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Enhanced Virulence of *Salmonella enterica* Serovar Typhimurium after Passage through Mice▽†

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The interaction between *Salmonella enterica* and the host immune system is complex. The outcome of an infection is the result of a balance between the *in vivo* environment where the bacteria survive and grow and the regulation of fitness genes at a level sufficient for the bacteria to retain their characteristic rate of growth in a given host. Using bacteriological counts from tissue homogenates and fluorescence microscopy to determine the spread, localization, and distribution of *S. enterica* in the tissues, we show that, during a systemic infection, *S. enterica* adapts to the *in vivo* environment. The adaptation becomes a measurable phenotype when bacteria that have resided in a donor animal are introduced into a recipient naïve animal. This adaptation does not confer increased resistance to early host killing mechanisms but can be detected as an enhancement in the bacterial net growth rate later in the infection. The enhanced growth rate is lost upon a single passage *in vitro*, and it is therefore transient and not due to selection of mutants. The adapted bacteria on average reach higher intracellular numbers in individual infected cells and therefore have patterns of organ spread different from those of nonadapted bacteria. These experiments help in developing an understanding of the influence of passage in a host on the fitness and virulence of *S. enterica*.

Salmonella enterica is a facultative intracellular pathogen capable of causing a spectrum of diseases in humans and animals. Current treatments for *S. enterica* infections are insufficiently effective, and there is a need to develop novel vaccines and therapeutics. Development of these control strategies would benefit from a more sophisticated evaluation of how the bacteria maintain their growth/survival throughout the infection in the face of changes in the host environment that may also include activation of host immune responses.

Infection of mice with *Salmonella enterica* serovar Typhimurium is an established model of systemic typhoid fever in humans. In systemic infections, *S. enterica* resides within phagocytes. Early after intravenous (i.v.) infection, a decrease in total viable bacterial numbers is observed in infected livers and spleens, as the result of a high rate of reactive oxygen radical-mediated killing that exceeds the bacterial division rate (6). After this initial phase, killing becomes negligible and the bacteria divide intracellularly at variable rates, depending on both the virulence of the infecting isolate and the level of resistance of the host (6, 7, 18). Growth of virulent *S.* Typhimurium *in vivo* is also associated with escape from infected macrophages and dissemination to other uninfected cells (1, 18). This is a necessary step for the overall net growth of the bacteria in the organs, results in the formation of new infection foci, and is likely to occur mainly as a consequence of necrosis of infected cells and establishment of new foci of infection by the released bacteria (1, 5).

Despite activation of the immune system, *S. enterica* grows at a constant rate in the spleen and liver during the first week of a systemic infection. This suggests that there is an exquisitely balanced process by which *S. enterica* adapts to allow the bacteria to retain their characteristic rate of growth in the face of the increasingly hostile environment. We have also found that the host immune response poses restraints and bottlenecks that do not equally and/or simultaneously affect all the bacteria present in the various body compartments (6, 13). Taken together these results suggest that the maintenance of bacterial fitness *in vivo* may be due to the stochastic emergence of microbial populations that have adapted to the *in vivo* environment.

In this study we set out to explore the possibility that during exposure to the *in vivo* environment, *S*. Typhimurium develops an increased ability to survive host killing and grow when reintroduced into a second naïve recipient host. We also tested whether the ability to phenotypically adapt to the *in vivo* environment is triggered by some of those factors that are known to be at the basis of the escalation of the host antimicrobial response. We have explored bacterial adaptation by transferring *in vivo*-grown bacterial populations from infected donor animals into naïve animals and by observing their growth/survival behavior and intracellular distributions within the tissues of the naïve recipient hosts. This work is a vital contribution toward our knowledge about the spatial and temporal

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dynamics of *S.* Typhimurium adaptation to the *in vivo* environment and the influence of growth and passage in a host on the fitness and virulence of *S.* Typhimurium.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *S. enterica* serovar Typhimurium strain SL1344 is a virulent wild-type strain which has a 50% lethal dose (LD_{50}) by the i.v. route of <20 CFU for BALB/c mice (9). *S.* Typhimurium SL5559 and SL5560 are sister transductants of *S.* Typhimurium C5 that differ only in O antigen type, allowing their differential identification after immunostaining (10). Preparation of electrocompetent *Escherichia coli* and *S. enterica* cells and transformations were performed as previously described (4). Media were supplemented with the appropriate antibiotic for selection (ampicillin at 100 μg/ml, kanamycin at 50 μg/ml, or chloramphenicol at 10 μg/ml). *In vitro* growth rates of *Salmonella* strains in LB broth were determined by both optical density and viable count.

Recombinant DNA techniques. Standard methods were used for molecular cloning (17). Chromosomal and plasmid DNA purifications and routine DNA modifications, including restriction endonuclease digestion of DNA, modifications of DNA, and ligations, were carried out using commercial kits and supplies, according to the manufacturers' instructions (Qiagen, Crawley, United Kingdom; Promega, Southampton, United Kingdom; Invitrogen, Paisley, United Kingdom; Roche, Lewes, United Kingdom; New England Biolabs, Hitchin, United Kingdom). DNA concentration and purity were measured using a Nanodrop ND-1000 spectrophotometer. PCR primers were designed using Primer3 software (http://frodo.wi.mit.edu/) and were purchased from Sigma-Genosys (United Kingdom). PCRs were performed in 25-μl reaction volumes in 0.2-ml Eppendorf tubes in a Perkin Elmer Gene Amp 2400 thermal cycler. Reaction mixtures contained 200 μM deoxynucleoside triphosphates, 2 mM Mg^{2+}, 0.01 volume of Proof Start DNA polymerase (2.5 U μl^{-1}; Qiagen), 0.1 volume of polymerase buffer (10×), 1 μM forward and reverse primers, and template DNA (~50 ng plasmid DNA or ~100 ng chromosomal DNA). Thermal cycler conditions were 94°C for 10 min and then 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

