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BACTERIAL INFECTIONS

Salmonella enterica Requires Lipid Metabolism Genes To Replicate in Proinflammatory Macrophages and Mice

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ABSTRACT To survive and replicate during infection, pathogens utilize different carbon and energy sources depending on the nutritional landscape of their host microenvironment. *Salmonella enterica* serovar Typhimurium is an intracellular bacterial pathogen that occupies diverse cellular niches. While it is clear that *Salmonella* Typhimurium requires access to glucose during systemic infection, data on the need for lipid metabolism are mixed. We report that *Salmonella* Typhimurium strains lacking lipid metabolism genes were defective for systemic infection of mice. Bacterial lipid import, β-oxidation, and glyoxylate shunt genes were required for tissue colonization upon oral or intraperitoneal inoculation. In cultured macrophages, lipid import and β-oxidation genes were required for bacterial replication and/or survival only when the cell culture medium was supplemented with nonessential amino acids. Removal of glucose from tissue culture medium further enhanced these phenotypes and, in addition, conferred a requirement for glyoxylate shunt genes. We also observed that *Salmonella* Typhimurium needs lipid metabolism genes in proinflammatory but not anti-inflammatory macrophages. These results suggest that during systemic infection, the *Salmonella* Typhimurium that relies upon host lipids to replicate is within proinflammatory macrophages that have access to amino acids but not glucose. An improved understanding of the host microenvironments in which pathogens have specific metabolic requirements may facilitate the development of targeted approaches to treatment.

KEYWORDS *Salmonella*, lipid metabolism, macrophage

Microbial pathogens encounter diverse niches throughout infection (1). Spatially, infection spreads to new tissues or cell types. Temporally, the innate immune response gives way to adaptive effectors, and infection may evolve into a chronic state. Within each unique pathogen niche, the availability of nutrients is influenced by factors such as the host metabolic state (2–4), the host inflammatory state (5–8), interactions with other microbes (9–11), and stochastic heterogeneity (12–14). Together, these factors define the nutritional microenvironment and influence pathogen replication, persistence, and transmission and the outcome of infection.

Salmonella enterica subspecies *enterica* serovar Typhimurium naturally infects mice and has been used to model the acute and chronic stages of human typhoid fever, which is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi and is responsible for 200,000 deaths globally each year (15). *Salmonella* Typhimurium is acquired through contaminated food or water and reaches the anaerobic small intestine, where it traverses the epithelial cell layer (16). The bacteria are taken up by and replicate within professional phagocytes, which facilitate the colonization of tissues, including Peyer's patches, mesenteric lymph nodes, spleen, and liver (17). *Salmonella* Typhimurium has been observed in humans and animals within lipid-rich macrophages that accumulate during systemic infection (18–21). In addition, the bacteria likely encounter many types of macrophages over the course of infection, including those that are

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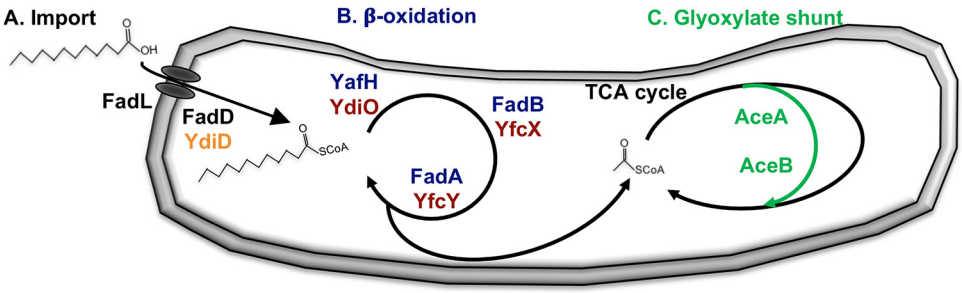

FIG 1 Schematic of lipid metabolism pathways in *Salmonella* Typhimurium. (A) Long-chain free fatty acids are imported via FadL (outer membrane translocase) and activated by FadD or YdiD (acyl-CoA synthetases). Canonical import genes are black; putative secondary import gene is orange. (B) Resulting acyl-thioesters are serially β-oxidized via YafH or YdiO (acyl-CoA dehydrogenases), FadB or YfcX (3-hydroxyacyl-CoA dehydrogenases), and FadA or YfcY (3-ketoacyl-CoA thiolases), producing acetyl-CoA, which is metabolized via the TCA cycle. Canonical β-oxidation genes are blue; putative secondary β-oxidation genes are red. (C) The glyoxylate shunt (green) consists of AceA (isocitrate lyase) and AceB (malate synthase), which bridge the TCA cycle to preserve carbon and cofactors during growth in the absence of glycolytic energy sources.

polarized toward either a pro- or anti-inflammatory state (5, 22, 23). How the pathogen may exploit these different cellular environments is not clear.

Salmonella Typhimurium has been demonstrated to require access to glucose during systemic infection (24), but data on its use of lipids are mixed. Some studies of mutant strains lacking key enzymes in lipid metabolism report no *in vivo* defect (25–27), but others demonstrate attenuation (28, 29). While bulk expression profiling and proteomics do not point to a significant induction of lipid metabolism genes during infection of macrophages or mice (30–33), recent single-cell work with fluorescent reporters and metabolomics suggests some bacteria use lipids (34, 35). We examined the requirement for *Salmonella* Typhimurium lipid uptake, β-oxidation, and glyoxylate shunt genes in mice and in polarized macrophages. We found that the nutritional environment of the macrophage, specifically access to amino acids and glucose, dictates whether bacteria require lipid metabolism genes to replicate and/or survive. Moreover, we determined that that *Salmonella* Typhimurium uses lipid metabolism genes within proinflammatory, but not anti-inflammatory, macrophages.

