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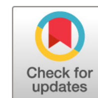

The Ethanolamine Permease EutH Promotes Vacuole Adaptation of *Salmonella enterica* and *Listeria monocytogenes* during Macrophage Infection

Christopher J. Anderson,[a] John Satkovich,[a] Volkan K. Köseoğlu,[a] Hervé Agaisse,[a] Melissa M. Kendall[a]

[a]Department of Microbiology, Immunology, and Cancer Biology, University of Virginia School of Medicine, Charlottesville, Virginia, USA

ABSTRACT Ethanolamine is a ubiquitous and essential molecule within a host. Significantly, bacterial pathogens exploit ethanolamine during infection to promote growth and regulate virulence. The ethanolamine permease EutH is dispensable for growth *in vitro* under standard conditions, whereas EutH is required for ethanolamine utilization at low pH. These findings suggested a model in which EutH facilitates diffusion of ethanolamine into the bacterial cell in acidic environments. To date, the ecological significance of this model has not been thoroughly investigated, and the importance of EutH to bacterial growth under physiologically relevant conditions is not known. During infection, immune cells internalize invading bacteria within an acidic, nutrient-depleted vacuole called the phagosome. Here, we investigated the hypothesis that EutH promotes bacterial survival following phagocytosis. Our findings indicate that EutH is important for survival and replication of the facultative intracellular pathogens *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* during prolonged or transient exposure to the phagosome, respectively. Furthermore, in agreement with EutH being important in the acidic environment, neutralization of the vacuole abolished the requirement for EutH. Significantly, consistent with a role for EutH in promoting intramacrophage survival, EutH was not required during *S*. Typhimurium local intestinal infection but specifically conferred an advantage upon dissemination to peripheral organs. These findings reveal a physiologically relevant and conserved role for EutH in spatiotemporal niche adaptation during infection.

KEYWORDS *Listeria*, *Salmonella*, ethanolamine, macrophage, pathogenesis, vacuole

Ethanolamine (EA) is a ubiquitous molecule within a host as a base constituent of phosphatidylethanolamine (PE), an abundant lipid in mammalian and bacterial cell membranes. Additionally, free EA is present within cells and in bodily fluids (1–5). In mammals, EA-containing lipids as well as EA itself are essential for health by modulating immune processes, energy balance, cell growth, regulated cell death pathways, and cardioprotection (4, 6–12). In bacteria, EA plays a dynamic role as a metabolite that promotes growth of pathogens during infection as well as a signal that modulates virulence (13–20). Genes encoding EA metabolism and signaling are carried in the EA utilization (*eut*) locus. *eut* loci have been identified in nearly 100 fully sequenced bacterial genomes and can be generally classified according to the number of genes within a respective locus (short versus long) (21). The short loci may contain only the genes encoding the EA-ammonia lyase EutBC, which catalyzes the first step in the breakdown of EA. In contrast, the long loci may also encode autoregulatory components, auxiliary proteins involved in EA catabolism, as well as genes encoding a microcompartment (22). Significantly, most short and long *eut* loci encode an EA permease—EutH or Eat (21). *In vitro* studies have shown that EutH and Eat are

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Address correspondence to Melissa M. Kendall, melissakendall@virginia.edu.

A *S*. Typhimurium

B *L. monocytogenes*

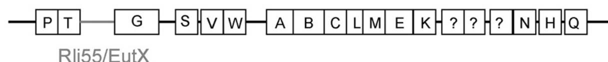


FIG 1 Schematic of the *eut* locus in (A) *S*. Typhimurium or (B) *L. monocytogenes*.

dispensable for bacterial growth *in vitro* under standard conditions (23–27), whereas EutH is required for EA utilization at low pH, suggesting a model in which EutH facilitates EA diffusion into the bacterial cell in acidic environments (25). To date, an ecologically relevant role for EutH in EA utilization and/or signaling has not been established.

Salmonella enterica serovar Typhimurium and *Listeria monocytogenes* are facultative intracellular pathogens that cause acute or mild gastroenteritis, respectively, and can cause invasive systemic disease in susceptible individuals. Importantly, both *S*. Typhimurium and *L. monocytogenes* exploit EA to promote pathogenesis (13, 18, 20, 28). In *S*. Typhimurium, the *eut* locus contains 17 genes, including the transcription factor EutR (23, 29, 30) (Fig. 1A). In response to EA and vitamin B_{12}, EutR directly activates *eut* expression to promote EA utilization (16, 29). In the intestine, *S*. Typhimurium exploits EA as a noncompetitive metabolite to sidestep nutritional competition from the microbiota and establish infection (13, 20). Subsequently, *S*. Typhimurium invades the epithelial barrier and penetrates to the lamina propria, where *S*. Typhimurium is internalized by macrophages. In the intramacrophage environment, EutR promotes *S*. Typhimurium survival and replication by directly activating expression of *ssrB* (13), which encodes SsrB, the master regulator of *Salmonella* pathogenicity island 2 (SPI-2) (31–33). SPI-2 encodes the type 3 secretion system 2 (T3SS-2) and effectors that convert the phagosome into a replicative niche called the *Salmonella*-containing vacuole (SCV) (34–37). Significantly, EutR-mediated regulation of SPI-2 during systemic infection is independent of EA metabolism (13), suggesting a dynamic role for EutR in *S*. Typhimurium host colonization. In *L. monocytogenes*, the *eut* locus contains at least 19 genes (Fig. 1B) that are regulated by a two-component system, EutVW, and a riboswitch-containing small RNA (sRNA), Rli55/EutX, that functions as an antiterminator (18, 38, 39). Similar to *S*. Typhimurium, EA metabolism also promotes *L. monocytogenes* host colonization, as disruption of *eutB* impacts growth of *L. monocytogenes* in HeLa cells as well as during systemic infection (18, 28). Although the disease progressions of *S*. Typhimurium and *L. monocytogenes* share common features, *L. monocytogenes* uses a distinct strategy for replicating within host cells. *L. monocytogenes* secretes the pore-forming toxin listeriolysin O (LLO) and the phospholipases C to escape the phagosome and replicate in the cytosol (40–44). Regardless of prolonged or transient exposure to the vacuole, *S*. Typhimurium and *L. monocytogenes* must be able to withstand the acidic, nutrient-limiting environment of the phagosome to cause disease. Here, we provide evidence that the EA permease EutH contributes to fitness of these pathogens during macrophage infection. Furthermore, in agreement with EutH being important in the acidic environment, neutralization of the vacuole abolished the requirement for EutH. Additionally, although EutH was dispensable during *S*. Typhimurium local intestinal infection, EutH specifically conferred an advantage during dissemination to peripheral organs. These findings reveal a conserved and physiologically relevant role for EutH in spatiotemporal niche adaptation during infection.

