grown overnight in 20 μg/mL of L-arginine was collapsed to media pH levels

after incubation in fresh EG minimal medium without L-arginine. However, when moving from 20 μg/mL to 80 μg/mL L-arginine media, Δ*argCBH Salmonella* and wild-type controls had identical intracytoplasmic pH values. Interestingly upon H_2O_2 treatment, Δ*argCBH Salmonella* grown in 20 μg/mL L-arginine suffered a rapid and complete collapse of the intracellular pH below the media pH, regardless of the presence of L-arginine in the fresh media or not. Moreover, Δ*argCBH Salmonella* grown overnight in EG minimal medium with 80 μg/mL L-arginine had a similar starting pH as wild-type controls when incubated in fresh media containing L-arginine, but a lower starting pH when incubated in fresh media without L-arginine. However, in striking contrast to the results of the 20 μg/mL L-arginine cultures, 80 μg/mL L-arginine cultures of Δ*argCBH Salmonella* were able to maintain their intracellular pH at or above media pH levels when challenged with H_2O_2. Combined, these data indicate that a collapse in intracellular ΔpH is more predictive of the susceptibility of Δ*argCBH Salmonella* to oxidative stress than changes in respiratory flux through the ETC.

DISCUSSION

L-arginine metabolism is an essential determinant of virulence in *Salmonella* (28–30). However, we are still lacking a mechanistic understanding of why L-arginine metabolism is important for *Salmonella* during infection. Herein, we demonstrate that *Salmonella* relies on L-arginine metabolism both *in vivo* and *in vitro* to resist the antimicrobial actions of ROS produced by the phagocyte NADPH oxidase. We show that increased L-arginine concentrations protect the pathogen from peroxide-induced cytoplasmic ΔpH collapse and subsequent killing. Thus, L-arginine metabolism contributes to the resistance of *Salmonella* to oxidative killing by preserving pH homeostasis.

Defects in L-arginine biosynthesis impair the virulence of diverse pathogenic bacteria in the host, despite the possession of functional L-arginine transport systems (36–39). Our study shows that Δ*argCBH S. Typhimurium* auxotrophic for L-arginine are attenuated for virulence in the host. The restoration of virulence of Δ*argCBH Salmonella* to wild-type levels in *Cybb*$^{-/-}$ mice that lack ROS generated by the phagocyte NADPH oxidase suggests that L-arginine biosynthesis plays a role in bacterial resistance to oxidative stress. Consistent with this observation, Δ*argCBH Salmonella* are hypersusceptible to both the bacteriostatic and bactericidal activity of exogenous H_2O_2. The dispensability of L-arginine biosynthesis for *Salmonella* virulence in the absence of oxidative stress demonstrates either a specific necessity for biosynthesis during oxidative stress or an overall higher L-arginine requirement during oxidative stress that cannot be met by transport alone. The hypersusceptibility of Δ*argCBH S. Typhimurium* to peroxide stress can be rescued if the bacterial cells are grown in higher concentrations of L-arginine (80 μg/mL) prior to challenge, which lends credence to the idea that *Salmonella* experiences high L-arginine requirements during the oxidative stress engendered in the innate response in C57BL/6 mice.

During periods of high electron flow through the ETC, the killing activity of the phagocyte NADPH oxidase acts through a collapse in the intracellular ΔpH (15). In response to ROS impairing the proton-pumping NDH-I isoform of NADH dehydrogenase, *Salmonella* shifts redox balance and ATP production from the ETC and oxidative phosphorylation to fermentation and substrate-level phosphorylation. Synthesis of acetate from acetyl-CoA helps *Salmonella* to maintain a ΔpH by consuming intracellular protons that accumulate in the absence of sufficient NDH-I activity. The work herein demonstrates that L-arginine metabolism must be considered as an additional component by which *Salmonella* maintain ΔpH during periods of oxidative stress. *Salmonella* possesses three different catabolic pathways that could be functioning to preserve pH homeostasis through either direct consumption of intracellular protons or production of ammonia to act as a proton sponge during oxidative stress: the L-arginine decarboxylase (ADC) pathway, the L-arginine deiminase (ADI) pathway, and the L-arginine succinyltransferase (AST) pathway. *Salmonella* bearing deletions in the inducible, degradative form of L-arginine decarboxylase (*adiA*) are known to be impaired in their ability to tolerate acidic pH (18, 21). Interestingly, research in *E. coli* has already demonstrated a role for *adiA* in resistance to both acid and oxidative stress

