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Competition and Resilience between Founder and Introduced Bacteria in the *Caenorhabditis elegans* Gut

Cynthia Portal-Celhay and Martin J. Blaser

Department of Medicine and Microbiology, New York University School of Medicine, New York, New York, USA

The microbial communities that reside within the intestinal tract in vertebrates are complex and dynamic. In this report, we establish the utility of *Caenorhabditis elegans* as a model system for identifying the factors that contribute to bacterial persistence and for host control of gut luminal populations. We found that for N2 worms grown on mixed lawns of bacteria, *Salmonella enterica* serovar Typhimurium substantially outcompeted *Escherichia coli*, even when *E. coli* was initially present at 100-fold-higher concentrations. To address whether innate immunity affects the competition, the *daf-2* and *daf-16* mutants were studied; their total gut bacterial levels reflect overall capacity for colonization, but *Salmonella* outcompeted *E. coli* to an extent similar to wild-type worms. To address the role of virulence properties, *Salmonella* Δ*spi-1* Δ*spi-2* was used to compete with *E. coli*. The net differential was significantly less than that for wild-type *Salmonella*; thus, *spi-1 spi-2* encodes *C. elegans* colonization factors. An *E. coli* strain with repeated *in vivo* passage had an enhanced ability to compete against an *in vitro*-passed *E. coli* strain and against *Salmonella*. Our data provide evidence of active competition for colonization niches in the *C. elegans* gut, as determined by bacterial factors and subject to *in vivo* selection.

The microbial communities that reside within the intestinal tract in vertebrates are complex, and their populations are dynamic (11, 25, 43–45, 49). However, little is known about how microorganisms establish and maintain stable colonization of their hosts. How do different species found and maintain a presence in the intestinal ecosystem? How do they interact with their host and with other microbial community members? In which regions of the intestine is colonization preferred? Why are some strains favored over others? Attempts to establish suitable animal models to address these questions using pigs, chicks, mice and other hosts are limited by difficulties in animal handling, including needs for xenobiosis, and establishing colonization with limited numbers of bacterial species is expensive (51, 59). However, reliable animal models are needed for assessing the contribution of both bacterial and host factors to the process of intestinal colonization.

Simplified invertebrate model systems facilitate the understanding of the mechanisms underlying the interactions between gut microorganisms and their host. *Caenorhabditis elegans*, a free-living soil nematode that feeds primarily on bacteria (10), possesses important attributes, permitting it to serve as a model host to address such questions. Its rapid generation time, ease of propagation, well-defined cell lineage, fully sequenced genome containing a large number of vertebrate orthologues (50), and genetic tractability has aided the study of many biological processes, including microbial pathogenesis (1, 2, 8, 14, 15, 21, 22, 24, 29, 31, 39, 57) and immunity (26, 30, 32, 36, 40, 41, 52, 55, 56). *C. elegans* possesses evolutionarily conserved signaling pathways for innate immunity, especially those involving the DAF-2 insulin/IGF-I-like receptor (12, 19, 20, 48), p38 MAP kinase (37, 38, 58), and transforming growth factor β (TGF-β) (16, 46), which regulate an array of antibacterial effector molecules, including lysozymes, lipases, and C-type lectins (18, 26, 47).

The *C. elegans* gut, composed of 20 epithelial cells arranged to form a tube with a central lumen (53), can harbor large bacterial populations during adult life and thus can be used as a model for commensalism. When *C. elegans* is grown on bacterial lawns, most of the ingested bacterial cells are killed by the pharyngeal grinder (6, 7); however, some escape and can retain viability within the *C. elegans* gut, phenomena observed for both *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (3, 42). We sought to harness this property as a way to understand both bacterial persistence and competition within the *C. elegans* lumen. We hypothesized that selection occurs within the *C. elegans* gut, leading to dominance, coexistence, or replacement of competing bacteria. After hypothesizing that both host selection and particular strain characteristics affect the outcome of the competition, we used competition between *E. coli* and *Salmonella* in hosts with specific immune phenotypes to address questions that pertain to the establishment of commensalism.

MATERIALS AND METHODS

***C. elegans* strains and growth conditions.** All strains were provided by the *Caenorhabditis* Genetic Center and maintained on modified (0.35% peptone) nematode growth medium (mNGM), using standard procedures (54).

Bacterial strains, plasmids, and growth conditions. *E. coli* OP50 (10) and *S. enterica* serovar Typhimurium SL1344 (60) have been described. *S.* Typhimurium SL1344 containing plasmid pSMC21 (9) and the Δ*spi-1* Δ*spi-2* mutant were kindly provided by Fred Ausubel (3) and Heran Darwin, respectively. Cultures were grown in Luria-Bertani (LB) broth at 37°C supplemented or not with ampicillin (100 μg/ml), kanamycin (50 μg/ml), streptomycin (60 μg/ml), and tetracycline (20 μg/ml). Bacterial lawns used for *C. elegans* life span assays were prepared by spreading 25 μl of an overnight culture of the bacterial strains on 3.5-cm-diameter

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Address correspondence to Martin J. Blaser, Martin.Blaser@med.nyu.edu.