Chromosomal integration of antibiotic resistance cassettes in *Salmonella* strains. Individually tagged kanamycin or chloramphenicol resistance gene constructs were integrated into the chromosomes of *S.* Typhimurium strains SL5559 (C5, O4) and SL5560 (C5, O9) using a modification of the ET cloning procedure (15, 20), as previously described (14). A fragment containing the kanamycin resistance gene cassette was amplified from pACYC177 (2), using the primers ajg636 (5′-CCGCAGGTTCAGTCGGTAAAAGATGAAATGGTTGGCCTGATGAATACCGTTCAGGCATAACTCAAAATCTCTGATGTTACATTGC-3′) and ajg637 (5′-CTACGTACACCATGTCCCGCGTCGGTCAACTTCCTGTGAAAAATAGAACATATCCCTTCCTTAGAAAAACTCATCGAGCATC-3′), and a fragment containing the chloramphenicol resistance gene cassette was amplified from pACYC184 (3), using the primers ajg638 (5′-CCGCAGGTTCAGTCGGTAAAAGATGAAATGGTTGGCCTGATGAATACCGTTCAGGCATAACGACGCACTTTGCGCCGAAT-3′) and ajg639 (5′-CTACGTACACCATGTCCCGCGTCGGTCAACTTCCTGTGAAAAATAGAACATATCCCTTCCTTACGCCCCGCCCTGCCACT-3′). Approximately 1 μg of each linear PCR product was used for integration into the chromosome (between two pseudogenes, *malX* and *malY*) using a modification of the lambda red method (3), as previously detailed (14). Loss of the pBADλred helper plasmid was essentially as previously described (8), using ID Intralactam circles (MAST Diagnostics, Bootle Merseyside, United Kingdom) to screen for the absence of beta-lactamase in bacterial colonies. The resultant strains with a chromosomally located individually tagged kanamycin (SL5559Kan, O4) or chloramphenicol (SL5560Cm, O9) resistance gene cassette were confirmed by PCR and sequencing. We confirmed that the *in vitro* and *in vivo* net growth rates of the two strains, individually and in competition, were the same. Each mixed-inoculum experiment was repeated using the reciprocal combination of strains; i.e., group A received SL5559Kan grown *in vivo* and SL5560Cm grown *in vitro*, whereas group B received SL5560Cm grown *in vivo* and SL5559Kan grown *in vitro* (there was no difference between the trends in the two groups, and for conciseness only data from group A are provided).

Animals. All animals were handled in strict accordance with good animal practice as defined by the relevant international (directive 543/5 of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes, Brussels, Belgium) and local (Department of Veterinary Medicine, University of Cambridge) animal welfare guidelines. All animal work was approved by the Ethical Review Committee of the University of Cambridge and was covered by a project license granted by the Home Office under the Animal (Scientific Procedures) Act 1986. C57BL/6 mice were purchased from Harlan Olac Ltd. (Blackthorn, Bicester, United Kingdom). Mice homozygous for deletions in gp91*phox*$^{-/-}$, recombinant gamma interferon (IFN-γR$^{-/-}$), and NO synthase (NOS2$^{-/-}$) were bred at the Wellcome Trust Sanger Institute (Hinxton, Cambridge, United Kingdom). Mice homozygous for deletions in tumor necrosis factor alpha (TNF-α$^{-/-}$) were obtained from Catherine Lawrence (University of Strathclyde, Glasgow, United Kingdom). Sex- and age-matched mice between 9 and 14 weeks old were used for the experiments. Bacterial cultures were grown from single colonies in 10 ml LB broth incubated overnight without shaking at 37°C and then diluted in phosphate-buffered saline (PBS) to the appropriate concentration for inoculation. Inocula were enumerated by the use of LB agar pour plates. Mice were inoculated by injection into a lateral tail vein in a volume of 0.2 ml.

Enumeration of viable salmonellae in mouse tissues. Mice were killed by cervical dislocation, and livers and spleens were aseptically removed. Each organ was placed into a stomacher bag and homogenized in 10 ml sterile water in a Colworth stomacher 80. The resulting homogenate was diluted in a 10-fold series in PBS, and LB agar pour plates were used to enumerate viable bacteria.

