Quantitative PCR-Based Competitive Index for High-Throughput Screening of Salmonella Virulence Factors††

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Salmonella enterica serovar Typhimurium is an intracellular pathogen and a main cause of food-borne illness. In this study, a quantitative PCR (qPCR)-based competitive index (CI) method was developed to simultaneously compare the growth of multiple Salmonella strains. This method was applied to a mixture of 17 Salmonella mutants lacking regulator genes, and their survival ratios were compared based on expression of natural resistance-associated macrophage protein 1 (Nramp1). Nramp1, as a major host innate immune component, controls the intracellular replication of pathogens. Deletion strains containing unique DNA barcodes in place of regulator genes were mixed with the parental control, and the bacteria were inoculated into congeneric mice differing only at Nramp1. Most of the deletion strains were outcompeted by wild-type bacteria in either mouse strain, and the lack of Nramp1 didn't increase the tested strain/parent control replication ratios. When the same collection of mutants was tested in congenic mouse-derived primary macrophages, a major Nramp1-expressing cell type, six strains (ΔhimD, ΔphoP/phoQ, ΔrpoE, ΔrpoS, ΔompR/envZ, and Δhfq strains) grew better in Nramp1−/− than in Nramp1+/+ macrophages, suggesting that these six regulators may play roles in overcoming Nramp1-mediated bactericidal activity in primary macrophages. The discrepancy in survival of macrophages and that of mice suggests either that there are differences in macrophage populations or that other cell types expressing Nramp1 control Salmonella proliferation in the host. The method described allows competitive infection analysis to be carried out on complex mixtures of bacteria and provides high reproducibility from independent biological replicates.

Salmonella enterica serovar Typhi, hereafter S. Typhimurium, causes gastroenteritis and a self-limiting disease in humans but a typhoid fever-like systemic disease in mice. S. Typhimurium has been studied as a model for typhoid fever because the host range of S. enterica serovar Typhi is limited to humans. Salmonella has been equipped with a plethora of virulence factors to resist hostile host intracellular milieu. Genes that encode virulence factors are widely distributed around the entire chromosome of pathogenic Salmonella, and their expression is tightly controlled by at least 20 different regulators that sense environmental cues during infection. In a previous study, we identified 17 of 83 regulator genes tested to be required for systemic infection in mice (61). Salmonella strains deleted in those 17 regulator genes were significantly attenuated in virulence in BALB/c mice (61). The virulence phenotype can be influenced by several parameters, including the route of administration, the inoculation dose, the organs examined, and the genotype of the host animal (25, 34, 53, 54). The BALB/c strain used in the previous virulence study lacks a functional Nramp1 protein, a major host innate immune component, and cannot control Salmonella replication, thereby succumbing to low infectious doses (27, 43). We reasoned that the Salmonella regulator mutants that were attenuated in BALB/c mice might exhibit different phenotypes in the presence of Nramp1.

Nramp1 (also known as Slc11a1), a highly hydrophobic protein with 12 transmembrane domains, is expressed in cells of myeloid origin and is localized mainly to the phagosomal membrane of macrophages, neutrophils, and dendritic cells (11, 22, 55). This protein is required for resistance against taxonomically unrelated pathogens, including Mycobacterium, Salmonella, and Leishmania (28, 58, 60, 64). The mechanism by which Nramp1 restrains pathogens from proliferating within host tissue cells is likely to be linked to its role as an iron and manganese antiporter, because these are essential nutrients promoting the growth of microorganisms (17, 28, 64). Besides depletion of divalent metals from the phagosomal space, Nramp1 has been reported to exert a variety of other functions. Nramp1 increases major histocompatibility complex (MHC) class II expression and antigen presentation (33, 51) and induces rapid proinflammatory responses such as upregulation of gamma interferon (IFN-γ), interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), and keratinocyte chemoattractant (KC) (31, 32, 46, 55, 56). As well as inducing higher production of cytokines and chemokines, Nramp1 facilitates the formation of reactive oxygen and nitrogen species as an antimicrobial defense mechanism (2, 5, 21). Expression of Nramp1 in macrophages also increases expression of Salmonella pathogenicity island 2 (SPI-2)-associated virulence genes, providing increased bacterial defenses to counteract host immunity (63).