RESULTS

Deletion of *Salmonella* Typhimurium lipid metabolism genes abrogates growth on lipids. To verify the requirement for *Salmonella* Typhimurium lipid metabolism genes, we examined growth in broth (see Fig. S1 and S2 in the supplemental material) or on plates (see Table S1 in the supplemental material). *Salmonella* Typhimurium fatty acid degradation involves multiple steps, some of which can be executed by gene products encoded by either a canonical or a secondary lipid metabolism pathway (36–38). First, medium- and long-chain fatty acids are transported across the outer membrane and activated by the addition of coenzyme A (CoA) to produce acyl-CoA (Fig. 1A). We found that the fatty acid import genes *fadL* and *fadD* were required for growth on oleic acid as a sole carbon source, but the *Salmonella* Typhimurium paralog of *fadD*, denoted *ydiD*, was not. In *Escherichia coli*, *ydiD* contributes to anaerobic growth (39). However, our data showed that *fadL* and *fadD*, but not *ydiD*, were required for *Salmonella* Typhimurium anaerobic growth on lipids (Table S1). These defects were complemented by a plasmid containing the target gene (Fig. S1 and S2; Table S1).

In the second step of lipid metabolism, acyl-CoA is oxidized by a series of enzymes to produce acetyl-CoA, which enters the tricarboxylic acid (TCA) cycle (37, 40) (Fig. 1B). As in *E. coli* (41, 42), *Salmonella* Typhimurium carries both canonical (*yafH*, *fadB*, and *fadA*) and secondary (*ydiO*, *yfcX*, and *yfcY*) β-oxidation pathways. For growth on lipids, *Salmonella* Typhimurium required the secondary pathway under anaerobic conditions. In the presence of oxygen, *Salmonella* Typhimurium relied largely upon the canonical pathway (Fig. S1; Table S1).

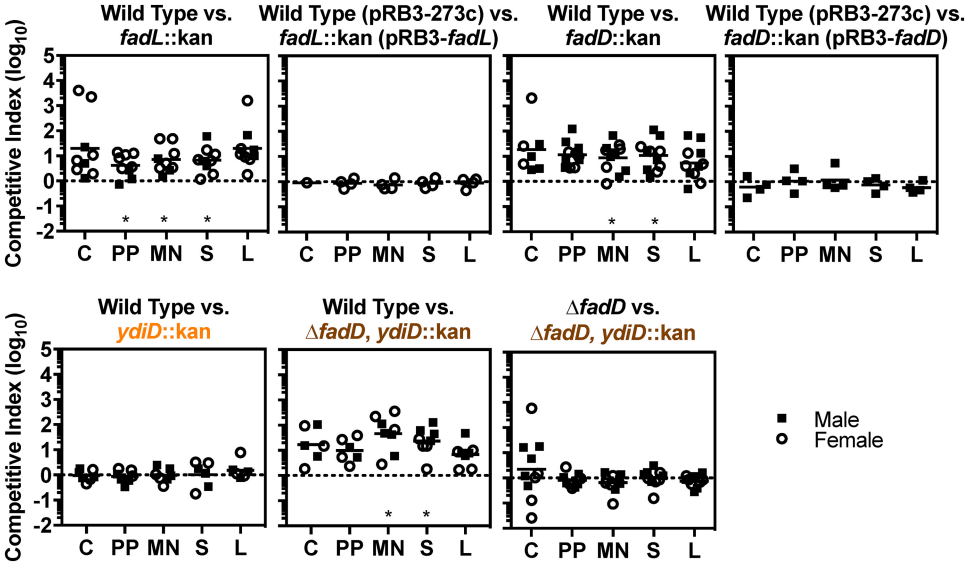

FIG 2 Lipid import genes are needed for *Salmonella* Typhimurium to competitively colonize mouse tissues. Mice were orogastrically inoculated with equivalent numbers of wild-type or mutant bacteria, as determined by CFU (1×10^9 or 2×10^9 [Δ*fadD* versus Δ*fadD, ydiD::kan* experiment only]). After 2 weeks, the following tissues were harvested to enumerate CFU: cecum (C), Peyer's patches (PP), mesenteric lymph nodes (MN), spleen (S), and liver (L). The competitive index was calculated as the ratio of the indicated strains at harvest divided by the ratio of the inoculum. Each symbol represents a tissue from one mouse. *, $P < 0.05$ by one-sample *t* test.

During the third step of lipid metabolism, the glyoxylate shunt pathway feeds the acetyl-CoA derived from β-oxidation into a truncated version of the TCA cycle (Fig. 1C). When lipids are the sole carbon source, the glyoxylate shunt is needed to preserve carbon for incorporation into biosynthetic pathways. The glyoxylate shunt consists of two enzymes encoded in an operon, isocitrate lyase (*aceA*) and malate synthase (*aceB*) (43). As anticipated, nonpolar deletions of either gene eliminated aerobic and anaerobic growth on oleic acid as a sole carbon source, and these defects were complemented by a plasmid containing the target gene (Fig. S1 and S2; Table S1).

***Salmonella* Typhimurium requires lipid degradation genes for orogastric infection of mice.** Several groups have found differing requirements for *Salmonella* Typhimurium lipid metabolism during infection. However, many of these studies were conducted in mouse strains that develop acute lethal infection due to a genetic lesion in innate immunity (25–29). To establish whether *Salmonella* Typhimurium requires lipid metabolism genes in immunocompetent mice, we performed mixed inoculations in Sv129S6/SvEvTac (Sv129) mice, which typically survive infection (44). Mice were orogastrically infected with equivalent numbers of wild-type and lipid-metabolism mutant bacteria and euthanized 2 weeks after infection, when bacteria have disseminated systemically and begun transitioning to a chronic infectious state (5, 19, 44). Tissues were harvested to enumerate CFU, and the competitive index was calculated as the ratio of recovered wild-type to mutant CFU, normalized to the ratio in the inoculum. Strains lacking the canonical import gene *fadL* or *fadD* were attenuated in competition with wild type (Fig. 2). Complementation of each of these strains with a plasmid containing the target gene restored colonization to wild-type levels. A strain lacking *ydiD*, the secondary paralog of *fadD*, competed equivalently with wild type, and loss of *ydiD* did not further attenuate a *fadD* mutant. These data indicate that *ydiD* is dispensable and that the canonical import genes *fadL* and *fadD* contribute to infection. Thus, *Salmonella* Typhimurium lipid uptake and activation are needed for tissue colonization in mice.