Transfer of bacteria. Mice were killed by cervical dislocation, and their livers and spleens were removed aseptically. The infected organ was homogenized, using an Ultra-Turrax T25 blender, in 10 ml of distilled water. The organ homogenate was diluted in PBS prior to i.v. inoculation. For some experiments, the organ homogenate was filtered through a 0.22-μm-pore-size filter (Millipore) to remove *in vivo*-grown bacteria and was then subsequently spiked with *in vitro*-grown bacteria. The transfer of bacteria to the first naïve animal of the group was completed in less than 1 min from the time of death of the donor. To prepare the *in vivo*-grown bacteria for the experiment whose results are shown in Fig. 1, bacteria were prepared from mice at 5 h, 24 h, 48 h, or 72 h postinfection (p.i.), and this entailed infecting the donors with different doses of bacteria initially so that the numbers of bacteria per organ at these different time points p.i. were approximately the same. Thus, a C57BL/6 mouse was infected i.v. with $\log_{10}$ 6.16 CFU of *S.* Typhimurium SL1344 and killed at 5 h p.i., when the bacterial load was $\log_{10}$ 5.50 CFU in the spleen. Also, a C57BL/6 mouse was infected i.v. with $\log_{10}$ 5.92 CFU of *S.* Typhimurium SL1344 and killed at 24 h p.i., when the bacterial load was $\log_{10}$ 5.79 CFU in the spleen. Similarly, a mouse was infected with $\log_{10}$ 4.92 CFU and killed at 48 h p.i., when the splenic load was $\log_{10}$ 5.86 CFU, and another was infected i.v. with $\log_{10}$ 3.92 CFU and killed at 72 h p.i., when the load in the spleen was $\log_{10}$ 6.13 CFU. To prepare the *in vivo*-grown bacteria for the experiment whose results are shown in Fig. 2, a C57BL/6 mouse was infected i.v. with $\log_{10}$ 3.12 CFU of *S.* Typhimurium SL5559Kan and killed at 72 h p.i., when the bacterial load was $\log_{10}$ 6.36 CFU in the spleen. To prepare the *in vivo*-grown bacteria for the experiment whose results are shown in Fig. 3A and B, a gp91*phox*$^{-/-}$ mouse was infected i.v. with $\log_{10}$ 1.25 CFU of *S.* Typhimurium SL5559Kan and killed at 72 h p.i., when the bacterial load was $\log_{10}$ 6.06 CFU in the spleen. To prepare the *in vivo*-grown bacteria for the experiment whose results are shown in Fig. 3C and D, a NOS2$^{-/-}$ mouse was infected i.v. with $\log_{10}$ 4.25 CFU of *S.* Typhimurium SL5559Kan and killed at 72 h p.i., when the bacterial load was $\log_{10}$ 6.45 CFU in the spleen. To prepare the *in vivo*-grown bacteria for the experiment whose results are shown in Fig. 3E and F, a TNF-α$^{-/-}$ mouse was infected i.v. with $\log_{10}$ 2.54 CFU of *S.* Typhimurium SL5559Kan and killed at 72 h p.i., when the bacterial load was $\log_{10}$ 5.10 CFU in the spleen. To prepare the *in vivo*-grown bacteria for the experiment whose results are shown in Fig. 3G and H, an IFN-γR$^{-/-}$ mouse was infected i.v. with $\log_{10}$ 3.23 CFU of *S.* Typhimurium SL5559Kan and killed at 72 h p.i., when the bacterial load was $\log_{10}$ 6.66 CFU in the spleen.

Serum preparation. Blood was collected from the lateral tail vein, incubated for 90 min at room temperature, and then centrifuged at 4,000 × *g* in a microcentrifuge for 3 min. Serum was removed and then incubated at 56°C for 45 min.

Immunostaining for fluorescence microscopy. Half of each organ was fixed overnight in 4% paraformaldehyde diluted in PBS, washed for 90 min in three changes of PBS, and then immersed in 20% sucrose (in PBS) for 16 h at 4°C before being embedded in optimal cutting temperature compound (OCT; Raymond A Lamb Ltd., Eastbourne, United Kingdom) in cryomolds (Park Scientific, Northampton, United Kingdom). Samples were frozen and stored at −80°C. Sections of 30 μm were cut, blocked, and permeabilized for 10 min in a permeabilizing solution containing 10% normal goat serum and 0.02% saponin in PBS (Sigma, Poole, United Kingdom). Liver sections were stained with a 1:500 dilution of rat anti-mouse CD18^{+} monoclonal antibody (clone M18/2; BD Pharmingen), together with a 1:200 dilution of Alexa Fluor 488-conjugated rabbit anti-lipopolysaccharide O4 (anti-LPS O4) monoclonal antibody (custom synthesis; Molecular Probes, OR) and a 1:500 dilution of rabbit anti-LPS O9 agglutinating serum diluted in permeabilizing solution, for 16 h at 4°C. Subsequently, sections

were washed in PBS (three 30-min washes) and then incubated with 1:200 Alexa Fluor 568-conjugated goat anti-rat antibody (Invitrogen-Molecular Probes, Paisley, United Kingdom) and a 1:200 dilution of Alexa Fluor 647-conjugated goat anti-rabbit antibody (Invitrogen-Molecular Probes). All sections were washed in PBS (three 30-min washes) and mounted onto Vectabond-treated glass slides (Vector Laboratories Ltd.) using Vectashield containing 4′,6-diamidino-2-phenylindole (Vector Laboratories Ltd.). The analysis of tissue sections was by multicolor fluorescence microscopy (MCFM) using a Leica DM6000B fluorescence microscope running FW4000 acquisition software. Intracellular bacterial distributions per group were based on the counts obtained from 600 infected liver phagocytes from tissue obtained from four mice per group.

Statistical analysis. All data analysis and figures were produced using the open-source R statistical language (16). Three replicate measurements were obtained for each mouse, and the data represent the means of these measurements. Where the *in vivo*-adapted and *in vitro*-grown bacteria were inoculated into different animals (e.g., see Fig. 1), the data consist of five mice per bacterial type (*in vitro* grown or *in vivo* grown) per time point. Where a mixture of the bacterial types (*in vitro* grown and *in vivo* grown) was inoculated into the same animal (e.g., see Fig. 2 and 3), each mouse contributes a difference in the bacterial counts calculated by taking the mean for the *in vitro*-grown replicates from the mean for the *in vivo*-adapted replicates. This is necessary to account for within-host correlation. Hence, in this case there are four data points per time point rather than at each time point for each bacterial type (we used only four rather than five mice for the double-infection experiments). Data for the liver and spleen were analyzed separately.

In order to explore how the net bacterial counts differed between different groups, we fitted a linear regression model to each data set. For the experiments in which the mixture of bacterial types is inoculated into the same animal (see Fig. 2 and 3) the model included a categorical time effect, and for the experiments where different mice were inoculated for each bacterial type (see Fig. 1), we included time and group main effects and a time-group interaction effect. In all cases, likelihood ratio tests required the presence of the main effects, and where appropriate, the time-group interaction effect was also selected in all except one analysis (in the spleen for the 5-h *in vivo*-adapted bacteria [see Fig. 1B], though for comparative purposes we included the mean and 95% confidence interval [CI] for this difference in Fig. 1F).