To better understand the interaction between Salmonella and host innate immune responses mediated by Nramp1, we compared replication of a variety of Salmonella regulator mu-
saints in mice with or without Nramp1, as well as primary macrophages derived from these same mice. A traditional method to compare growth between wild-type and mutant strains has been the competitive index (CI) assay (3, 18, 52). The conventional CI test is performed by infecting animals or cells with a mixture of mutants and wild-type bacteria that can be distinguished based on specific phenotypic differences. The number of each strain is enumerated in the input inoculum and in the output organ to compare persistence between test strains and the wild-type strain. The competitive index has become a standard for measuring virulence because it is more sensitive than the 50% lethal dose (LD50) assay and less prone to animal-to-animal differences. However, it has several disadvantages, including the excessive animal usage and the limited selection markers between the strains tested. The phenotypic traits, such as antibiotic resistance and metabolic characteristics, must be able to distinguish parent from mutant bacteria without influencing bacterial virulence (3, 18, 52). In this study, we developed a novel competitive index method using DNA barcode-tagged mutant strains, thus enabling us to determine the survival rates of numerous mutants in a single experiment by quantitative PCR (qPCR). Using the qPCR-based competitive index method (CIqPCR), we identified six *Salmonella* regulator mutants whose growth was more attenuated than that of the wild type in response to Nramp1 in primary macrophages.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** All *Salmonella* strains used in this study are *Salmonella enterica* serovar Typhimurium 14028s and its isogenic derivatives. Mutant strains with deletions in regulator genes were constructed using modified pKD13 (pKD13-mod) plasmids (pKD13; GenBank accession no. AY048744), which were designed to replace genes of interest with 135-nucleotide (nt) barcode sequences following homologous recombination. Linearized PCR products amplified from a pKD13-mod plasmid contain a kan cassette in the middle and a 40-nt sequence at each terminus. The 40-nt termini are homologous to a gene amplified from a pKD13-mod plasmid contain a FRT site left after FLP-mediated excision of the kan cassette. Barcode sequences following homologous recombination. Linearized PCR products which were designed to replace genes of interest with 135-nucleotide (nt) bar- cassettes in the middle and kan cassette was confirmed by PCR. Prior to elimination of the kan location (15), Replacement of a target gene with a of interest and facilitate homologous recombination at the correct chromosomal location. Site-directed mutagenesis was performed by the T7 polymerase system using touchdown PCR (15), resulting in in-frame, nonpolar deletions of the target genes. The sequences recognized by primers scarF and scarR in nested PCR are underlined and indicated by priming sites 4 and 1, respectively. T7 promoter sequences are shown between square brackets.

**ISOlation and culture of bone marrow-derived macrophages from mouse.** Bone marrow was extracted from the femurs of 5-week-old female 129SvJ mice as previously described (14) and incubated for 7 days in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen), which was complemented with 10% fetal bovine serum (Invitrogen) and 20% L929 cell supernatant containing macrophage colony-stimulating factor. Bone marrow-derived macrophages (BMDM) were counted and 2 × 10^6 cells were seeded in each well of a 6-well plate 1 day before infection. BMDM were incubated in DMEM containing 10% fetal bovine serum at 37°C with 5% CO2 overnight.

**ClqPCR in macrophages.** *Salmonella* strains were grown individually in Luria-Bertani (LB) broth overnight and equivalent amounts of each strain were mixed as an inoculum. A mix of bacteria strains was osonized with 10% mouse serum (Innovative Research) for 20 min prior to infection (6). Bacterial cells were added to BMDM monolayers at an input multiplicity of infection (MOI) of 10, and infection was initiated by centrifugation at 1,000 × g for 5 min. In order to estimate the threshold cycle (CT) value of each strain in the input, the same dose of bacteria mix, which was inoculated into a single macrophage well, was washed with distilled water and used as a template in nested PCR (2 × 10^5 CFU/PCR). The resulting PCR products were used as template DNAs in quantitative PCR after a serial dilution. Following 30 min of incubation at 37°C with 5% CO2, the medium was replaced with DMEM containing 100 μg/ml gentamicin, and cells were incubated for 1 h to remove extracellular bacteria. After treatment with 100 μg/ml gentamicin, BMDM were washed with PBS twice and overlaid with DMEM containing 20 μg/ml gentamicin for the remainder of the experiment. In order to enumerate intracellular bacteria at appropriate time points after infection, BMDM were lysed with 1% Triton X-100 for 10 min after several PBS washes, and the lysate was spun to collect bacterial cells at 4,500 × g for 5 min. A pellet of bacterial mix from each well was washed three times with PBS and resuspended in 30 μl of distilled water. A bacterial mix from a single well of a 6-well plate was regarded as an individual output sample, and the cells in suspension from a single well (30 μl) were used as a template in nested PCR. Serially diluted nested PCR products were subjected to quantitative PCR to measure the CT value of each strain in the output samples.