in vitro (31). Although the role of the ADI pathway in acid stress has not yet been evaluated in *Salmonella*, the ADI pathway is both active and critical for full virulence in mice (28). Additionally, work in *Staphylococcus epidermidis* has identified the importance of the ADI pathway in ameliorating endogenous oxidative stress conferred during acid stress (32). The second step in the AST pathway catalyzed by succinylarginine dihydrolase degrades the guanidino group of succinylarginine yielding succinylornithine and two ammonia. Based on the current literature, the likelihood of the AST pathway acting as a major pH homeostasis mechanism seems low as the pathway appears to mainly function as a mechanism to regenerate glutamate during nitrogen-limiting conditions (40). While either the ADI or ADC pathway could be the primary contributor to pH homeostasis during oxidative stress, the identification of the proton-consuming enzymes in the ADI pathway in our fitness screen may suggest that under *in vivo* conditions, the ADI pathway is more relevant than the ADC pathway.

In addition to considering that catabolism of L-arginine may be acting to directly preserve pH homeostasis during oxidative stress, other alternative or parallel mechanisms could explain the role arginine biosynthesis plays in *Salmonella* pathogenesis and resistance to oxidative stress. L-arginine may also serve as a source of nitrogen during infection, catabolized through the AST pathway to generate glutamate for diverse anabolic reactions such as the production of the antioxidant glutathione. However, this particular explanation seems unlikely given the absence of any fitness defect in AST pathway genes seen in our *in vivo* screen. While our results indicate a specific role for L-arginine biosynthesis during oxidative stress, the identification of L-arginine transporters in our fitness screen may suggest that L-arginine metabolism is also important for dampening the effects of reactive nitrogen species generated by inducible nitric oxide (NO) synthase (iNOS). Many pathogens have evolved strategies not only to detoxify NO, but also to divert and deplete host L-arginine to limit NO production (41). *Salmonella* upregulates both host arginase II (42) and host cationic amino acid transporters (30) during infection, facilitating a shift in host L-arginine catabolism away from iNOS and transport of host-derived L-arginine into the SCV. Stealing L-arginine from the host not only dampens NO production, but may also serve as a means to regulate T cell proliferation (43). Interestingly, amino acid transport mutants have been previously shown to exacerbate virulence defects tied to amino acid biosynthesis (44). Lastly, the role of L-arginine metabolism in *Salmonella* pathogenesis may be explained by the downstream formation of polyamines through the ADC pathway. Polyamines have been implicated in resistance to oxidative stress in diverse organisms (45); however, in *S. Typhimurium*, it appears that polyamines play only a minor role in oxidative stress resistance and are more important for defense against nitrosative stress (46). These alternative explanations are not incompatible with the arginine catabolism theory and could be functioning in parallel.

Respiratory flux may be functionally linked to resistance of *Salmonella* to oxidative stress, as strongly suggested by the fact that aerobically and anaerobically respiring *Salmonella* are more sensitive to peroxide killing than anaerobically fermenting cultures (15). *Salmonella* undergoing oxidative stress increases glucose consumption to fuel energetic and redox needs through a combination of substrate-level phosphorylation and aerobic respiration (16). Adding to these observations, we demonstrate that anaerobic fermentation can rescue hypersusceptibility to oxidative stress; however, our results also indicate that respiration alone is not fully predictive of increased sensitivity to oxidative stress. *Salmonella* that has stopped respiring after removal of L-arginine is not less sensitive to oxidative stress than actively respiring cells. What appears to be more predictive of hypersusceptibility to oxidative stress is the collapse of ΔpH associated with low levels of L-arginine. This observation is in line with the recently proposed model of oxidative killing by which a failure to maintain pH homeostasis and membrane energetics affects the redox status of proteins that rely on the ETC to catalyze the folding of periplasmic proteins (15). Inability to maintain pH homeostasis may also have profound effects on fundamental aspects of bacterial physiology, shifting the cytoplasmic pH into a range that is not compatible with enzymatic activity or transcription in general (13).