RESULTS AND DISCUSSION

EutH promotes *S*. Typhimurium survival with macrophages. *eutH* was originally identified as an open reading frame within the *eut* locus (27). Sequence analysis and

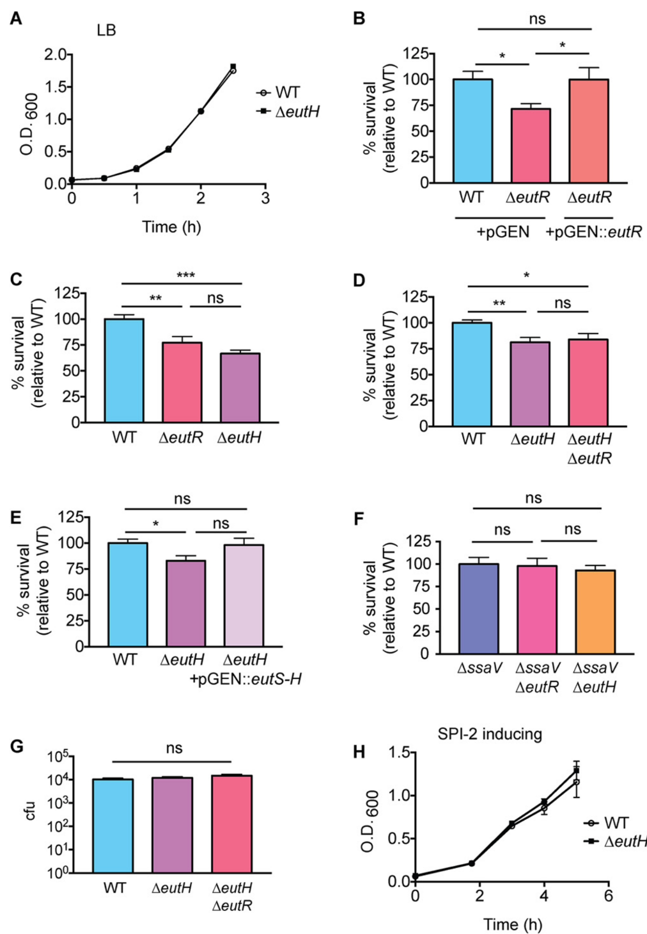


FIG 2 EutH promotes *S*. Typhimurium survival within macrophages. (A) *In vitro* growth curve of the WT *S*. Typhimurium (SL1344) and the Δ*eutH* (CJA052) strains grown in LB broth. Each data point shows the average from three biological replicates. Error bars represent the mean ± standard deviation (SD). (B) Intramacrophage survival and replication of WT (CJA034), Δ*eutR* (CJA032), or *eutR* complemented (CJA033) strains in peritoneal exudate macrophages after 7 h postphagocytosis. $n = 9$ replicates per strain. (C) Intramacrophage survival and replication of WT (AJK61), Δ*eutR* (CJA023), or Δ*eutH* (CJA043) *S*. Typhimurium strains in RAW macrophages after 5 h postphagocytosis. $n = 18$ replicates per strain. (D) Intramacrophage survival and replication of WT *S*. Typhimurium (AJK61), Δ*eutH* (CJA043), and Δ*eutH* Δ*eutR* (CJA168) strains after 5 h postphagocytosis in BMDMs. $n = 15$ replicates per strain. (E) Intramacrophage survival and replication of WT *S*. Typhimurium (CJA034), Δ*eutH* (CJA087), and *eutH* (CJA184) complemented strains after 5 h postphagocytosis in BMDMs. $n = 18$ replicates per strain. (F) Intramacrophage survival and replication of Δ*ssaV*, Δ*ssaV* Δ*eutR* (CJA064), and Δ*ssaV* Δ*eutH* (CJA172) *S*. Typhimurium strains after 5 h postphagocytosis in BMDMs. *ssaV* encodes an essential component of the T3SS-2, and the Δ*ssaV* strain is completely defective for T3SS-2-mediated secretion (48). $n = 9$ replicates per strain. (G) Initial phagocytosis of *S*. Typhimurium WT (AJK61), Δ*eutH* (CJA043), and Δ*eutH* Δ*eutR* (CJA168) strains at time zero (see Materials and Methods). $n = 24$ replicates per strain. (H) *In vitro* growth curve of the *S*. Typhimurium WT (SL1344) and Δ*eutH* (CJA052) strains grown in SPI-2 inducing medium. Each data point shows the average from three biological replicates. Error bars represent the mean ± SD. For panels B to G, the mean and standard error of the mean (SEM) are shown. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$. P values of >0.05 are not significant (ns).