In a nested PCR of input and output samples, two primers recognizing priming sites 4 and 1 (see Fig. 1), scarF (5'-ATTCCCGGAGATCCGTGACCT-3') and scarR (5'-GTGTAGGCTGGAGCTGCTCC-3'), respectively, were used to amplify 24-nt barcode sequences from a variety of deletion strains. Nestered PCR was performed by one cycle of 95°C for 10 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 30 s, and one cycle of 72°C for 5 min. Serially diluted nested PCR products were used as template DNAs in quantitative PCR using primer scarF and scarR and primers specific to the barcode sequence. Duplex DNA products resulting from quantitative PCR were detected with SYBR green reagent (Applied Biosystems) by using a StepOnePlus real-time PCR instrument (Applied Biosystems). Real-time PCR was carried out by 50 cycles of 95°C for 15 s and 60°C for 1 min, following 95°C for 10 min. In order to normalize CT values, the amplification efficiency of each barcode primer during PCR was calculated from the standard curve of each strain by using serially diluted template DNAs. Mutant strain/wild-type strain survival ratios were quantified using PCR amplification efficiency (E) and CT values of strains in input and output qPCR analyses by using the ClqPCR formula below (42, 62). At threshold, Q_{input} = E_{input}^{CT_{mutant} - CT_{wild}} Q_{output} = E_{output}^{CT_{mutant} - CT_{wild}} (where Q is template DNA quantity and wt is wild type).

**ClqPCR in mouse.** *Salmonella* strains were grown individually in LB medium overnight as described above and mixed equivalently in phosphate-buffered sa-

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**FIG. 1.** Scar sequences in deletion strains. The 135-nt scar sequences, replacing coding sequences of a target gene, are shown. The scar site or FLP recognition target (FRT) site left after FLP-mediated excision of the kan cassette is highlighted in dark gray. Barcode sequences inserted via SacI and AvrII are composed of 24 random nucleotides and are outlined in the schematic diagram. N, any nucleotide with either a purine or pyrimidine base; V, any nucleotide except thymine-based nucleotides. Sequences recognized by primers scarF and scarR in nested PCR are underlined and indicated by priming sites 4 and 1, respectively. T7 promoter sequences are shown between square brackets.
line (PBS) based on optical density at 600 nm (OD_{600}) values. A bacterial mixture was washed with PBS and diluted to 10^7 CFU/ml. Female 4- to 5-week-old Nrarpl1^-/- , 129SvJ mice and congenic Nrarpl1^-/- , 129SvJ (57, 58) mice were intraperitoneally injected (ip) with 100 μl of the mixed Salmonella strains at a final dose of 10^8 CFU/mouse. The number of injected bacteria was confirmed by plating diluted inoculum on LB agar plates. A portion of the inoculation mixture, corresponding to 2 × 10^6 CFU, was resuspended in distilled water and used in nested PCR to evaluate C_{F} values of the strains used as the input. At desired time points after infection, mice were euthanized to compare bacterial persistence between the strains. The liver and spleen were homogenized and plated on LB agar to isolate intracellular Salmonella. Colonies were scraped and collected in a tube with PBS. In order to assess C_{F} values in the output, a mixture of bacterial cells corresponding to 2 × 10^6 CFU was resuspended in distilled water and used as the template for nested PCR (2 × 10^6 CFU/PCR). Barcode DNAs amplified from nested PCRs of input and output samples served as template DNAs in the qPCR step, as above.