To extract the relevant comparisons of interest and to adjust for multiple comparisons, we used the multcomp package in R (11). Note that we are looking to compare the relative mean differences in $\log_{10}$ bacterial counts at each time point (after adjusting for the previous time point) between each of the bacterial types. The size of the relative differences and the corresponding 95% CIs are provided in Fig. 1 to 3, and Table S1 in the supplemental material presents all of these results.

To explore the trends in the proportions of intracellular bacteria between the two competing groups, we performed chi-square tests for trend. These are more powerful than conventional chi-square tests when there is an implicit ordering to the groups (in this case, the number of intracellular bacteria), since it explicitly tests for linear trends in the data. We classified intracellular bacterial numbers into six groups (1, 2, 3 to 5, 6 to 10, 11 to 20, and ≥21). We can summarize the relationships through the use of odds ratios (ORs). For example, for a single group (e.g., *in vitro*-grown *S. enterica*), the odds of having two bacteria per cell relative to one bacterium per cell is simply the number of cells containing two bacteria divided by the number containing a single bacterium. Therefore, the odds give a comparative measure of how likely the two events (two bacteria per cell versus one bacterium per cell) are within each group. If we have more than one group (i.e., *in vivo*- versus *in vitro*-grown *S. enterica*), we can use the ratio of the odds between each group to summarize the relative odds. Therefore, an OR of 1 means that the odds of having two bacteria per cell compared to one bacterium per cell is the same for both *in vivo*- and *in vitro*-grown *S. enterica*. An OR value of >1 would indicate that there are increased odds of having more bacteria per cell with the *in vivo*-grown than the *in vitro*-grown bacteria, and an OR value of <1 indicates the opposite. All ORs in Table 1 are relative to the group containing a single bacterium.

TABLE 1. Test for differences between groups[a]

OR for the following no. of bacteria per phagocyte:						*P* value
1	2	3–5	6–10	11–20	≥21	
1.00	1.49	3.16	4.4	5.89	Infinite	2.2×10^{-16}

[a] Test for differences between groups (i.e., *in vivo*-grown versus *in vitro*-grown *Salmonella*) in the proportion of total infected host $CD18^+$ phagocytes containing 1, 2, 3 to 5, 6 to 10, 11 to 20, or ≥21 bacteria by a chi-square test for trend. Each column shows the OR between the groups (i.e., SL5559Kan *in vivo*-grown versus SL5560Cm *in vitro*-grown *Salmonella*) relative to the data in the first column (i.e., one bacterium per cell). The chi-square test for trend assesses the existence of this trend in the ORs.

RESULTS AND DISCUSSION

Identification of an increased net growth rate of *in vivo*-adapted *Salmonella*. We tested the effect of *in vivo* bacterial growth on the ability of *S.* Typhimurium to survive host killing and grow when it was reintroduced into a second naïve recipient host. C57BL/6 mice were infected i.v. with either *in vivo*- or *in vitro*-grown *S.* Typhimurium. The *in vivo*-grown bacteria were prepared from donor mice at 5 h, 24 h, 48 h, or 72 h p.i. and reintroduced into naïve animals, and bacterial numbers in livers and spleens were determined at various times p.i. Note that the analysis for the *in vitro*- versus *in vivo*-grown bacteria at 5 h p.i. was conducted separately due to the fact that the experiment was conducted independently and the $\log_{10}$ count for the inoculum was slightly different. Figures 1A to D show the absolute $\log_{10}$(CFU) counts for each of the comparisons. However, it is of key interest here to compare relative differences between the bacterial types (*in vitro* grown and *in vivo* grown) between consecutive time points (Fig. 1E to H). At between 0 and 6 h p.i., we observed that there was little evidence to suggest that exposure to the *in vivo* environment conferred on *Salmonella* an increased ability to resist the early *in vivo* bacterial killing within spleen and liver phagocytes compared to that of the *in vitro*-grown bacteria (top panels of Fig. 1E and F). A possible exception might be in the liver for the comparison of the bacteria grown *in vivo* for 5 h, though this is not matched by the results for the spleen. For this reason and because of the results of the later experiments, we consider that it is likely to be an anomaly and due to the low number of bacteria counted at the 6-h time point. At between 6 and 48 h p.i., we found that with the exception of the bacteria grown *in vivo* for 5 h, each of the other *in vivo*-grown bacteria (obtained from donor mice at 24 h, 48 h, and 72 h p.i.) exhibited higher growth rates than the *in vitro*-grown bacteria (bottom panels of Fig. 1E and F). On average, the $\log_{10}$(CFU) values at between 6 and 48 h were larger by 0.44, 0.84, and 0.89 in the liver for bacteria grown *in vivo* for 24 h, 48 h and 72 h, respectively, compared to the bacteria grown *in vitro* and likewise were larger by 1.12, 1.32, and 1.24, respectively, in the spleen. Furthermore, there was some evidence to suggest that there might be a further advantage conferred by the *in vivo*-grown bacteria harvested from donor mice at 48 and 72 h p.i. over that conferred by the bacteria grown *in vivo* for 24 h at between 6 and 48 h (Fig. 1G and H) in the liver, but this was not mirrored in the spleen. In any case, this additional interaction effect becomes negligible between the groups grown *in vivo* for 48 h and 72 h. This suggests that the adaptation of *Salmonella* in the donor animal most likely occurs at between 5 h and 24 h p.i. and is not further enhanced by continued growth in the donor animal.