In summary, *Salmonella* L-arginine metabolism is an important determinant of pathogenesis during the selective pressure imposed by the phagocyte NADPH oxidase. However, in the absence of oxidative stress, L-arginine biosynthesis appears to be dispensable for *Salmonella* pathogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Wild-type *Salmonella enterica* subsp. *enterica* serovar Typhimurium strain 14028s and its mutant derivatives were used for all assays. The deletion mutant was constructed using the λ-Red homologous recombination system as previously described (47). The chromosomal deletion was confirmed by genotyping PCR with external check primers followed by Sanger sequencing to confirm the correct orientation of the antibiotic cassette. The complementation plasmid was generated by amplification of the gene of interest from the wild-type chromosome followed by ligation of the gene fragment into the low-copy number plasmid pWSK29 (48). The complementation plasmid was confirmed by Sanger sequencing with internal and external check primers. Strains were stored at −80°C in LB + 7% DMSO broth. See Tables S2 to S4 for the details of all strains and plasmids used in this study.

Cultures were routinely started from either a small amount of −80°C DMSO stock or a single colony, which was inoculated into the appropriate liquid growth medium and grown overnight (16 h to 20 h) aerobically at 37°C with shaking. The liquid growth media used for all assays was either Lysogeny broth (LB) or essential salts minimal media with glucose (EG) (1.7 mM $MgSO_4$, 9.5 mM citric acid, 57.4 mM K_2HPO_4, 16.7 mM H_5NNaO_4P, 0.4% d-Glucose, pH 7.0 unless otherwise noted). As appropriate, penicillin or chloramphenicol was added at final concentrations of 250 or 20 μg/mL, respectively. To determine CFU, bacterial cultures were serially diluted 10-fold in phosphate-buffered saline (PBS) and spread onto LB agar plates followed by growth at 37°C for 16 h.

Growth assays. *Salmonella* grown overnight in LB were diluted to 2.5×10^6 CFU/mL into fresh EG minimal media in the presence or absence of 80 μg/mL supplemental L-arginine. For growth assays in the presence of hydrogen peroxide, 150 μM H_2O_2 was added directly to the cultures after resuspension into fresh media. The optical densities at 600 nm (OD_{600}) of 200 μL aliquots of bacterial cultures were recorded with a Biotek Synergy H1 plate reader (Agilent, Santa Clara, CA) every 15 min for 40 h. The time (h) at which each culture reached an OD_{600} of 0.2 was calculated to determine statistical significance.

Ethics statement and murine model. All mice were used according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado Anschutz Medical Campus School of Medicine. C57BL/6J and congenic *Cybb*$^{-/-}$ mice deficient in the gp91*phox* membrane subunit of the phagocyte NADPH oxidase (49) originated from breeders obtained from The Jackson Laboratory. *Cybb*$^{-/-}$ mice were regularly verified by genotyping PCR against the C57BL/6J background controls. All mice were housed in static cages in groups of three to five mice per cage under specific pathogen-free conditions. During experiments, mice were housed in static cages in groups of three to five mice per cage under BSL2 and specific pathogen-free conditions. All experiments used 6- to 8-week-old male and female mice, and animals were assigned randomly to experimental groups. Animal subjects were not involved in any previous procedures prior to experimentation.

Library construction, sequencing, and analysis. The targeted ARG gene deletion library consisted of 54 specific single gene deletion mutants representing genes participating in *Salmonella's* arginine metabolism. Barcodes were introduced into the respective mutants of a previously published collection of Kanamycin resistant mutants (50) using Lambda-red recombination of a Tetracycline resistance cassette amplified from the *S. enterica* sv Typhimurium LT2 strain TT25401 (https://rothlab.ucdavis.edu/drugs/tetra.shtml). Recombination relied on extensive homology between the original mutants and the barcode-containing primers PCR_L1 and PCR_R1 in Table S3. The ARG pool also contained seven differently barcoded malXY mutants as neutral controls with no expected phenotypes.

Five mice were given an intraperitoneal injection of 3,000 CFU of the input pool. The input pools and output pools from spleens and livers were prepared for sequencing essentially as previously described (51). Briefly, bacteria were harvested and grown in LB + Tet, pelleted, washed with water, and lysed. The lysate was subsequently used as template for PCR using indexed Illumina sequencing primers that annealed directly, flanking both sides of the barcodes in primer PCR _L1. The frequency of each barcode was then determined by Illumina sequencing. Raw outputs were normalized to the median of the barcoded *malXY* neutral controls. Significance was determined using a two-tailed *t* test. The ratio of the medians of the organs and inputs were reported as $\log_2$ fold changes.