As an alternative, total DNAs were isolated from the spleen or liver homogenates using GeneElute bacterial genomic DNA kit (NA2110, Sigma) and used as templates in nested PCR instead of isolating bacterial colonies on agar plates. The C_{F}qPCR values were comparable between two methods, although interestingly a larger deviation was observed between specimens when a small piece of liver was applied, as reviewed by Mastroeni et al. (38).

Phenotype-based conventional CI. Bacterial strains were cultivated individually overnight in LB prior to infection. Each strain was washed and diluted in PBS to 2 × 10^6 CFU/ml. The test strain was mixed with a reference strain (MA6054) at a ratio of 1:1, and 100 μl (10^7 CFU/mouse) of the mixed cells was used to infect Nrarpl1^-/- , 129SvJ mice intraperitoneally. The inoculum, used in infection, was diluted in PBS and spread on plates to enumerate the injected dose of bacteria. The reference strain, MA6054, produces an arabinose-inducible β-galactosidase and can be distinguished from test strains on LB agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (26). Infected mice were sacrificed at days 2, 5, and 7 postinfection to isolate the spleens. The spleens were homogenized by mechanical disruption, and the suspensions were plated on LB agar with X-Gal and arabinose (1 mM) (26). Infected mice were sacrificed at days 2, 5, and 7 postinfection to isolate the spleens. The spleens were homogenized by mechanical disruption, and the suspensions were plated on LB agar with X-Gal and arabinose.

The conventional competitive index was then calculated as [number of test strains × number of reference strains]/number of test strains/number of reference strains. The competitive index of the pKD13 (pKD13-mod) plasmids harboring unique barcodes were constructed and tested in qPCRs using primers specific to the inserted barcodes. A pool containing equivalent DNA from each pKD13-mod was analyzed in qPCR to examine the efficiency of the barcodes during PCR. Of the 102 pKD13-mod derivatives containing 24-nt barcodes, 88 were found to perform well in test qPCRs, producing comparable C_{F} values at a threshold level. Thereafter, these 88 pKD13-mod plasmids were employed as templates in the construction of deletion strains. The allelic replacement strategy was to replace all codons between the translational initiation codon and the last seven codons with a 135-nt sequence containing a DNA barcode and T7 promoter. The final sequence following FLP-mediated recombination encodes a 45-amino-acid sequence without stop codons that is in frame and therefore not likely to be polar upon expression of downstream genes in the same operon. The T7 promoter upstream of the barcode sequences can be used to identify strains using in vitro transcription from the T7 promoter. Small labeled RNAs produced via AvrII digestion followed by in vitro transcription can be applied to microarray hybridization for quantification of strains. An additional advantage of this approach was the ability to leave the last 7 amino acids of the deleted gene to avoid the frequent problem of overlapped gene coding sequences (B. L. Wanner, personal communication). The scar sequence remaining after FLP-mediated excision of the kan cassette is shown in Fig. 1. Using the pKD13-mod library with a variety of barcodes, genes of interest were deleted by λ Red recombination and replaced with a 135-nt sequence, including a unique barcode. For the parental control in competitive infection studies, we inserted a barcode in the pseudogene STM0314, which did not affect Salmonella virulence (see Fig. S1 in the supplemental material).

Calculation of the competitive index utilizing quantitative PCR in mixed populations. Quantitative reverse transcription-PCR (qRT-PCR) has been a strong tool for measuring transcription levels and expression changes of genes of interest (40, 51, 61). We applied qRT-PCR to a mixed bacterial population to enumerate each bacterial strain and compare growth and survival between strains during a single infection. Each mutant strain was distinguished via a 24-nt barcode with a specific primer in the mixed population. The quantity of PCR products is theoretically proportional to the quantity of initial template DNAs under the exponential phase of increase, when PCR reagents are not limited (24). The amounts of PCR products are deducible from the initial template quantities and amplification efficiencies of barcode primers and are equivalent between strains at a threshold level. If the PCR efficiency is ideal, the amount of PCR product will double for each cycle during the exponential phase of PCR amplification (37). However, due to differences in primer specificity among the barcode sequences, the amount of PCR products will not be increased twice every cycle (29, 44). Therefore, it was necessary to take account of the PCR efficiency of each barcode primer in enumeration by using RT-PCR (44, 62). The relative ratio of a mutant strain to the wild-type strain was determined from the efficiency-calibrated mathematical model, which has been broadly used for relative quantification in RT-PCR (42, 62). To calibrate C_{F} values between strains, the amplification efficiency (E) of each barcode primer was determined using the slope of
the standard curve of each strain, as demonstrated in Fig. S2 in the supplemental material (9, 42). As shown in the equation in Materials and Methods, a competitive index formula using qPCR (CIqPCR) was computed based on PCR amplification efficiency and \( C_T \) values of strains in input and output samples.