Having shown that lipid import is required for *Salmonella* Typhimurium colonization of tissues, we asked whether β-oxidation is required. In mixed infection experiments, strains lacking a single gene or operon from either the canonical or secondary β-oxidation pathway competed equivalently with wild type (Fig. 3). However, strains lacking genes

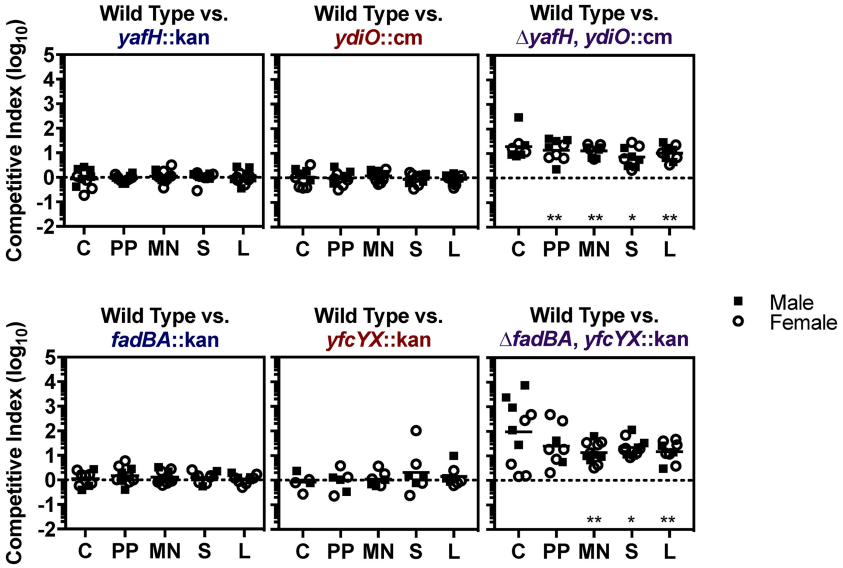

FIG 3 β-Oxidation genes are needed for *Salmonella* Typhimurium to competitively colonize mouse tissues. Mice were orogastrically inoculated with equivalent numbers of wild-type or mutant bacteria (1×10^9 CFU). After 2 weeks, the following tissues were harvested to enumerate CFU: cecum (C), Peyer's patches (PP), mesenteric lymph nodes (MN), spleen (S), and liver (L). The competitive index was calculated as the ratio of the indicated strains at harvest divided by the ratio of the inoculum. Each symbol represents a tissue from one mouse. *, $P < 0.05$; **, $P < 0.01$ by one-sample *t* test.

encoding both cognate enzymes were attenuated. Thus, the canonical and secondary β-oxidation pathways are redundant during infection, and β-oxidation of lipids is necessary for tissue colonization.

Since both lipid import and β-oxidation contribute to *Salmonella* Typhimurium colonization of tissues, we addressed whether the incorporation of acetyl-CoA into the TCA cycle via the glyoxylate shunt is also important. Mutant strains lacking either glyoxylate shunt pathway gene were outcompeted by wild type in multiple tissues, a phenotype complemented by the corresponding gene on a plasmid (Fig. 4). Therefore, in some situations, lipids may be the only carbon source available to *Salmonella* Typhimurium during infection.

Lipid degradation genes are required for infection when *Salmonella* Typhimurium bypasses the gastrointestinal tract. Prior to dissemination to the spleen and liver, orogastric inoculation exposes *Salmonella* Typhimurium to the intestinal tract. Fatty acids within the intestine alter the invasion of strains with defects in lipid

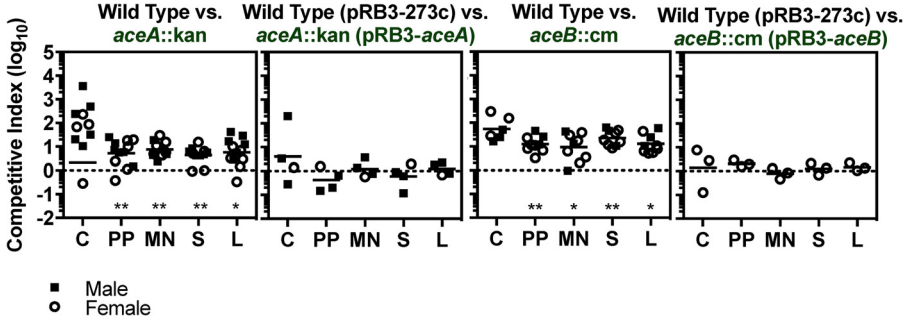

FIG 4 Glyoxylate shunt genes are needed for *Salmonella* Typhimurium to competitively colonize mouse tissues. Mice were orogastrically inoculated with equivalent numbers of wild-type or mutant bacteria (1×10^9 CFU). After 2 weeks, the following tissues were harvested to enumerate CFU: cecum (C), Peyer's patches (PP), mesenteric lymph nodes (MN), spleen (S), and liver (L). The competitive index was calculated as the ratio of the indicated strains at harvest divided by the ratio of the inoculum. Each symbol represents a tissue from one mouse. *, $P < 0.05$; **, $P < 0.01$ by one-sample *t* test.