structure predictions indicated that EutH was a hydrophobic protein containing at least six transmembrane helices, and thus EutH was hypothesized to function as a permease (27). Although deletion of *eutH* did not render *S*. Typhimurium unable to utilize EA *in vitro*, this deletion resulted in mild attenuation during intraperitoneal (i.p.) mouse infection, suggesting that EutH and EA were important for *S*. Typhimurium replication *in vivo* (27). Subsequent studies confirmed that *eutH* was dispensable for bacterial growth *in vitro* under standard conditions (23, 25) (Fig. 2A) but also showed that EA enters cells in a charge-dependent manner (25). These latter findings suggested that pH may influence the requirement for EutH in EA utilization (25). Consistent with this idea,

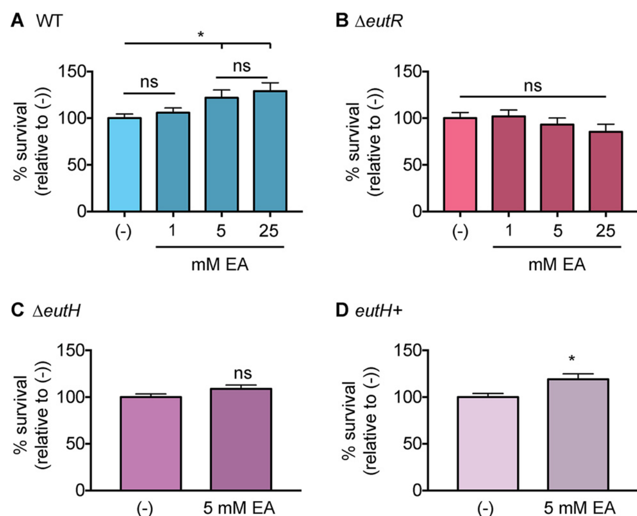


FIG 3 Bacterial survival following EA supplementation. (A) Intramacrophage survival and replication of WT *S*. Typhimurium (AJK61) after 5 h postphagocytosis in RAW macrophages without or with EA supplementation. $n = 9$. (B) Intramacrophage survival and replication of Δ*eutR S*. Typhimurium (CJA023) after 5 h postphagocytosis in RAW macrophages without or with EA supplementation. $n = 9$. (C) Intramacrophage survival and replication of Δ*eutH S*. Typhimurium (CJA043) after 5 h postphagocytosis in RAW macrophages without or with EA supplementation. $n = 9$. (D) Intramacrophage survival and replication of the *eutH* complemented strain (*eutH*+) of *S*. Typhimurium (CJA184) after 5 h postphagocytosis in RAW macrophages without or with EA supplementation. $n = 12$. For all values, the mean and SEM are shown. *, $P \leq 0.05$. *P* values of >0.05 are not significant (ns).

a Δ*eutH S*. Typhimurium strain was unable to grow on EA at low pH (with the addition of 20 or 41 mM EA to the culture medium) (25). These data suggested a model in which EutH facilitates EA diffusion into the bacterial cell in acidic environments (25), which we hypothesize includes the phagosome of macrophages. EA signaling through the transcription factor EutR promotes *S*. Typhimurium fitness within macrophages (Fig. 2B) (13); therefore, we examined the importance of EutH to *S*. Typhimurium survival during macrophage infection. In accordance with the proposed model, the Δ*eutH S*. Typhimurium strain was similarly attenuated compared to the Δ*eutR* or Δ*eutH* Δ*eutR* strains following infection of RAW macrophages or bone marrow-derived macrophages (BMDMs), respectively (Fig. 2C and D). Furthermore, *trans*-complementation with *eutS-H* expressed from the native *eut* P1 promoter restored survival of the Δ*eutH* strain within BMDMs to near-wild-type (WT) levels (Fig. 2E). In agreement with our previous findings (13), the macrophage survival phenotype was dependent on the T3SS-2 (Fig. 2F). Importantly, there were no differences in phagocytic uptake of the Δ*eutH* or Δ*eutH* Δ*eutR* strains compared to the WT (Fig. 2G) or during *in vitro* growth in a macrophage-like medium (SPI-2 inducing medium) (Fig. 2H).

As an alternative method to demonstrate the importance of EutH to EA-enhanced survival, we infected RAW macrophages and then supplemented the medium with EA following gentamicin treatment. The latter step ensured that the added EA was restricted to internalized bacteria. EA supplementation augmented WT *S*. Typhimurium survival within macrophages in a concentration-dependent manner (Fig. 3A). In contrast, EA addition did not affect survival of the Δ*eutR* and Δ*eutH* strains (Fig. 3B and C), and EA enhanced survival of the complemented Δ*eutH* strain (Fig. 3D). Collectively, these data indicate that EutH is required for EA-dependent survival and replication within macrophages.