In order to rule out the possibility of cross-reactivity between different barcode primers, a bacterial mixture composed of equivalent amounts of 8 barcode-tagged strains was subjected to qPCR-based relative quantification using 8 cognate barcode primers, specific to the 8 mixed strains and 8 unrelated primers, chosen at random but not specific to the 8 test strains (see Fig. S3 in the supplemental material). The unrelated barcodes showed a \( \Delta C_T \) value (reference barcode \( C_T \) – test barcode \( C_T \)) of less than \( -15 \) cycles \((-25.6 \leq \Delta C_T \leq -15.2\)), whereas barcodes used in the mixed strains exhibited \( C_T \) values similar to that of the reference barcode (see Fig. S3 in the supplemental material), indicating that these 8 unrelated barcodes gave a signal that was essentially undetectable compared to the signal from relevant barcodes.

Description of the CIqPCR method. A flowchart of the CIqPCR method in mice is shown in Fig. 2. A reference strain (the \( \text{STM0314 strain} \) and deletion strains, each with a unique barcode, were cultivated individually prior to infection. Strains were mixed equally in the inoculum and 10^4 CFU of the mixed cells were intraperitoneally administered to each mouse. The inoculum was verified by plating and counting the number of CFU. The mice were euthanized to isolate Salmonella-infected organs at the desired time point after infection (6). Organs were homogenized and spread on LB agar to grow bacteria (7). Bacterial colonies were scraped and collected the next day. The mixture was diluted in PBS buffer, and 2 \times 10^6 CFU were used as the template for nested PCR (8). Bacterial strains from the output populations were analyzed by qPCR using barcode-specific primers (9). CIqPCR was calculated by comparing \( C_T \) values between a wild-type strain and a mutant strain in input and output qPCRs, taking into account that the amplification efficiency of individual barcodes ranged from 1.53 to 1.94.

The quantitative PCR was performed using a two-step PCR procedure described in detail in Materials and Methods. In an initial nested PCR step, two outside primers corresponding to either end of the 135-bp scar sequences were used to amplify the barcode sequences. The products, each of which had the same ends but a unique barcode in the middle, were serially diluted and used as templates in a second round of amplification: qPCR in which one of the outside primers and the barcode-specific primer were used. By using nested PCR rather than qRT-PCR directly on the mixture, the specificity was greatly increased, as has been shown in other studies and observed in our laboratory (1, 23, 49).

The reliability of the CIqPCR assay was validated by comparison with the traditional CI method (Fig. 3). Four strains, including a reference strain (the \( \text{STM0314 strain} \) and three barcode-labeled strains (\( \text{STM2209, STM5096, and STM4333 strains} \)), were mixed equivalently, and the bacteria were inoculated intraperitoneally into mice (10^6 CFU/mouse). Intracellular bacteria residing in the spleens were enumerated...
using CI\qPCR at days 2, 5, and 7 postinfection. In parallel with the CI\qPCR assay, each deletion strain was mixed with a reference strain (MA6054 [26]), which has an arabinose-inducible β-galactosidase gene, at a 1:1 ratio, and 10⁴ CFU of the mixed bacteria was inoculated into each mouse. Mice were sacrificed at days 2, 5, and 7 postinfection, and the spleen homogenates were plated on LB agar with X-Gal and arabinose. The con-galactosidase gene, at a 1:1 ratio, and 10⁴ CFU of the mixed strain (MA6054 [26]), which has an arabinose-inducible regulator genes was compared within Nramp1 mice (Nramp1