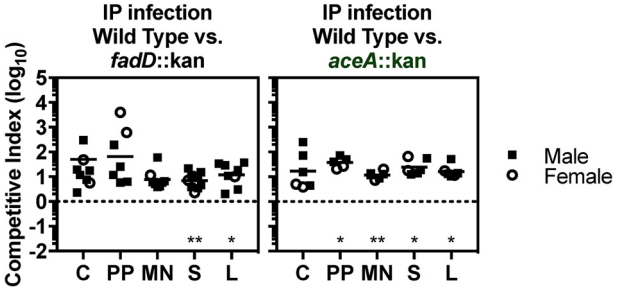

FIG 5 Upon intraperitoneal inoculation, lipid metabolism genes are needed for *Salmonella* Typhimurium to competitively colonize mouse tissues. Mice were intraperitoneally inoculated with equivalent numbers of wild-type or mutant bacteria (1×10^3 CFU). After 2 weeks, the following tissues were harvested to enumerate CFU: cecum (C), Peyer's patches (PP), mesenteric lymph nodes (MN), spleen (S), and liver (L). The competitive index was calculated as the ratio of the indicated strains at harvest divided by the ratio of the inoculum. Each symbol represents a tissue from one mouse. *, $P < 0.05$; **, $P < 0.01$ by one-sample *t* test.

metabolism and regulate the expression of *Salmonella* Typhimurium genes required for systemic infection (45–48). Therefore, we asked whether *Salmonella* Typhimurium requires lipid metabolism genes when mixed inoculations are delivered into the intraperitoneal cavity, a method that allows *Salmonella* Typhimurium to bypass the gastrointestinal tract and become rapidly engulfed by resident peritoneal macrophages (17). We found that mutant strains lacking *fadD* or *aceA* colonized tissues poorly compared with the wild-type (Fig. 5). These data indicate that *Salmonella* Typhimurium requires lipid metabolism genes to colonize tissues irrespective of passage through the gastrointestinal tract.

In macrophages cultured with nonessential amino acids, *Salmonella* Typhimurium requires lipid import and beta oxidation to replicate. A cell culture model of *Salmonella* Typhimurium lipid utilization would enable an examination of the intracellular niche(s) in which the bacterium uses lipids. Therefore, we infected bone marrow-derived macrophages (BMDMs) from Sv129 mice or RAW264.7 cell line macrophages and monitored bacterial load after 18 h. Two methods were used to quantify bacterial load, namely, standard plating for CFU and a flow cytometry-based fluorescence dilution assay (49). We also compared standard medium containing glucose (4.5 mg/ml) to glucose-free medium, with or without oleic acid, as bacteria may exploit the accumulated lipid droplets (50) (see Fig. S3 in the supplemental material). No differences in replication were observed between the wild type and mutant (see Fig. S4 in the supplemental material), indicating that under these conditions, *Salmonella* Typhimurium lipid metabolism genes are dispensable.

Since we observed a *Salmonella* Typhimurium need for lipid metabolism genes in mice but not macrophages, we hypothesized that a modified tissue culture medium may be required. A recent study demonstrated that RAW264.7 cells grown with nonessential amino acids (NEAAs) support *Salmonella* Typhimurium expression of the glyoxylate shunt operon, specifically, an *aceBA-gfp* reporter (34). We were not growing the macrophages under conditions similar to those *in vivo*. A recent study reported that RAW264.7 cells grown with NEAA restrict the replication of a *Salmonella* Typhimurium strain lacking a lipid uptake gene (*fadD*) (34). We found that primary and cell culture macrophages supplemented with NEAAs reduced the replication and/or survival of *Salmonella* Typhimurium lacking lipid import genes (*fadL* and *fadD*) or the canonical β-oxidation pathway (*fadBA* and *yafH*) (Fig. 6A and B; see Fig. S5A and B in the supplemental material). The presence of NEAAs also reduced the percentage of mutant bacteria that replicated (Fig. 6C; Fig. S5) but not the number of times replicating bacteria doubled (Fig. 6D; Fig. S5D). These results suggest that macrophage access to amino acids creates an intracellular environment in which a fraction of intracellular *Salmonella* Typhimurium import and oxidize lipids to replicate and/or survive. In the remainder of experiments, we supplemented the tissue culture media with NEAAs.