To rule out the possibility that any increased rate of growth of the *in vivo*-grown bacteria was due to a nonbacterial immunosuppressive or toxic soluble factor present in the tissue homogenate injected with the bacteria, we included groups of

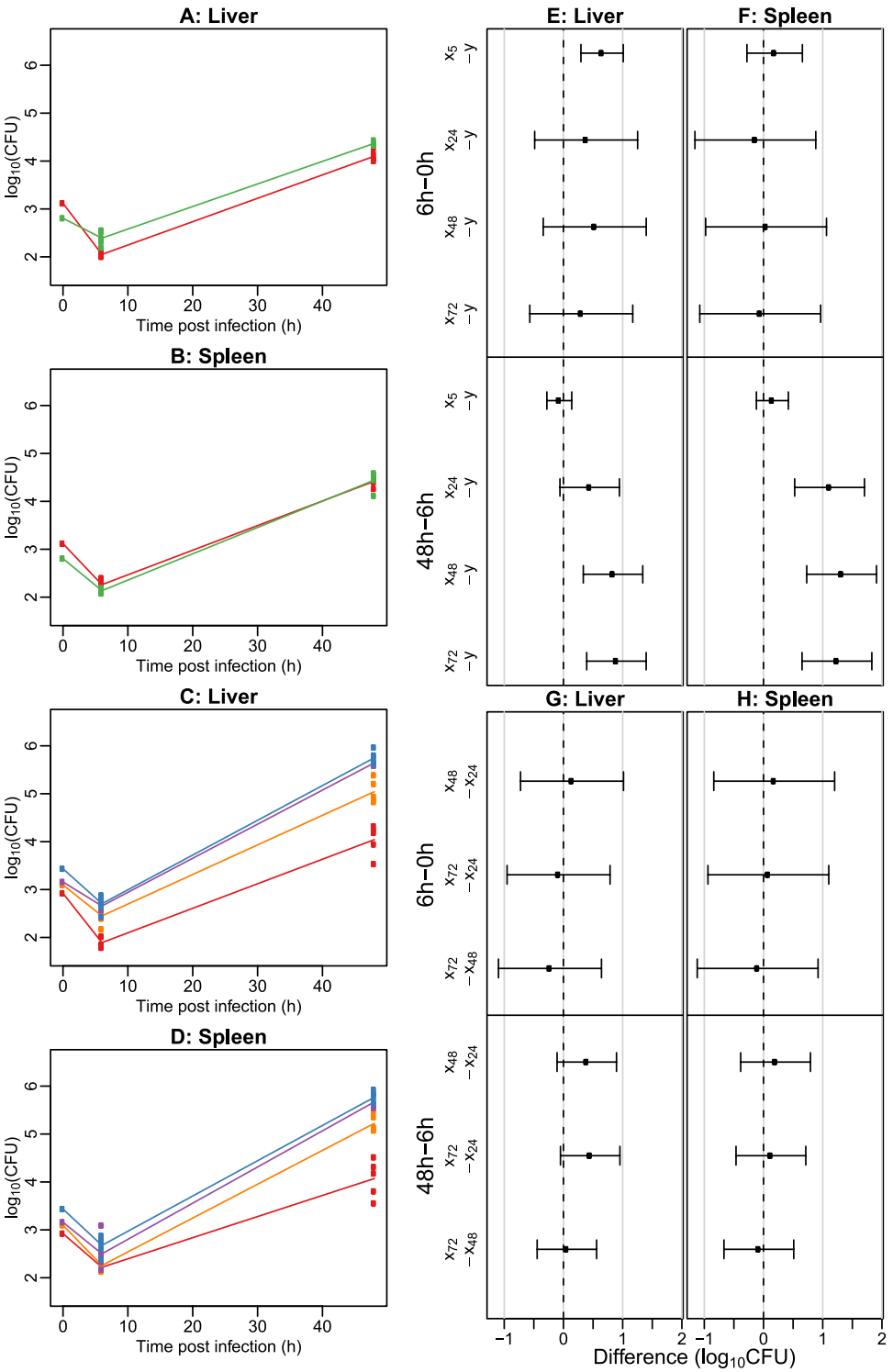

FIG. 1. Identification of an enhanced-net-growth-rate phenotype of *in vivo*-grown *S.* Typhimurium. C57BL/6 mice were infected i.v. with either *in vivo*-grown *S.* Typhimurium prepared from mice at 5 h p.i. (green) or *in vitro*-grown SL1344 (red) as a control (A and B) or *in vivo*-grown *S.* Typhimurium prepared from mice at 24 h (orange), 48 h (purple), or 72 h p.i. (blue) or *in vitro*-grown SL1344 (red) as a control (C and D). Bacterial numbers in livers and spleens were determined at 6 h and 48 h p.i. Results are expressed as mean $\log_{10}$ viable count (lines) and $\log_{10}$ viable counts from individual mice (circles). $n = 5$ mice per group. (E to H) Means and adjusted 95% CIs for the differences in $\log_{10}$(CFU) counts between each consecutive time point for each pair of bacterial types. X stands for *in vivo* grown and is followed by the amount of time that the bacteria were grown in the donor animal, and Y stands for *in vitro* grown. If the CIs do not cross zero, then this is equivalent to ascertaining statistical significance. Gray lines correspond to 1 $\log_{10}$ unit.

mice that received filtered organ homogenate spiked with *in vitro*-grown bacteria. No acceleration of the bacterial net growth rate was seen in mice receiving these spiked organ homogenates (data not shown), indicating strongly that growth of the bacteria themselves *in vivo* is a prerequisite for the enhanced-growth phenotype and that the organ homogenates are having no effect. We also observed that there were no differences in the net growth rates in the recipients of bacteria harvested from livers versus those isolated from spleens of donor mice (data not shown) and, therefore, that the adaptation is not a function of the organ from which the bacteria were recovered.

Thus, during a systemic infection, *Salmonella* bacteria grown *in vivo* are able to grow faster upon direct injection into naïve recipient mice. This enhanced growth becomes a measurable phenotype only when the bacteria have resided in the donor animal for more than 5 h. The organ from which the bacteria have been isolated is irrelevant to the final phenotype. When the bacteria are introduced into a recipient animal, the enhanced-growth phenotype does not appear to confer increased resistance to the early reactive oxygen intermediate (ROI)-mediated host killing mechanisms but can be detected as an enhancement in net growth rates in a phase of the infection (6 h onwards) when bacterial division is controlled by bacteriostatic host resistance mechanisms (6).