Survival of Salmonella mutants lacking virulence regulators in mice (Nramp1\textsuperscript{+/+} and Nramp1\textsuperscript{−/−} 129SvJ). In order to confirm the validity of the CI\qPCR method as a high-through-put screening tool, the persistence of 17 mutants lacking regulator genes was compared within Nramp1\textsuperscript{+/+} and Nramp1\textsuperscript{−/−} mice using quantitative PCR. Our previous work identified 17 regulators required for growth in BALB/c mice by either intragastric (i.g.) or intraperitoneal (i.p.) infection (61). While these regulators were clearly required for systemic infection in BALB/c mice, which is NRamp1\textsuperscript{−/−}, we thought it possible that some of these mutant strains might have a differential effect on survival of 129SvJ Nramp1\textsuperscript{+/+} and 129SvJ Nramp1\textsuperscript{−/−} mice. The 17 regulators required for survival in Nramp1\textsuperscript{−/−}/BALB/c mice are SpvR, FrsR, YbdM, HimD, PhoP/PhoQ, SsrA/SsrB, SlyA, Hnr, STM2575, RpoE, SmgB, CsrA, STM2912, RpoS, Cpr, OmpR/EnvZ, and Hfq (see Table S1 in the supplemental material). Two genes (STM2575 and STM2912) annotated as regulators but not characterized further were included. Genes encoding these 17 regulators were replaced with unique bar-code DNAs listed in Table S2 in the supplemental material. A mixture containing equal amounts (around 556 CFU/strain) of the 17 mutants and the reference strain (the ΔSTM0314 strain) was inoculated i.p. into mouse strain 129SvJ with or without Nramp1 at 10⁴ CFU/mouse. Bacterial cells in the input inoc-ulum and the output splenic homogenates were subjected to a qPCR-based CI test as described above, and the survival rate of each strain was enumerated at day 5 postinfection (Fig. 4). Considering that a CI\qPCR value of 1 indicates a comparable growth between the parent strain and a mutant, most of the deletion strains showed growth attenuation in both congenic Nramp1\textsuperscript{+/+} and Nramp1\textsuperscript{−/−} mice, which is consistent with our single-infection results (see Table S1 in the supplemental material) and the results reported elsewhere by other investiga-tors. Some mutant strains, including ΔphoP, ΔhimD, ΔSTM2912, ΔrpoS, and ΔompR/envZ strains, were outcom-peted by wild-type bacteria more strongly in Nramp1\textsuperscript{−/−} mice than in Nramp1\textsuperscript{+/+} mice. The mutant strains with lower CI\qPCR values in Nramp1\textsuperscript{−/−} mice are likely to be attenuated in intra-cellular replication independent of Nramp1, whereas the wild-type reference bacteria proliferate better in Nramp1\textsuperscript{−/−} mice.