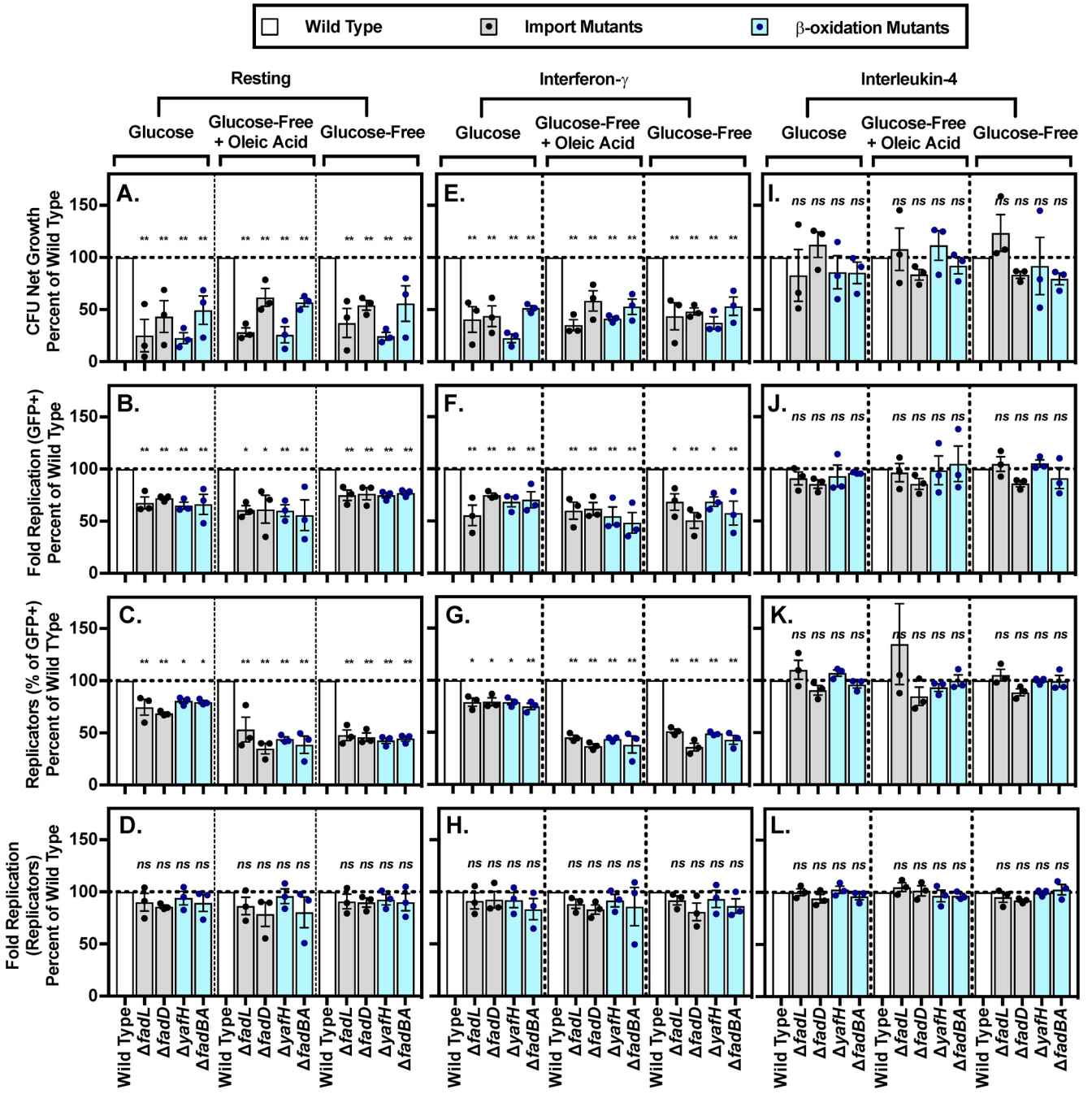

FIG 6 Supplementation of macrophage media with NEAA reveals requirement for lipid import and β-oxidization genes in resting and interferon-γ-pretreated BMDMs. BMDMs were transferred into defined medium with the indicated carbon source for 18 to 24 h prior to infection with the indicated strains. Net growth was calculated as CFU at 18 h postinfection divided by CFU at 2 h postinfection (A, E, I). Fluorescence dilution was used to calculate fold replication for all GFP+ bacteria (B, F, J), percentage of GFP-low replicating bacteria (C, G, K), and fold replication of the GFP-low population of replicating bacteria (D, H, L). GFP-low replicating bacteria were gated based on the fluorescence of the inoculum. Data were normalized to wild type (100%). Average of triplicate samples from each of three independent biological replicates (circles) is superimposed on mean and SEM. *, $P < 0.05$; **, $P < 0.01$ by one-way ANOVA.

In macrophages cultured without glucose, *Salmonella* Typhimurium requires the glyoxylate shunt pathway. Having found macrophage growth conditions that require intracellular *Salmonella* Typhimurium to use the first two steps of lipid metabolism, we sought conditions that also required the glyoxylate shunt pathway (*aceA* and *aceB*). We found that in macrophages grown without glucose, *Salmonella* Typhimurium requires the glyoxylate shunt pathway to replicate and/or survive (Fig. 7A and B; Fig. S6A and B). Under these conditions, the percentage of mutant bacteria that replicated declined, but the number of times replicating