Vacuole acidification drives the requirement for EutH. Uncharged EA freely diffuses across the bacterial membrane, whereas protonated EA cannot. The pH of the environment influences the ratio of uncharged to charged EA. At low pH, the protonated form of EA predominates, and EutH facilitates diffusion of EA into the bacterial cell (25). Therefore, to further test the proposed model that EutH mediates EA diffusion

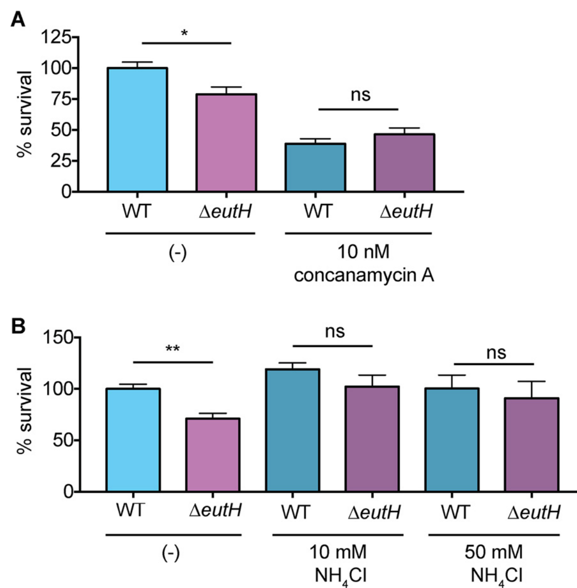
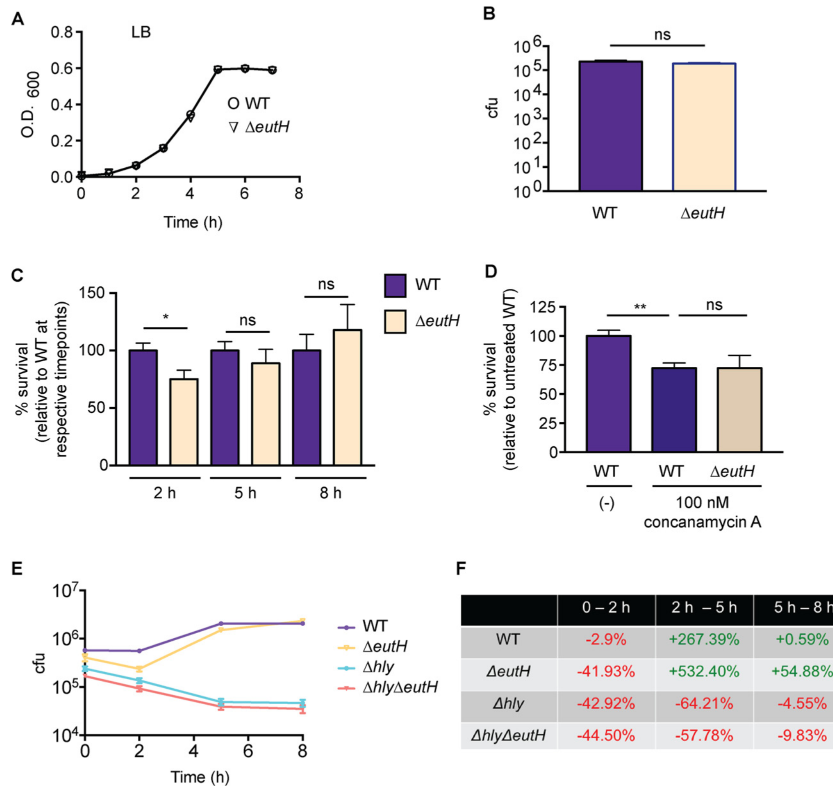


FIG 4 EutH enhances *S*. Typhimurium fitness in the acidified SCV. (A) Intramacrophage survival and replication of the WT (AJK61) or Δ*eutH* (CJA043) strain in BMDMs without or with concanamycin A treatment. $n = 9$ replicates per strain per condition. (B) Intramacrophage survival and replication of the WT (AJK61) or Δ*eutH* (CJA043) strain in BMDMs without or with NH_4Cl. $n = 9$ replicates per strain per condition. The mean and SEM are shown. *, $P \leq 0.05$; **, $P \leq 0.005$. P values of >0.05 are not significant (ns).

specifically at low pH, we examined how neutralization of the vacuole affected the requirement for EutH in enhancing *S*. Typhimurium survival within macrophages. For the initial experiments, BMDMs were treated with concanamycin A, an inhibitor of vacuolar ATPases that prevents acidification. Consistent with our previous data, in the untreated BMDMs, the Δ*eutH* strain was significantly less fit than the WT. However, after concanamycin A treatment, both strains were significantly attenuated compared to infection of untreated BMDMs and survived similarly to each other (Fig. 4A). Acidification of the SCV is required for expression of SPI-2 and formation of the T3SS-2 (45–50), and complete ablation of vacuole acidification with concanamycin A renders *S*. Typhimurium susceptible to macrophage killing (47). Thus, these data further underscore a role for EA signaling in enhancing T3SS-2-mediated survival and replication. Next, we repeated these experiments using NH_4Cl, a mild base. Treatment with NH_4Cl did not impact replication of WT *S*. Typhimurium during BMDM infection; however, neutralization using 10 or 50 mM NH_4Cl restored survival of the Δ*eutH* strain to nearly WT levels (Fig. 4B). These findings reveal that EutH contributes to *S*. Typhimurium intramacrophage survival and replication specifically in response to vacuole acidification.