Virulence-attributed phenotypes can vary depending on the origin of host cells (7). After i.p. administration into host animals, Salmonella migrates quickly to the filtering organs, the spleen and liver (45, 47). To assess whether the survival and growth of the regulator mutant strains differed in the spleen and liver, the same mixture of 17 deletion strains and a refer-ence strain was injected into Nramp1\textsuperscript{+/+} or Nramp1\textsuperscript{−/−} mice, and the persistence in the spleen was compared to the persis-tence in the liver in each mouse strain (see Fig. S4 in the supplemental material). With the exception of 6 strains (ΔphoP/ΔphoQ, ΔsrrA/ΔsrrB, ΔslyA, ΔcstA, ΔSTM2912, and Δhfsq strains), the regulator mutants exhibited similar growth pro-files between the two organs, suggesting that the spleen and liver provide a similar milieu to the survival of the tested strains. Notably, the ΔsrrAΔsrrB strain had a lower CI\qPCR in Nramp1\textsuperscript{−/−} liver than in Nramp1\textsuperscript{+/+} liver but comparable values in the spleen. Overall, strains (including ΔhimD, ΔslyA, Δhnr, ΔrpoE, ΔcstA, and Δhfsq strains) appeared to be more defective for growth in the liver than in the spleen. The lower CI\qPCR in the liver might be attributable to a more restrictive environment in the liver for the growth of some mutants, as observed in the infection with Listeria monocytogenes (10, 13). The more significant attenuation of the ΔslyA strain in the liver has been noted before and is consistent with our observation (35).
Survival of *Salmonella* mutants lacking virulence regulators in primary bone marrow-derived macrophage from *Nramp1*+/+ and *Nramp1*−/− 129SvJ mice. Comparing the persistence of *Salmonella* mutants lacking regulators in *Nramp1*+/+ and *Nramp1*−/− mice to that of the parent control, we did not find any regulator mutants that survived better in *Nramp1*−/− than in *Nramp1*+/+ mice (see Fig. 4). Most of the deletion strains were defective for growth in either mouse strain. After phagocytosis of pathogenic bacteria, *Nramp1* is targeted to the membrane of the pathogen-containing phagosome in macrophages, neutrophils, and myeloid-derived dendritic cells (11, 22, 48, 55). In order to further investigate the effects of the 17 regulators on *Salmonella* survival in the presence of *Nramp1*, we also performed the assay in a cell culture model of infection. BMDM were prepared from both *Nramp1*+/+ and *Nramp1*−/− 129SvJ mice and infected with equal mixtures of the 17 deletion strains and the parent strain at an input MOI of 10 to 1. At 30 min, 6 h, and 18 h after infection, primary macrophages were lysed and the intracellular bacteria were subjected to two-step qPCR to enumerate each deletion strain (Fig. 5; see also Fig. S5 in the supplemental material). CIqPCR values at 18 h postinfection are shown in Fig. 5. Some *Salmonella* strains, including the ΔhimD, ΔphoP, ΔompR, ΔosR, ΔompRΔenvZ, and Δhfq strains, survived better (i.e., a higher CIqPCR value) in *Nramp1*−/− macrophages than in *Nramp1*+/+ macrophages, indicating that these strains were more sensitive to the effects of *Nramp1*. Additionally, *Salmonella* strains not expressing SlyA, RpoE, CsrA, and Hfq were attenuated more than 5-fold compared to the parent strain in both *Nramp1*+/+ and *Nramp1*−/− mouse-derived cells, implying significant roles of SlyA, RpoE, CsrA, and Hfq during bacterial survival in macrophages. However, two mu-
tant strains, the ΔspvR and ΔfruR strains, replicated significantly better than a wild-type strain in both Nramp1+/+ and Nramp1−/− macrophages. Different phenotypes between macrophages and animal models may be attributed to the function of other cell types in controlling replication of pathogenic bacteria in the whole animal, as described further in Discussion.

**DISCUSSION**

In this work, we demonstrate the efficacy of a novel qPCR-based CI method to distinguish the effect of *Salmonella* regulators in the presence or absence of Nramp1. By tagging mutant strains with unique DNA sequences, we were able to evaluate growth of multiple strains by quantitative PCR. Using far fewer mice than what would be required for LD50 experiments, the qPCR-based competitive index method was robust and resulted in reduced variation between animals (average standard deviation of 0.41 among mice and 0.36 among macrophage cultures). As in other high-throughput screening methods, the CISP method required several parameters to be optimized for successful reproducibility in an animal model inoculated with the same pool of mutant strains. Using too high of an inoculum may overwhelm the host immune response, quickly killing the mice and leading to the growth of avirulent mutants that could otherwise be attenuated. However, using too small of an inoculum may result in complete clearance of individual mutants, leading to a spuriously low competitive index. Higher-dose inoculation has been suggested to obtain consistent phenotypic characteristics of mutant strains unless it causes a physical burden irrelevant to phenotypic results (39). We used a mixture of 10^5 CFU as an inoculum in Nramp1+/+ or Nramp1−/− mice. The larger the number of strains to be tested together, the smaller the inoculum of any single mutant. Consistent results were obtained regardless of the number of tested strains when the number of mixed strains was between 2 and 36 in the inoculum of 10^4 CFU (data not shown). However, titration of optimal inoculum should be carried out depending on the genotype of the host animal, the route of administration, and the period of infection. Some mutant strains are attenuated for growth in intragastric infection but not in intraperitoneal infection or vice versa (see Table S1 in the supplemental material). Bacterial persistence profiles can also be influenced by the infection period as well. We observed that some *Salmonella* mutant strains (ΔSTM2281, ΔhidD, and ΔbarA strains) were attenuated at day 7 postinfection but were comparable with a wild-type strain at day 2 postinfection (data not shown). A short infection period may not provide a sufficient time for some mutants to exhibit their phenotype if the deleted genes play roles in long-term systemic infection in the host, such as resistance to the adaptive immune system.