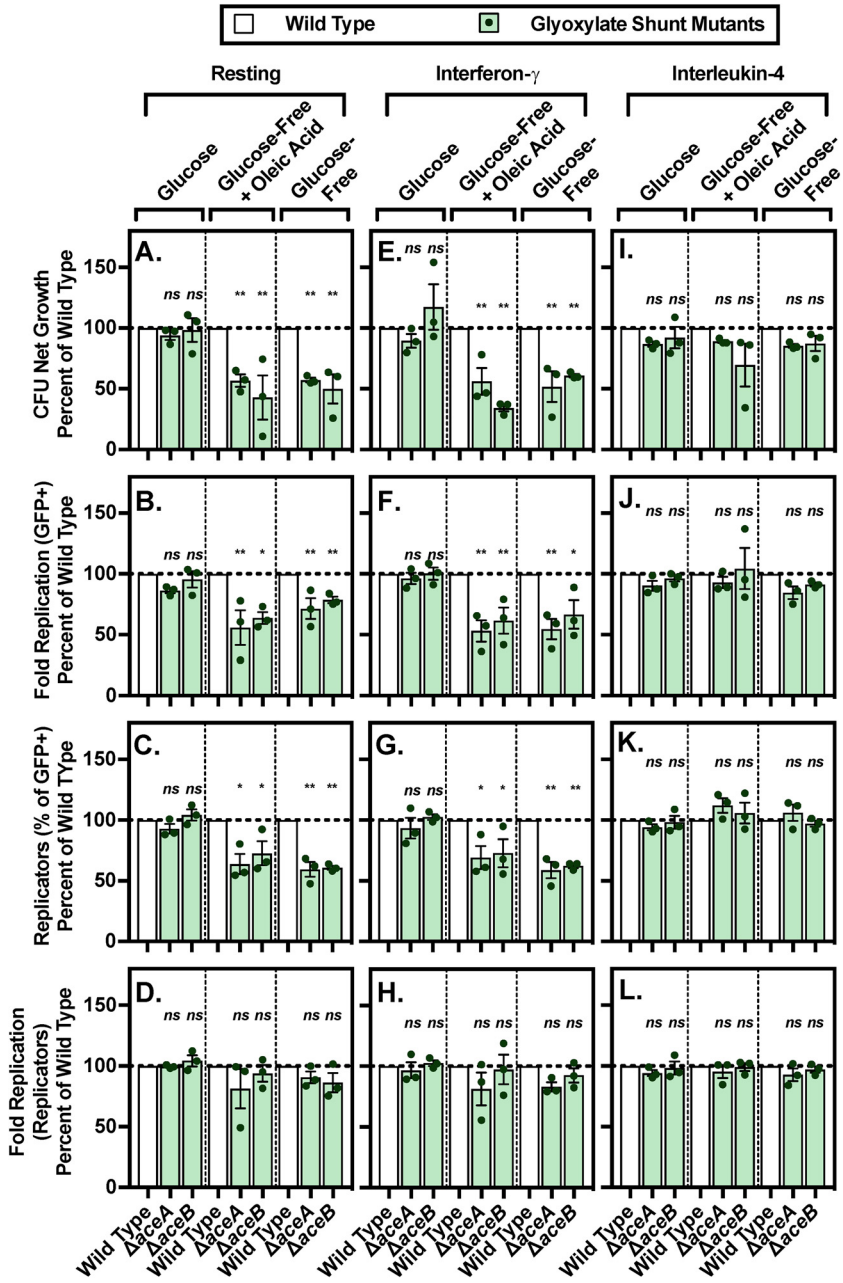

FIG 7 A lack of glucose in the macrophage culture media uncovers a *Salmonella* Typhimurium requirement for the glyoxylate shunt genes in resting and interferon-γ-pretreated BMDMs. BMDMs were transferred into defined medium with the indicated carbon source for 18 to 24 h prior to infection with the indicated strains. Net growth was calculated as CFU at 18 h postinfection divided by CFU at 2 h postinfection (A, E, I). Fluorescence dilution was used to calculate fold replication for all GFP+ bacteria (B, F, J), percentage of GFP-low replicating bacteria (C, G, K), and fold replication of the GFP-low population of replicating bacteria (D, H, L). GFP-low replicating bacteria were gated based on the fluorescence of the inoculum. Data were normalized to wild type (100%). Average of triplicate samples from each of three independent biological replicates (circles) is superimposed on mean and SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by one-way ANOVA.

bacteria doubled remained steady, compared with that in the presence of glucose (Fig. 7C and D; Fig. S6C and D). That glucose obviates the need for the glyoxylate shunt pathway is consistent with the role of this pathway in anaplerosis in the absence of glucose (43). Moreover, the data indicate that the presence of NEAAs within tissue culture media changes the metabolism of the macrophages and/or the bacteria to reflect or mimic conditions that the bacteria experience within an animal. That is, when NEAAs are supplied, a fraction of *Salmonella* Typhimurium use

lipids to replenish the TCA cycle, consistent with the requirement for the glyoxylate shunt genes for the colonization of mice.

***Salmonella* Typhimurium requires lipid metabolism to replicate in proinflammatory but not anti-inflammatory macrophages.** In animals, *Salmonella* Typhimurium likely encounters many kinds of activated macrophages, particularly those polarized toward a classical proinflammatory (M1), microbiocidal state, or toward an alternative, anti-inflammatory (M2) phenotype (5, 7). The experiments described above were carried out with resting macrophages, meaning that they were not pretreated with cytokines. However, resting macrophages become proinflammatory upon exposure to lipopolysaccharide (LPS), which occurs upon infection with *Salmonella* Typhimurium (51). To clearly establish whether pro- or anti-inflammatory macrophages restrict the growth of *Salmonella* Typhimurium lacking lipid metabolism genes, we pretreated BMDMs or RAW264.7 cells with interferon gamma (IFN-γ) or interleukin-4 (IL-4), which polarize macrophages toward pro- and anti-inflammatory states, respectively (5, 7). We obtained similar results in resting and IFN-γ pretreated macrophages, namely, lipid import and β-oxidation contributed to replication whether glucose was present or not, and the glyoxylate shunt pathway was only needed in the absence of glucose (Fig. 6 and 7; Fig. S5 and S6 in the supplemental material). These observations are consistent with the proinflammatory activation state that resting macrophages acquire upon infection with *Salmonella* Typhimurium (51). IL-4-stimulated macrophages did not require any *Salmonella* Typhimurium lipid genes under any conditions tested. Thus, *Salmonella* Typhimurium lipid metabolism genes appear to be required for replication and/or survival in proinflammatory macrophages.

DISCUSSION

Diverse pathogens use fatty acids during infection (52–56), but little is known about the specific microenvironments that enable or necessitate lipid acquisition. Our study used *Salmonella* Typhimurium mutants lacking genes in every step of lipid metabolism, and we examined their phenotypes in microbiological media, in mice, and in cultured macrophages. Sv129 mice, which develop a systemic, nonfatal infection (44, 57), were used to test for lipid gene requirements. The glyoxylate shunt gene *aceA* is needed by *Salmonella* Typhimurium in Sv129 mice for the colonization of mesenteric lymph nodes, but this same study reported no phenotype for an *aceA* mutant strain in C3H/HeN mice, which develop fatal *Salmonella* Typhimurium infections (29). BALB/c mice infected with *Salmonella* Typhimurium also develop fatal infections, which do not require that *Salmonella* Typhimurium have lipid import genes (*fadL* [46] or *fadD* [25]), β-oxidation genes (*yafH* [27]), or the glyoxylate shunt gene *aceA* (25, 26). These data may suggest that lipids are dispensable in mouse models of fatal acute infection but contribute to long-term colonization of tissues.

We tested lipid mutants for growth in macrophages with the aim of modeling phenotypes observed in mice. Macrophages supplemented with NEAAs curbed the replication of *Salmonella* Typhimurium lacking genes for the first two lipid metabolism steps, uptake and β-oxidation. These results echo a report that in NEAA-grown RAW264.7 cells, a lipid uptake gene (*fadD*) is required for replication by the *Salmonella* Typhimurium strain NCTC 12023 (ATCC 14028) (34), which is genetically and phenotypically distinct from SL1344 (58, 59). We also observed a NEAA-dependent increase in *Salmonella* Typhimurium intracellular replication in primary and cell line macrophages from mice with different genetic backgrounds, namely, Sv129 and BALB/c, respectively (see Fig. S7 in the supplemental material). NEAAs may enable the observation of a macrophage phenotype for *Salmonella* Typhimurium with fatty acid metabolism gene mutations because it supports better bacterial growth. Alternatively, macrophage access to NEAAs may enable some bacteria to metabolize lipids based on access to lipids and/or changes in bacterial metabolic capabilities. The presence of NEAAs may also change the metabolism of the macrophage and/or of the bacteria such that a subset of bacteria now requires lipids for growth. Regardless, when macrophages have access to NEAAs, the nutritional microenvironment and/or capabilities of intracellular *Salmonella* Typhimurium change in a manner(s) that influences the outcome of infec-

tion. These observations highlight the fact that growing host cells under different conditions may enable *in vivo* phenotypes to be studied in cell culture.