The adaptation seen in *in vivo*-grown bacteria is reversible by *in vitro* passage. To test whether the increased net growth rate of the *in vivo*-grown bacteria was due to a reversible phenotype or to selection of a stable trait (e.g., selection of a hypervirulent bacterial mutant), we passaged the *in vivo*-grown bacteria in LB broth for 16 h and then reintroduced the passaged bacteria into C57BL/6 mice. The experiment revealed that the increased-net-growth-rate phenotype was transient, with a single overnight passage in LB broth being sufficient to reverse the increased net growth rate of the *in vivo*-grown *S.* Typhimurium bacteria (data not shown). We investigated whether a soluble factor in serum was responsible for the observed increase in growth by comparing systemic infections in conventional C57BL/6 mice infected i.v. with *S.* Typhimurium grown for 16 h in either LB broth or mouse serum. The enhanced-growth phenotype was not conferred by growing the bacteria in serum. Moreover, the increased net growth rate of the *in vivo*-grown bacteria could not be further exacerbated by repeated passage through a second donor animal. Thus, the enhanced-growth phenotype does not breed true, in that it is lost upon passage *in vitro*. It is therefore most likely to be due to a transient phenotypic change dependent on changes in gene regulation in response to environmental cues and not to selection of a mutant subpopulation with an inherited enhanced-growth-rate phenotype.

Enhanced growth of *in vivo*-adapted bacteria is not due to generalized suppression of the host immune system of the recipient animal. Our initial experiments were performed by injecting different groups of mice with either *in vitro*- or *in vivo*-grown bacteria. We initially concluded that the enhanced-growth phenotype was due to a change in the ability of the *in vivo*-grown bacteria to grow in the naïve recipients, but there is a formal possibility that the *in vivo*-grown bacteria are actually affecting the responses of the recipients, such that *in vitro*-grown bacteria coinjected simultaneously with those grown *in vivo* would also display enhanced growth. To test this we performed simultaneous infections where we inoculated *in vivo*- and *in vitro*-grown bacteria into the same animal via a single injection. To be able to do this experiment, we used two nearly isogenic *S.* Typhimurium sister transductants (strains SL5559Kan and SL5560Cm) that can be distinguished on plates by their different antibiotic resistance profiles and which express different LPS O antigens and that can thus be differentially visualized in tissue sections by immunostaining (9, 10).

In all of these experiments, it was clear that the growth rates of the different bacteria, when they were injected simultaneously, were similar to those seen when the two strains had been injected individually. Figures 2A and C show the absolute $\log_{10}$(CFU) counts in recipient animals for the *in vivo*-grown bacteria harvested from the donor mouse at 72 h p.i. These plots are slightly misleading, because each mouse contributed two measurements to the plot, one for the *in vitro*-grown bacteria and one for the *in vivo*-grown bacteria. In reality, we are interested in comparing the differences between the *in vivo*- and *in vitro*-grown bacteria within each animal, and these are shown in Fig. 2B and D. It can be seen from these plots that the viable counts of *in vitro*- and *in vivo*-grown bacteria decreased to similar levels (6 h p.i.) in the recipient naïve mice, but the *in vivo*-grown bacteria recovered from the donor animal at 72 h p.i. showed an enhanced growth rate at between 6 and 48 h. On average, these differences were 1.28 and 1.04 $\log_{10}$(CFU) greater for the *in vivo*-grown bacteria recovered from the liver and spleen, respectively (Fig. 2E and F). The growth rates (in the naïve mice) were similar for both the *in vitro*- and *in vivo*-grown bacteria at between 48 and 72 h p.i. Thus, the *in vitro*-grown and the *in vivo*-grown bacteria grow at different rates in the same recipient animal, and the enhanced growth rate of the *in vivo*-grown bacteria is very unlikely due to a generalized effect of the *in vivo*-grown bacteria on the host immune system.

Intracellular distributions of *in vivo*- and *in vitro*-grown *Salmonella* within phagocytes in infected livers. Our previous work on the spread and distribution of *S.* Typhimurium in the tissues of infected mice indicated that bacterial growth is paralleled by increases in the number of infected cells resulting in dispersive infections (6, 7, 17). This is indicated by the fact that intracellular bacterial loads per cell remain generally skewed toward low numbers, despite the observed increases in total bacterial numbers in a given organ. Mathematical modeling predicts that this is due to stochastic density-dependent lysis of the infected cells (1). In addition, we have found that intracellular bacterial densities correlate with the net growth rate of a particular *Salmonella* isolate in a given mouse strain (i.e., faster-growing strains have distributions of intracellular bacteria that are more skewed toward higher counts) (1). Therefore, it might be expected that the higher division rate of the *in vivo*-grown bacteria should lead to intracellular bacterial loads per cell in the tissues of a recipient naïve mouse higher than those of the slower-dividing *in vitro*-grown bacteria.