EutH promotes *L. monocytogenes* vacuole adaptation. To examine whether EutH plays a more general role in bacterial fitness within the acidic environment, we assessed the requirement for EutH in *L. monocytogenes* during *in vitro* growth as well as during BMDM infection. Similar to *S*. Typhimurium, EutH did not impact *L. monocytogenes* growth *in vitro* (Fig. 5A) or uptake by macrophages (Fig. 5B). Although *L. monocytogenes* can begin to escape the vacuole within 30 min following internalization (51, 52), the majority of *L. monocytogenes* bacteria are contained within the vacuole during the first 2 to 3 h following uptake (53, 54) and then escape to the cytosol within 5 to 10 h (54–57). Therefore, we assessed the contribution of EutH to *L. monocytogenes* survival and replication at 2, 5, and 8 h postinfection (hpi) as time points reflective of vacuolar containment (2 h) and cytosolic replication (5 and 8 h). At 2 hpi, the Δ*eutH L. monocytogenes* strain was significantly attenuated compared to the WT (Fig. 5C). Correlating with increased access to the neutral, nutrient-replete cytosol (58), similar numbers of WT and Δ*eutH* cells were recovered as infection progressed (Fig. 5C). Consistent with the *S*. Typhimurium neutralization assays, treatment of macrophages



	0 – 2 h	2 h – 5 h	5 h – 8 h
WT	-2.9%	+267.39%	+0.59%
ΔeutH	-41.93%	+532.40%	+54.88%
Δhly	-42.92%	-64.21%	-4.55%
ΔhlyΔeutH	-44.50%	-57.78%	-9.83%

FIG 5 EutH contributes to survival of WT *L. monocytogenes* during macrophage infection. (A) *In vitro* growth curve of WT (10403S) and Δ*eutH* (VK01) *L. monocytogenes* strains grown in LB broth. Each data point shows the average from three biological replicates. Error bars represent the mean ± SD. (B) Initial phagocytosis of WT (10403S) and Δ*eutH* (VK01) *L. monocytogenes* strains at time zero (see Materials and Methods). (C) Intramacrophage survival and replication of WT (10403S) or Δ*eutH* (VK01) *L. monocytogenes* strains in BMDMs at the indicated times postphagocytosis. 2 hpi, $n = 18$ replicates per strain; 5 hpi, $n = 12$ replicates per strain; 8 hpi, $n = 9$ replicates per strain. For each time point, WT survival and replication were set to 100%. (D) Intramacrophage survival and replication of WT (10403S) or Δ*eutH* (VK01) *L. monocytogenes* strains in BMDMs without or with concanamycin A treatment at 2 h postphagocytosis. $n = 9$ replicates per strain per condition. (E) Intramacrophage CFU per milliliter of the WT (10403S), Δ*eutH* (VK01), Δ*hly* (DP-2161), or Δ*hly* Δ*eutH* (VK06) strains at time zero and 2, 5, and 8 h postphagocytosis. $n = 12$ to 18 replicates per strain per time point. (F) The percentage of change in intramacrophage survival and replication over time is calculated from data shown in panel E. Each percentage is indicative of the change between the two time points listed and was calculated as (later time point – earlier time point)/(earlier time point). For panels B to E, the mean and SEM are shown. *, $P \leq 0.05$; **, $P \leq 0.005$. P values of >0.05 are not significant (ns).

with concanamycin A ablated attenuation of the *eutH L. monocytogenes* strain in comparison to the WT strain (Fig. 5D).

LLO is essential for *L. monocytogenes* escape from the vacuole (43, 59). Therefore, to further interrogate the importance of EutH to *L. monocytogenes* fitness within the vacuole, we assessed time-dependent survival and replication of *L. monocytogenes* carrying a deletion of *hly* (that encodes LLO) in the WT or Δ*eutH* background strains. At 2 hpi, we again measured a significant decrease in bacterial recovery (CFU) of the Δ*eutH* strain compared to the WT, and no significant differences in CFU were measured at 5 or 8 hpi (Fig. 5E). Notably, the Δ*eutH*, Δ*hly*, and Δ*hly* Δ*eutH* strains displayed similar survival defects within the first 2 hpi (Fig. 5E and F). In agreement with previous studies (43, 60), our data show that the Δ*hly* strain did not replicate within the macrophages, and we did not measure any further attenuation in the context of the *eutH* deletion (Fig. 5E and F). A caveat to this experiment is that LLO functions in many aspects of *L. monocytogenes* pathogenesis (61), such as intracellular growth and survival within the phagosome (43, 60); therefore, further attenuation may not be detectable. Alternatively, EutH- and LLO-dependent survival may be functionally linked. Additional studies are needed to fully understand how EutH impacts *L. monocytogenes* survival within the