We did not document any *Salmonella* Typhimurium requirement for glyoxylate shunt genes unless the culture media lacked glucose. These observations are consistent with the *Salmonella* Typhimurium requirement for glyoxylate shunt genes in microbiological media without glucose (43). These data suggest that macrophage access to NEAAs affects *Salmonella* Typhimurium access to and/or the ability to use glucose versus lipids as a carbon source. The *Salmonella* Typhimurium that requires the glyoxylate shunt to colonize mouse tissues may reside within macrophages that have little access to glucose but significant access to other nutrients. Thus, the host cell nutritional microenvironment has the potential to determine which intracellular pathogens replicate and contribute to infection.

Macrophage access to nutrients is not the only microenvironmental factor that affects the intracellular replication of *Salmonella* Typhimurium lipid metabolism mutants. Macrophage exposure to pro- or anti-inflammatory cytokines determines whether these cells rely upon glycolytic or oxidative metabolism, respectively (5, 7). We found that macrophages pretreated with pro- but not anti-inflammatory cytokines required *Salmonella* Typhimurium to have intact lipid metabolism genes to survive and/or replicate. Supplementation with NEAAs was essential to observe the effect of macrophage activation state on *Salmonella* Typhimurium replication, and all three steps of lipid metabolism, including the glyoxylate shunt, were needed in media lacking glucose. Thus, proinflammatory macrophages may provide *Salmonella* Typhimurium with access to lipids and/or enable, for instance, the expression of bacterial lipid metabolism genes. The bacteria within mice that need lipids to replicate may reside within proinflammatory macrophages.

One difference we observed between *Salmonella* Typhimurium lipid requirements in mice versus cell culture is that the canonical and secondary β-oxidation genes compensated for each other only in mice. In broth culture, anaerobiosis allows for the expression of the secondary pathway, suggesting *Salmonella* Typhimurium in mice may use lipids under anaerobic conditions, for example in granulomas.

Future efforts to define the specific tissue or cellular environment(s) in which *Salmonella* Typhimurium requires exogenous lipids to replicate may improve our understanding of the microenvironments in which the pathogen resides. It may also be of use to test whether *Salmonella* Typhimurium lipid metabolism genes are required in murine genetic and pharmacological models that respond to *Salmonella* Typhimurium infection with predominantly M1 or M2 macrophage production. This may be addressable by, for example, comparing the survival and cellular residency of wild-type and lipid-mutant *Salmonella* Typhimurium strains in C57BL/6 (M1) and BALB/c (M2) mice (60). However, interpretation of results is likely to be confounded by differences in the genetic backgrounds of these two strains (61, 62). Alternatively, mice can be pretreated with pharmacological agents that drive them to produce macrophages that are largely M1 (e.g., CpG oligodeoxynucleotides [63, 64]) or M2 (e.g., all-*trans*-retinoic acid [65] and melatonin [66]). Since these methods have primarily been examined in Nramp1$^{-/-}$ mouse backgrounds, their effect on Nramp1$^{+/+}$ mice over time will need clarification prior to testing the hypothesis that lipid metabolism genes are needed for tissue colonization in animals with an M1 phenotype.

In conclusion, the entire lipid metabolism pathway is required for *Salmonella* Typhimurium replication in proinflammatory macrophages when glucose is limited and amino acids are abundant, consistent with bacterial requirements for tissue colonization in Sv129 mice. Thus, pathogen nutritional strategy can be strongly affected by the polarization state of the host cell type and by the availability of nutrients to that host cell.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium wild-type strain SL1344 (67) and derivatives were cultured in LB media containing 30 μg/ml streptomycin, 50 μg/ml ampicillin, 30 μg/ml kanamycin, or 34 μg/ml chloramphenicol. Liquid cultures were incubated overnight at 37°C with aeration. Deletion strains were marked at the indicated locus with kanamycin or chloram-

phenicol resistance cassettes (68, 69), which were P22 phages transduced into SL1344 (70). The resistance cassette was removed by the induction of FLP recombinase or I-SceI to yield an 84-bp scar or no scar, respectively. Mutant strains were validated by PCR and growth on oleic acid.

Complementation plasmids were derived from medium copy number plasmid pRB3-273c (71). The target gene and its promoter were amplified from *Salmonella* genomic DNA (350 bp upstream and 50 bp downstream of the gene) using PCR primers containing restriction digest sites for BamHI and HindIII (*fadL*, *aceA*, and *aceB*) or BamHI and KpnI (*fadD*). For *aceA*, the following two amplicons were initially generated: the *aceBA* promoter region with a downstream KpnI recognition sequence and the *aceA* gene with *aceB* Shine-Dalgarno sequence and KpnI recognition sequence engineered into the upstream PCR primer. The two amplicons were digested using KpnI, ligated, and amplified as a single fragment using PCR. For *aceB*, the entire *aceBA* operon was amplified, digested with HpaI to remove 1,157 bp of the *aceA* gene, ligated, and amplified as a single fragment using PCR. The fragment thus contained the promoter, *aceB*, the 31-bp intergenic region, the initial 117 nucleotides, and final 31 bp of *aceA*. Amplicons were digested with BamHI and HindIII or KpnI, gel purified, ligated into linearized pRB3-273c, and verified by sequencing of the entire insertion (Fig. S1; Table S1). This approach was used because the promoter-*aceB* amplicon only partially complemented mutants lacking *aceB* for unknown reasons.

For fluorescence dilution experiments, strains were transformed with pDiGi and chromosomally marked with green fluorescent protein (GFP) at the *rpsM* locus using P22 phage transduction (17). To induce the expression of dsRed prior to infection, strains were cultured overnight in media containing 170 mM morpholineethanesulfonic acid (MES) (pH 5.0), 5 mM KCl, 7.5 mM $(NH_4)_2SO_4$, 0.5 mM K_2SO_4, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 0.3% glycerol, 0.1% Casamino Acids, and 10 mM arabinose with appropriate antibiotics (49). We found that pDiGc, which encodes GFP under the *rpsM* promoter, significantly hindered *Salmonella* infection, presumably due to high GFP expression (data not shown).