Murine competition assays. Mice were inoculated intraperitoneally (i.p.) with ~500 CFU of a mixture containing equal numbers (250 CFU strain 1 + 250 CFU strain 2) of two strains of *Salmonella* that were each grown separately overnight in LB. Mice were humanely euthanized 3 days after inoculation by CO_2 inhalation followed by cervical dislocation. Spleens and livers were harvested from each mouse and emulsified in 5 or 3 mL PBS, respectively. CFU were quantified by serial dilution onto LB agar plates containing the appropriate antibiotics. Plates were incubated for 16 h at 37°C prior to determining colony counts. Competitive index was calculated as follows: $([\text{strain 1/strain 2}]_{output}/[\text{strain 1/strain 2}]_{input})$.

Susceptibility to H_2O_2. *Salmonella* grown overnight in EG media with either 20 μg/mL or 80 μg/mL L-arginine were resuspended to an OD_{600} of 0.4 in 3 mL of either fresh EG media or EG media + 80 μg/mL L-arginine. Cultures were then allowed to equilibrate in the fresh media for 30 min at 37°C with shaking prior to taking a "time zero" sample for serial dilution onto LB agar. The cells were challenged by adding 400 μM H_2O_2 directly to the cultures and incubating for another 30 min at 37°C with shaking. The number of surviving

bacteria at "time 2" were quantified after serial dilution onto LB agar and following incubation at 37°C for 16 h. Percent survival was calculated as follows: ([CFU from "time 2"/CFU from "time zero"] × 100).

Polarographic O_2 measurements. Consumption of O_2 was measured using an ISO-OXY-2 O_2 sensor attached to an APOLLO 4000 free radical analyzer (World Precision Instruments, Inc., Sarasota, FL) as described in Husain et al. (52). Briefly, *Salmonella* cultures grown overnight in EG media with either 20 μg/mL or 80 μg/mL L-arginine were resuspended to an OD_{600} of 0.4/mL in either fresh EG media or EG media + 80 μg/mL L-arginine. Cultures were then allowed to equilibrate in the fresh media for 30 min at 37°C with shaking. Then, 3 mL of bacterial cultures were rapidly withdrawn, vortexed for 1 min, and immediately recorded for O_2 consumption. A two-point calibration for 0% and 21% O_2 was done as per manufacturer's instructions.

Intracellular pH determination. To estimate the intracytoplasmic pH of *Salmonella*, the pH-sensitive pHluorin plasmid driven from the PBAD promoter was used (53). *Salmonella* grown overnight in EG media with 0.2% L-arabinose and either 20 μg/mL or 80 μg/mL L-arginine were resuspended to an OD_{600} of 0.2 in 3 mL of either fresh EG media or EG media + 80 μg/mL L-arginine. Cultures were then allowed to equilibrate in the fresh media for 30 min at 37°C with shaking. The cultures were scored for fluorescence using a Shimadzu R5300C spectrofluorometer with an excitation wavelength scan from 300 to 490 nm and emission at 510 nm for 5 min. The cells were then treated with 400 μM H_2O_2 and the excitation spectra was measured for an additional 15 min. To determine the value of internal pH from the obtained fluorescence ratio (390/480 nm), pHluorin-expressing *Salmonella* were incubated in EG media + 80 μg/mL L-arginine pH 5.0 to 8.5 in the presence of protonophore potassium benzoate. A Boltzmann sigmoid best-fit curve was applied to the plot of fluorescence ratio and the known pH values obtained using the standards (Fig. S3).

Statistical analysis. All statistical analyses were performed using GraphPad Prism Version 9.0 for macOS (GraphPad Software, La Jolla California USA, www.graphpad.com). *P* values of < 0.05 (two-tailed) were considered statistically significant. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. For analyzing *in vivo* data, the unpaired, parametric, two-tailed *t* test was used. Each dot within the scatterplot represents data from one animal (biological replicate). Box and whisker plots represent minimums to maximums, 25th and 75th percentiles, and medians. For analyzing *in vitro* data, the two-way ANOVA with Tukey's correction was used. Each dot within the interleaved scatterplot represents one biological replicate (average of two technical replicates per biological replicate). Bars represent mean ± SEM.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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A.M. and A.V.-T designed and wrote the study; W.C. and S.P. made the 54 mutant library; A.M., L.L., and J.K.A.T. performed investigations; S.P. and M.M. analyzed data; A.M. and A.V.-T. provided funding.

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We declare no conflicts of interest.

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