To test this hypothesis, we analyzed tissue sections from the livers of mice simultaneously infected with *S. enterica* grown *in vivo* or *in vitro*. We found that the distributions of the numbers of bacteria per cell were different between the two populations within the same animals, with the *in vivo*-grown bacteria reaching higher bacterial loads per cell than those grown *in vitro*, on

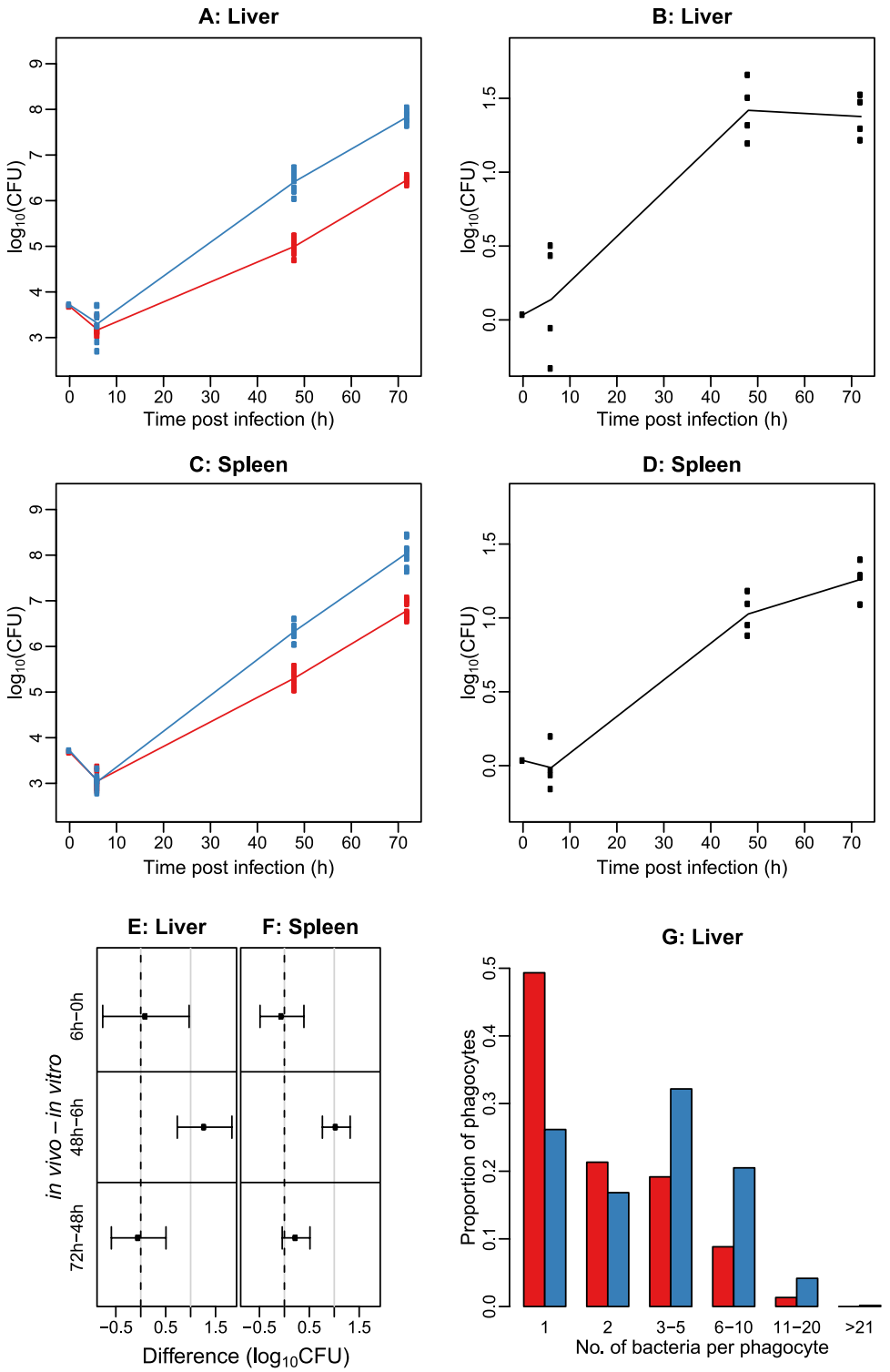

FIG. 2. We performed simultaneous infections where we inoculated *in vivo*- and *in vitro*-grown bacteria into the same animal via a single injection. C57BL/6 mice were infected i.v. with *in vitro*-grown SL5560Cm (red) and *in vivo*-grown (72 h p.i.) SL5559Kan (blue). Bacterial numbers in livers (A) and spleens (C) (using antibiotic-containing media) were determined at 6 h, 48 h, and 72 h p.i. Results are expressed as mean $\log_{10}$ viable counts (lines) and $\log_{10}$ viable counts from individual mice (circles). $n = 4$ mice per group. (E and F) Means and adjusted 95% CIs for the differences between the $\log_{10}$(CFU) counts at consecutive time points (shown in panels B and D, respectively). Gray lines correspond to 1 $\log_{10}$ unit. (G) Intracellular distributions of *in vivo*- and *in vitro*-grown *Salmonella* within phagocytes in infected livers. The figure shows the proportion of infected phagocytes in the liver relative to the numbers of bacteria contained within each phagocyte at 72 h p.i. Blue bars, SL5559Kan grown *in vivo*; red bars, SL5560Cm grown *in vitro*.