The composition of strains in the mixed population might also affect their survival phenotypes. The possibility of *trans* complementation has been an issue in previous coinfection experiments, in which one bacterial mutant could complement another during infection of the same mouse. *Trans* complementation is more likely to occur in high-dose inoculations where multiple bacteria may infect a common host cell. Recently, outer membrane vesicles have been highlighted due to their role in transferring virulence factors between adjacent bacteria and even between host cells (6, 30, 59) (Yoon et al., submitted for publication). Regulator mutants defective in the expression of virulence factors, which are secreted by outer membrane vesicles, might be complemented, at least in principle, by vesicles produced from coinfecting parental bacteria. However, in comparing individual infections to infections with a mixture, we have not yet observed the possibility of *trans* complementation. The importance of dose, combination and ratio of strains, infection time, site of recovery, and infection route has been previously emphasized in mixed infections using two or more strains (4, 53).

Nramp1 is expressed exclusively in dendritic cells, macrophages, and neutrophils—all cells of myeloid origin (22, 48, 51). Comparing bacterial survival betweenNramp1+/+ and Nramp1−/− macrophages, we identified 6 regulators (HimD, PhoP/PhoQ, RpoE, RpoS, OmpR/EnvZ, and Hfq) whose absence more strongly attenuated *Salmonella* survival in Nramp1−/− than in Nramp1+/+ macrophages. These 6 regulators may regulate virulence factors necessary for resistance to Nramp1-mediated bactericidal activities within macrophages. *Salmonella* has been reported to increase expression of *Salmo-

In the acute mouse infection model, *Salmonella* cells are rapidly disseminated to the spleen and liver and are present in several cell types (19). However, the survival phenotype within macrophages has been regarded as a barometer to infer virulence in the animal; therefore, we anticipated that there would be little difference between Nramp1−/+/Nramp1−/− mice and primary macrophages derived from the same strains of mice. Interestingly, *Salmonella* strains with deletions in *spvR*, *himD*, *phoP*/*phoQ*, *srrAssrB*, *smpB*, *crp*, and *ompR*/*envZ* were significantly attenuated in mice at day 5 postinfection (Fig. 4) and at a shorter infection time (less than 4 days [data not shown]) but not in bone marrow-derived macrophages (Fig. 5). This discrepancy might be attributable to several possibilities. The microbialicidal activity of macrophages may vary between the sources, as described in reference 36. Buchmeier and Heffron reported that the survival of *Salmonella* mutant strains is influenced by the origin of macrophages (7). Peritoneal macrophages are more microbialicidal than splenic and bone marrow-derived macrophages toward *Salmonella* (7). *Salmonella* strains inoculated into the peritoneal cavities of the mice appear to be cleared more efficiently than *Salmonella* in primary macrophage infections. Differential survival within other tested organs has been observed in infection with a variety of pathogenic bacteria, and thus the target organ has been another parameter in considering the survival ability of a mutant in the host (7, 10, 13, 20). Another possible explanation for the discrepancy in survival between macrophages and mice is the contribution of cell types other than macrophages in controlling bacterial proliferation. Geddes et al. determined the cell types targeted by *Salmonella* using flow cytometry and found that ~55% and ~18% of the infected cells were neutrophils

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and monocytes, respectively, whereas macrophages containing Salmonella were hardly detected (19). Furthermore, Salmonella was able to replicate intracellularly within neutrophils despite the short half-life and bactericidal properties of these cells. Surprisingly ~23% of infected splenic cells were B and T cells (19). Dendritic cells play a role in shunting Salmonella across the intestinal epithelial barrier and are the only cell type capable of stimulating naive T cells (41, 50). Recently, it was reported that Nramp1 expression is increased in intestinal, splenic, and bone marrow-derived dendritic cells upon infection with Salmonella infection, although the increased expression did not trigger increased clearance of bacteria (55). Based on these diverse locations of Salmonella, the roles of Nramp1 in other pathogenic cells may be important for Salmonella to either manipulate host cellular functions or subvert host immune responses and cause systemic disease.

In conclusion, our results demonstrate that the CI qPCR method is a novel technique that can be employed to enumerate multiple strains in mixed pools of bacteria in a short time within the same animal, decreasing animal-to-animal variation. This new approach to investigating the role of bacterial regulators is applicable to other pathogenic bacteria and will help elucidate how virulence is coordinated in relation to specific host factors.

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