Bacterial growth assays. For broth growth experiments, overnight cultures were washed in phosphate-buffered saline (PBS) and diluted to an optical density at 600 nm (OD_{600}) of 0.01 in 300 μl of LB or M9 minimal medium supplemented with 0.004% histidine, 1 mM $MgSO_4$, 100 μM $CaCl_2$, and either 0.4% dextrose, 0.4% glycerol, or 0.1% sodium oleate and 1% IGEPAL CA-630, which was necessary to solubilize oleate. Bacteria were grown in 96-well plates with shaking in an Eon or Synergy H1 microplate spectrophotometer (BioTek) at 37°C, and the OD_{600} was recorded every 20 min.

For growth on solid media, overnight cultures were washed in PBS and resuspended in PBS to an OD_{600} of 1.0, and 3 μl was spread onto M9 minimal agar plates supplemented with 1 mM $MgSO_4$, 100 μM $CaCl_2$, 0.004% histidine, 1.5% agar, 1% IGEPAL CA-630, and either 0.4% dextrose, 0.1% sodium oleate, 0.1% sodium decanoate, or 0.1% sodium octanoate. No growth was observed in the absence of carbon. For anaerobic growth, plates were supplemented with 25 mM nitrate as an alternative electron acceptor; no anaerobic growth was observed in the absence of nitrate. Anaerobic plates were grown at 37°C within GasPak EZ anaerobe pouches (BD).

Cell culture and bone marrow-derived macrophage generation. Macrophages were routinely cultured in Dulbecco modified Eagle medium (DMEM) high glucose (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM β-mercaptoethanol. To generate bone marrow-derived macrophages, femurs and tibias from 4- to 10-week-old wild-type Sv129S6/SvEvTac mice (Taconic Laboratories) were flushed with PBS to recover bone marrow, layered over Histopaque-1083 (Sigma), and centrifuged for 25 min at 500 $\times$ *g*. The mononuclear cell fraction was recovered and washed in complete media. Cells were seeded at 1×10^5 to 2×10^5 cells/ml in complete medium supplemented with 30% to 35% conditioned media from 3T3 cells expressing macrophage colony-stimulating factor (MCSF) and fed 3 to 4 days later. After 1 week in culture, BMDMs typically replicated 5- to 10-fold under these conditions. Twenty-four hours prior to infection, macrophages were transferred into defined glucose-free DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 50 μM β-mercaptoethanol, and 1$\times$ NEAA (alanine, asparagine, aspartic acid, glycine, serine, proline, and glutamic acid; Sigma). Importantly, we observed less replication of *Salmonella* Typhimurium within macrophages in the absence of NEAAs (Fig. S7). Glucose-free medium was supplemented with 4.5 g/liter glucose or 600 μM oleic acid (conjugated 6:1 with bovine serum albumin [BSA]; concentrated oleic acid dissolved in 0.1 M Na_2CO_3 at 55°C was added to BSA dissolved in PBS, stirred for 1 h at 37°C, filter sterilized, and stored at −20°C). Supplementation with BSA vehicle control yielded results identical to glucose-free medium. Macrophages were activated with 2 ng/ml recombinant murine IFN-γ (PeproTech) or 20 ng/ml recombinant murine IL-4 (PeproTech) for 18 to 24 h prior to infection.

Infection of cell culture macrophages. For fluorescence dilution experiments, bacteria were added to BMDMs at a concentration of 3×10^7 CFU/ml in media. We found that this protocol reproducibly yielded infection of 70% to 80% of BMDMs with dsRed-expressing bacteria by flow cytometry (data not shown). After 45 min and 2 h, medium was exchanged for media containing 100 and 10 μg/ml gentamicin, respectively. At 2 and 18 h postinfection, parallel samples were washed three times with PBS and lysed with 0.1% Triton X-100. A portion of the lysate was diluted in PBS and plated to determine CFU. Net growth was calculated as the recovered CFU at 18 h divided by that at 2 h. The remainder of the lysate was centrifuged for 20 min at 2,500 $\times$ *g* at 10°C, fixed with 1.6% paraformaldehyde, and analyzed using a CyAn ADP cytometer (Beckman Coulter). A minimum of 30,000 GFP-positive events were collected for analysis. Data were analyzed with FlowJo. Samples were gated for GFP-positive bacteria. Bacterial fold replication was calculated as the dsRed geometric mean of the inoculum divided by that of the GFP-positive population at 18 h postinfection. Data were normalized to wild type (100%).

Mouse infections. Experimental protocols were approved by the University of Colorado Institutional Committee for Animal Care and Use. Seven-week-old male and female Sv129S6/SvEvTac mice (Taconic Laboratories) were used for competitive infection studies. Mice were inoculated with a 1:1 mixture of two differentially marked strains. For orogastric infections, animals were fasted 8 to 12 h prior to oral gavage

with 1×10^9 of each strain (2×10^9 total in 100 μl), except for the Δ*fadD* versus Δ*fadD;ydiD* coinfection, which was 2×10^9 each strain. For intraperitoneal infections, animals were inoculated with 1×10^3 of each strain (2×10^3 total in 100 μl). At 2 weeks postinfection, animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Cecum, Peyer's patches, mesenteric lymph nodes, spleen, and liver were harvested, homogenized in PBS, and serially diluted to enumerate CFU. The competitive index (CI) for each organ was calculated as ($CFU_{strain\ A}/CFU_{strain\ B}$) output/($CFU_{strain\ A}/CFU_{strain\ B}$) input.

Statistics. *P* values were calculated using one-way analysis of variance (ANOVA) or one-sample *t* test (GraphPad Prism) and considered significant if the *P* value was <0.05, as described in the figure legends.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.02 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 6, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 7, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 8, PDF file, 0.1 MB.

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