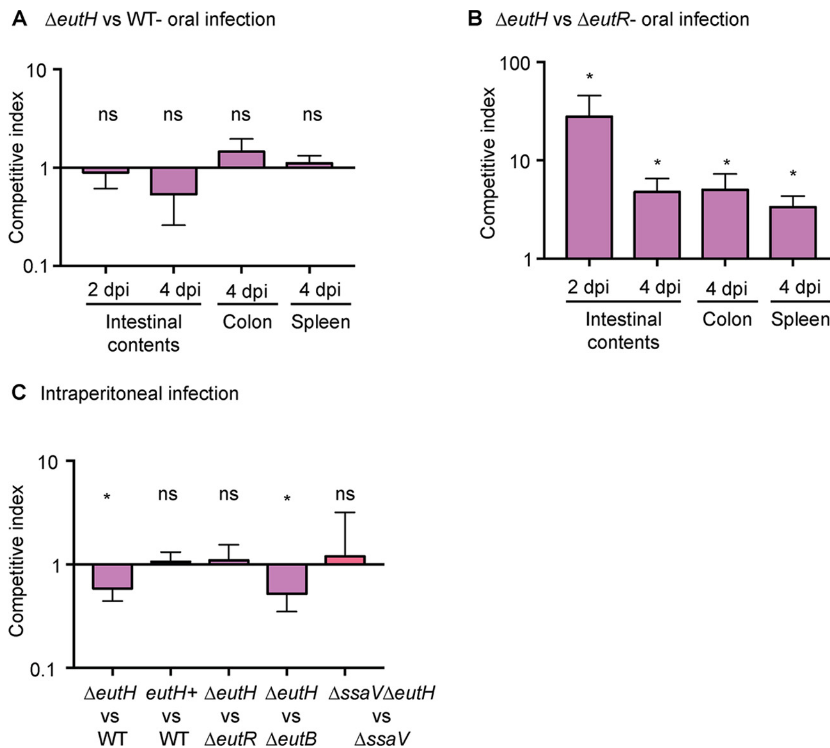


FIG 6 Contribution of EutH to *S*. Typhimurium fitness *in vivo*. (A) Competitive indices of the Δ*eutH* (CJA046) versus WT (SL1344) strain during colitis. $n = 7$ or 8 mice. (B) Competitive indices of the Δ*eutH* (CJA052) versus Δ*eutR* (CJA007) strain during colitis. $n = 8$ mice. (C) Competitive indices in the spleen of the Δ*eutH* (CJA046) versus WT (SL1344) strain, the Δ*eutH* complemented (CJA192) (*eutH*+, which contains pGEN::*eutS-eutH*) versus WT (CJA182, which contains pGEN empty vector) strain, the Δ*eutH* (CJA052) versus Δ*eutR* (CJA007) strain, the Δ*eutH* (CJA043) versus Δ*eutB* (CJA018) strain, and the Δ*ssaV* Δ*eutH* (CJA172) versus Δ*ssaV* strain at 6 hpi following intraperitoneal injection. $n = 8$ to 11 mice per competition. The median and interquartile range are shown *, $P \leq 0.05$. P values of >0.05 are not significant (ns).

phagosome. Nevertheless, these findings reveal a conserved role for EutH in pH-dependent vacuole adaptation in distinct intracellular pathogens.

EutH plays a spatiotemporal role in EA signaling during mammalian infection. The gastrointestinal (GI) tract and intramacrophage environments are primary niches for *S*. Typhimurium during host infection (62). The GI tract is characterized by neutral pH and millimolar concentrations of EA (20, 63, 64), indicating that during infection, EutH may play a niche-specific role in host adaptation. To investigate this idea, we performed competition experiments using *S*. Typhimurium-infected murine models of colitis and systemic infection (65). To examine the importance of EutH to *S*. Typhimurium intestinal colonization, we infected streptomycin-treated mice with equal numbers of cells of the WT and Δ*eutH* strains or with equal numbers of cells of the Δ*eutR* and Δ*eutH* strains and assessed bacterial burden in the intestinal contents as well as in the colon and spleen. The Δ*eutR* strain cannot metabolize EA, and this strain is attenuated for intestinal colonization (13). Equal numbers of cells of the WT and Δ*eutH* strains were recovered from intestinal contents at 2 and 4 days postinfection (dpi) as well as from the colon and spleen (4 dpi) (Fig. 6A), whereas the Δ*eutH* strain significantly outcompeted the Δ*eutR* strain under all conditions (Fig. 6B). These findings indicate that EutH is dispensable for EutR-dependent regulation of EA utilization during colitis.

To evaluate the role of EutH in *S*. Typhimurium dissemination, we intraperitoneally infected mice and assessed bacterial numbers recovered from the spleen at 6 hpi. Macrophages play a major role in *S*. Typhimurium dissemination, and in agreement with data shown in Fig. 2 to 4, the Δ*eutH* strain was recovered in significantly lower

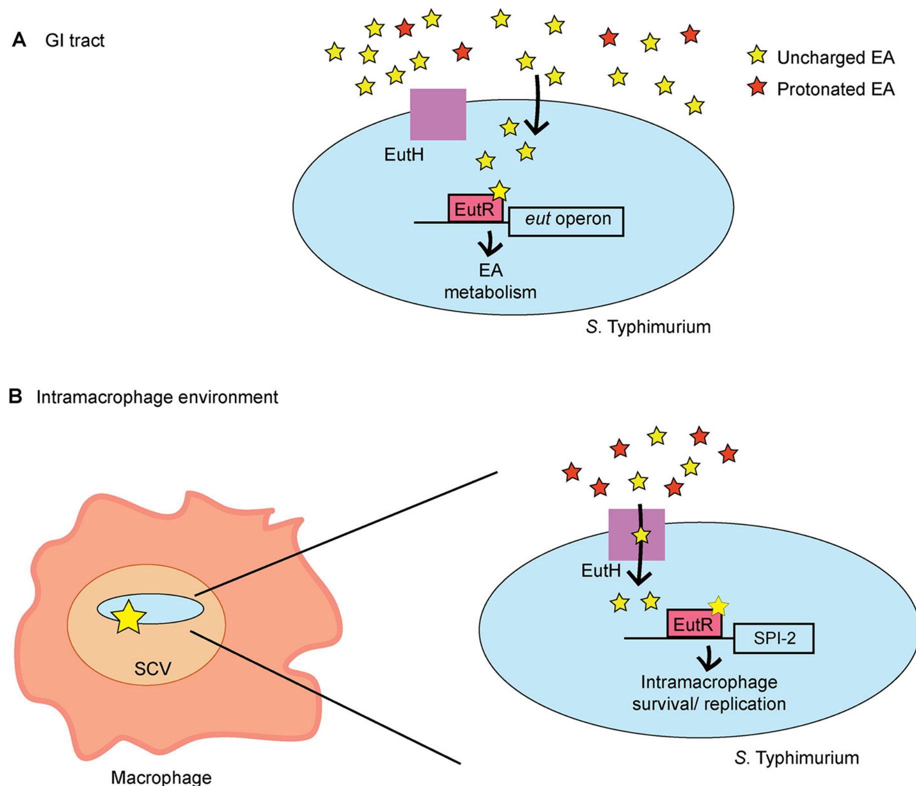


FIG 7 EutH plays a spatiotemporal role in *S.* Typhimurium niche adaptation. (A) EutH is dispensable for EA utilization in the intestine. (B) EutH contributes to EutR-dependent signaling and *S.* Typhimurium survival and replication during systemic infection.