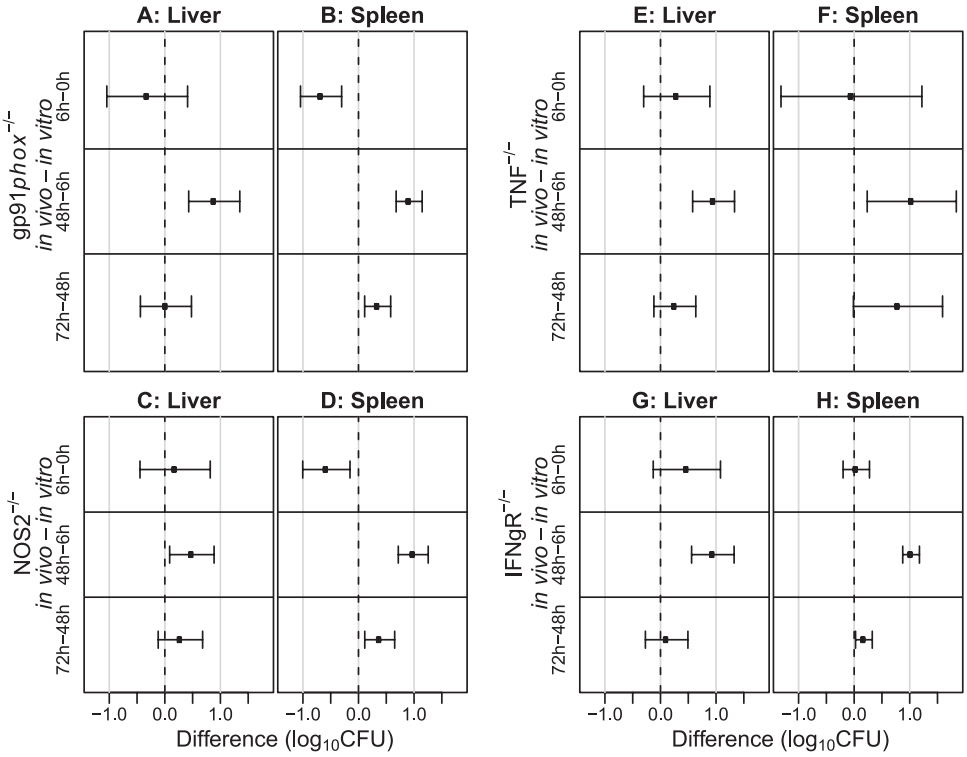

FIG. 3. We performed simultaneous infections where we inoculated *in vivo*-grown SL5559Kan and *in vitro*-grown SL5560Cm bacteria into the same animal (C57BL/6 mice) via a single injection. *In vivo*-grown bacteria were prepared from a gp91*phox*$^{-/-}$ mouse (A and B), an NOS2$^{-/-}$ mouse (C and D), a TNF-$\alpha^{-/-}$ mouse (E and F), and an IFN-γR $^{-/-}$ mouse (G and H). Bacterial numbers in livers and spleens (using antibiotic-containing media) were determined at 6 h, 48 h, and 72 h p.i. $n = 4$ mice per group. These plots show the means and adjusted 95% CIs for the differences between the $\log_{10}$(CFU) counts at consecutive time points. Gray lines correspond to 1 $\log_{10}$ unit.

average (Fig. 2G and Table 1). Thus, the enhanced-growth-rate phenotype seen with *in vivo*-grown bacteria is related to higher numbers of bacteria per cell. This is in line with predictions generated by our mathematical models of systemic infection with *S.* Typhimurium. The results also allow us to reject the hypothesis that the enhanced growth rates of the *in vivo*-grown bacteria are due to the acquisition of increased dispersiveness of the infection, which would have led to lower intracellular densities per cell.

The enhanced-growth-rate phenotype is not triggered by key known mechanisms of early resistance to *Salmonella*. The environment in the donor animals is highly likely to be modified by escalating inflammatory and immune responses. Environmental changes secondary to these responses may well be driving bacterial adaptation, leading to the enhanced-growth phenotype. We therefore tested whether the adaptation phenotype that determines faster bacterial net growth in recipient animals is triggered by a bacterial response to a number of known immunological mechanisms that are known to control the early phases of an *S.* Typhimurium infection in mice. This was tested for by evaluation of some of the more likely responses by using gene-knockout animals as donors for the transfer experiments. Mice with impaired ROI-dependent (gp91*phox*$^{-/-}$) or reactive nitrogen intermediate (RNI)-dependent (NOS2$^{-/-}$) antibacterial macrophage functions, those with deficient recruitment of phagocytes into the tissues (TNF-$\alpha^{-/-}$ mice), or those with deficient phagocyte activation (IFN-γR$^{-/-}$ mice) (6, 12, 19) were all tested. We found that bacteria grown in the tissues of these animals with gene-targeted immunodeficiency acquired the adapted phenotype as well as those grown in the tissues of immunocompetent mice did, resulting in a similar enhancement of bacterial net growth (>6 h p.i.) when the microorganisms were reintroduced into a naïve recipient mouse (Fig. 3A to H). This trend is true for the four knockout strains tested, although there are a couple of instances where the effect is not statistically significant (the full set of results is provided in Table S1 in the supplemental material). Taken together, it is unlikely that one of the key host resistance factors tested in this study is the trigger of the acquired phenotype that allows *S.* Typhimurium to grow faster in a recipient mouse. The basis of the adaptation is unclear, but it is clearly not in response to several of the most likely and important innate immune effector mechanisms, given that ablation of NADPH oxidase, NO synthase, IFN-γ, and TNF-α in donor animals had no effect on the enhanced-net-growth-rate phenotype observed using bacteria isolated from them.

Concluding remarks. In this study we have found that during infection *S. enterica* adapts such that when it is reintroduced into a naïve recipient animal it is able to grow at an increased net rate. This trait is not acquired during growth in serum and requires persistence in the donor animal for more than 5 h. Enhanced growth is thus not due simply to brief exposure to the *in vivo* environment *per se* but is acquired upon exposure to the host environment later in the infection process, which itself

is likely to be a consequence of an undetermined change that occurs in the host tissues. The enhanced growth is lost upon passage *in vitro*, and it is therefore most likely to be due to a transient adaptation of the organism in response to factors particular to the host and not to natural selection of a fitter subpopulation of the bacteria that has arisen through mutation. The adapted phenotype does not appear to prevent the early killing that normally occurs in the first 5 to 6 h after i.v. injection but confers a growth advantage in the later phase of the infection (>6 h p.i.), when the bacteria are exposed to bacteriostatic host responses. The fact that the adapted phenotype is not induced in the donor mice during the first 5 h of infection suggests that the as yet undetermined trigger for bacterial adaptation is absent in the host tissues in the very early stages of the disease. However, intriguingly, the host environment in these early stages of the infection must be providing signals that are sufficient for the bacteria to retain an already acquired adapted phenotype.

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