numbers compared to the WT, and this defect could be complemented when *eutH* was expressed in *trans* (Fig. 6C). A previous study reported that an *eutH* deletion strain did not significantly impact dissemination (27); however, the experimental details were not described and may contribute to differences between these studies. Additionally, because the difference in recovery of the WT and Δ*eutH* strains was ~2-fold, we substantiated the importance of EutH to systemic infection by performing competition infections between the Δ*eutH* and Δ*eutR* strains as well as between the Δ*eutH* and Δ*eutB* strains. We previously reported that the Δ*eutR* strain was attenuated during early systemic infection compared to the WT and the Δ*eutB* strains as EutR regulates SPI-2 independently of EA metabolism (13). In agreement with a role for EutH in EutR signaling and macrophage survival, the Δ*eutH* strain was recovered in similar numbers compared to the Δ*eutR* strain and was significantly outcompeted by the Δ*eutB* strain (Fig. 6C). Moreover, equal numbers of the T3SS-2-deficient Δ*ssaV* and Δ*ssaV* Δ*eutH* strains were recovered, which further underscores the requirement of the T3SS-2 in EA-dependent dissemination (Fig. 6C). Collectively, these data demonstrate that EutH contributes to *S.* Typhimurium dissemination during infection. Significantly, the initial study that focused on EutH concluded with the idea that EutH is selectively maintained within bacterial genomes because *S.* Typhimurium (and presumably other bacteria) frequently encounters EA at concentrations or under pH conditions that limit the external unprotonated EA—conditions under which EutH is required to enhance the ability to utilize EA (25). Our findings are consistent with the original *in vitro* model proposed nearly 15 years ago and reveal a physiologically relevant role for EutH in spatiotemporal niche adaptation during infection (Fig. 7A and B).

Conclusions Following entry into host cells, the majority of intracellular pathogens are at least initially trapped within a host vacuole. Although bacterial strategies to withstand or evade immune defenses within the phagosome may be redundant,

many are specific to a particular pathogen (66). In contrast, host-specific signals that promote virulence programs and/or metabolic processes may be shared among diverse pathogens. Indeed, EA is a seemingly ubiquitous molecule within the host, and diverse pathogens rely on EA as a metabolite to support growth during host infection as well as co-opt EA as a signal to modulate virulence. Although a deletion of *eutH* does not ablate bacterial virulence, EutH consistently and reproducibly enhanced bacterial survival and replication within the phagosome and during *S.* Typhimurium systemic infection. Altogether, our findings demonstrate that the EA permease EutH contributes to the ability of distinct bacterial pathogens to survive within the acidified vacuole of macrophages.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *S.* Typhimurium cultures were routinely grown overnight in LB broth with antibiotics when appropriate. Antibiotics were used in the following concentrations: ampicillin (100 μg/ml), streptomycin (100 μg/ml), chloramphenicol (20 μg/ml), and kanamycin (50 μg/ml). SPI-2-inducing medium was prepared as described: 100 mM Bis/Tris-HCl (pH 7.0), 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 38 mM glycerol, 0.1% Casamino Acids, and 8 μM $MgCl_2$ (67). Strains of *L. monocytogenes* were routinely grown overnight in brain heart infusion (BHI) broth without antibiotics.

Deletions of *eutR* and *eutH* were constructed in the WT *S.* Typhimurium SL1344, *invG* mutant, or *ssaV* mutant backgrounds using λ-red mutagenesis (68) using primers *eutR*_λ-red F/R or *eutH*_λ-red F/R listed in Table S2 in the supplemental material. To create the nonpolar deletions, the chloramphenicol or kanamycin cassettes were resolved using pCP20 (68). Unresolved strains were used as indicated for competition experiments *in vivo*. The *invG eutH eutR* mutant strain (CJA168) was left unresolved, as subsequent use of pCP20 removed all genomic DNA between *eutH* and *eutR*. The *eutH* mutant was complemented with pCJA031. pCJA031 was constructed by amplifying *S.* Typhimurium genomic DNA with primers *eutH*_complement F/R, which are specific to the *eut* operon to include the native P1 promoter through *eutH* (listed in Table S2). Amplified PCR product was digested with NdeI and NotI and inserted into pGEN-MCS (69) (Addgene MTA). When appropriate, WT and deletion strains were transformed with empty pGEN-MCS vectors as controls.

An in-frame *L. monocytogenes* 10403S *eutH* deletion mutant strain and listeriolysin O mutant *hly eutH* deletion strain were generated as described previously (70) with modifications. Briefly, primer pairs Δ*eutH*.PA/Δ*eutH*.PB and Δ*eutH*.PC/Δ*eutH*.PD (Table S2) were used to amplify two 400-bp fragments upstream and downstream of *eutH*. Subsequently, an 800-bp deletion fragment was constructed via joining these 400-bp DNA fragments by overlap extension PCR with the Δ*eutH*.PA/Δ*eutH*.PD pair. The 800-bp deletion fragment was cloned into the pMAD vector after restriction digestion with EcoRI and BamHI (New England BioLabs) and ligation reactions with T4 ligase (New England BioLabs). The resulting pMAD::Δ*eutH* deletion construct was introduced into the *L. monocytogenes* 10403S and Δ*hly* strains via electroporation (71). Following electroporation, transformants were selected on BHI plates supplemented with 0.5 M sucrose, 10 μg/ml erythromycin, and 20 μg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) after 48 h of growth at 30°C. After reisolation on a BHI-erythromycin plate, blue colonies were picked for growth in BHI containing erythromycin at 39°C for 24 to 48 h. This culture was plated on BHI containing erythromycin and X-Gal and incubated at 39°C to isolate transformants that harbor the chromosomally integrated deletion construct. Blue colonies were used to start serial passages (1 per day) in BHI at 30°C to mediate excision of pMAD plasmid from the chromosome. After 3 to 5 passages, the temperature was shifted to 39°C and incubation carried out for an additional 5 h. Final cultures were plated on BHI–X-Gal plates and incubated at 39°C for 24 h. White colonies, which are cured of pMAD plasmid, were screened for erythromycin sensitivity. In-frame deletion mutants were identified from these white erythromycin-sensitive clones by PCR. Final verification of all deletions was performed by sequencing.

Animal experiments. All animal experiments were approved by the Animal Care and Use Committee at the University of Virginia. For all infections, 10- to 12-week-old, female C57BL/6 mice were used (Envigo). For the mouse colitis model, 24 h prior to infection, mice received a single dose via oral gavage of 20 mg streptomycin (72). Mice were infected via oral gavage with an equal mixture of 5×10^8 CFU of each of the indicated *S.* Typhimurium strains. Fresh fecal samples were collected daily, and mice were euthanized at 4 dpi to assess bacterial burden in the indicated tissues. Tissue samples were weighed and then homogenized in 1 ml phosphate-buffered saline (PBS), and bacterial burden was quantified by plating serial dilutions of homogenized tissue on MacConkey agar with streptomycin to obtain total bacterial CFU and MacConkey agar with chloramphenicol to obtain unresolved mutant CFU. The competitive index for each tissue was calculated as the ratio of indicated strains recovered from tissue normalized to the ratio in the inoculum.

For i.p. infections, mice were infected with an equal mixture of 5×10^4 CFU of each of the indicated *S.* Typhimurium strains. Spleens were collected at 6 h postinfection. Spleens were homogenized and processed and competitive infections calculated as described above. For complementation studies, samples were plated on LB agar with ampicillin to obtain total bacterial CFU and LB agar with ampicillin and chloramphenicol to obtain unresolved mutant CFU.

Tissue culture. RAW cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1× penicillin-streptomycin-glutamine. Peritoneal exudate macrophages (PEMs) from 8- to 12-week-

old C57BL/6 mice were isolated as described previously (13, 73). Bone marrow-derived macrophages (BMDMs) were isolated and cultured as described previously (73, 74). Briefly, 8- to 12-week-old C57BL/6 (*S.* Typhimurium infections) or BALB/c (*L. monocytogenes* infections) mice were euthanized, and bone marrow was harvested from femurs. The extracted bone marrow was incubated with 0.84% ammonium chloride solution at room temperature for 10 min to lyse red blood cells. Bone marrow cells were centrifuged at 1,500 rpm for 5 min and resuspended in the indicated medium. Additional medium was provided to cell cultures 3 days after harvest, and medium was replaced after 6 days of culture before cells were used in assays. PEMs and BMDMs were cultured in RPMI 1640 supplemented with 10% FBS, 20% L-929 conditioned medium, and 1× penicillin-streptomycin-glutamine. All cells were seeded into 12-well plates at 5×10^5 cells/well for experiments.

Gentamicin protection assays were performed as described previously (13, 35, 73–77). We used the *invG* mutant and indicated *eut* mutants for macrophage infections as invasive *S.* Typhimurium strains kill macrophages *in vitro* (76). Furthermore, expression of invasion-associated genes is decreased upon phagocytosis, and thus these strains more closely mimic *S.* Typhimurium encountering macrophages during systemic infection (78). Overnight cultures of *S.* Typhimurium or *L. monocytogenes* were washed and resuspended in PBS before incubation with macrophages at a multiplicity of infection (MOI) of 10. RAW cells were infected using DMEM supplemented with 10% heat-inactivated FBS without antibiotics. PEMs and BMDMs were infected using RPMI 1640 supplemented with 10% heat-inactivated FBS without antibiotics. After 30 min of incubation, extracellular bacteria were killed with 100-μg/ml gentamicin treatment for 30 min before replacement with medium containing 10 μg/ml gentamicin for the remainder of the assay. Cells were lysed at the indicated time points in 1% Triton X-100, and CFU were determined by serial dilutions and plating onto LB agar (*S.* Typhimurium) or BHI agar (*L. monocytogenes*). Percentage of survival was calculated as viable CFU at indicated time points as a percentage of CFU following 30 min of incubation with 100 μg/ml gentamicin (time zero) and normalized such that the wild type was equal to 100%.

For vacuole neutralization, BMDMs were incubated for 1 h prior to infection with the indicated concentration of concanamycin A (Sigma), dimethyl sulfoxide (DMSO) vehicle, or ammonium chloride (79). Following a 1-h pretreatment, cells were washed twice to remove concanamycin A or DMSO vehicle before continuing with a standard gentamicin protection assay as described above. Ammonium chloride was maintained in the medium throughout infection.

For ethanolamine supplementation to RAW cell infection, ethanolamine was added to culture medium at the indicated concentration following the 30-min incubation with 100 μg/ml gentamicin (time zero) until cells were lysed at 5 h postinfection.

Statistical analysis. Statistical significance of *in vivo* competitive indexes was determined by Wilcoxon's signed-rank test with a theoretical median of 1. Student's *t* test was used for the comparison of viable CFU in tissue culture experiments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00172-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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