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype	Source and reference
Strains		
E. coli		
OP50	Uracil auxotroph	Caenorhabditis Genetics Center (10), University of Minnesota
OP50mgh	Uracil auxotroph, Str^r	F. M. Ausubel, Harvard Medical School
S. Typhimurium		
SL1344	Wild type, Str^r	F. M. Ausubel (3)
spi-1 spi-2 strain	ΔSPI-I and ΔSPI-II, Km^r Cm^r	K. H. Darwin, NYU School of Medicine
Plasmids		
pSMC21	Amp^r Km^r, GFP	G. V. Bloemberg (9), Harvard Medical School
pGB5	Amp^r Tet^r, GFP	G. V. Bloemberg (9)
pRZT3	Tet^r, DsRed	W. Bitter (49), VU Medical Centre, Amsterdam, Netherlands

mNGM agar plates. Plates were incubated overnight at 37°C and cooled to room temperature before use. Bacterial strains and plasmids used in this study are listed in Table 1.

***In vitro* competition assays.** To determine *in vitro* competition between bacterial strains at 25°C, the temperature used for the *in vivo* studies, single colonies of *E. coli* and *S.* Typhimurium SL1344 or the *Salmonella* Δ*spi-1* Δ*spi-2* mutant were used to inoculate LB broth. Cultures were incubated overnight at 37°C and, the next morning, adjusted to the same optical density (0.250). One milliliter of each culture was then used to inoculate 10 ml of fresh LB broth supplemented with streptomycin (60 μg/ml) to control contamination, since both strains are streptomycin resistant. Tubes then were incubated at 25°C, and samples were obtained every 3 to 6 h to determine the number of bacterial CFU. Dilutions were plated on MacConkey agar supplemented with streptomycin (60 μg/ml) to identify the two competing bacterial populations.

Life span assays. *C. elegans* life span determinations essentially followed established methods (3, 23). However, to avoid competition between introduced bacterial strains, nematodes were age synchronized by a bleaching procedure as described previously (54), and then embryos were incubated at 25°C on mNGM agar plates containing *E. coli* OP50, *S.* Typhimurium SL1344, or its Δ*spi-1* Δ*spi-2* mutant. The fourth larval stage (L4) was designated day 0 for our studies, and worms were transferred daily to fresh plates to eliminate overcrowding by progeny and until they laid no further eggs. Worm mortality was scored over time, with death defined when a worm no longer responded to touch (35). Worms that died of protruding/bursting vulva, bagging, or crawling off the agar were excluded from the analysis (5). Kaplan-Meier survival analysis was performed using GraphPad Prism. For each bacterial lawn, the time required for 50% of the worms to die (TD_{50}) in each mutant population was compared to that in the wild-type population, using a paired *t* test. A *P* value of <0.05 was considered to reflect significant differences from the control. A total of 100 worms were used in each life span experiment, and all experiments were performed at least in duplicate.

Bacterial colonization assay. Nematodes were age synchronized by bleaching (54), and embryos were incubated at 25°C on mNGM agar plates containing *E. coli* OP50, *S.* Typhimurium SL1344, or SL1344 Δ*spi-1* Δ*spi-2*, as described above, to prepare for the bacterial colonization assays. Bacterial colonization of *C. elegans* was determined using a method adapted from Garsin et al. (24) and others (4; R. A. Alegado, personal communication). At each time point tested, 10 worms were picked and placed on an agar plate containing 100 μg/ml gentamicin to remove surface bacteria. The worms then were washed in 5-μl drops of 25 mM levamisole in M9 buffer (LM buffer) for paralysis and inhibition of pharyngeal pumping and expulsion, were washed twice more with LM buffer containing 100 μg/ml gentamicin, and washed twice more with M9 buffer alone. The washed nematodes then were placed in a 1.5-ml Eppendorf tube containing 50 μl of phosphate-buffered saline (PBS) buffer with 1% Triton X-100 and mechanically disrupted using a motor pestle. Worm lysates were diluted in PBS buffer and incubated overnight at 37°C on MacConkey agar. Lactose-fermenting (*E. coli*) and nonfermenting (*Salmonella*) colonies were quantified and used to calculate the number of bacteria per nematode. We have previously reported the bacterial intestinal colonization of wild-type, *daf-2*, and *daf-16* worms (C. Portal-Celhay, E. R. Bradley, and M. J. Blaser, submitted for publication).

Shift and competition assays. To examine the persistence of colonization after a change ("shift") in bacterial lawns, *C. elegans* embryos were grown on NGM agar plates seeded with a founder bacterial strain (either *E. coli* OP50-pSMC21 or *S.* Typhimurium SL1344-pSMC21). At either day 2 (48 h) or day 4 (96 h) of adulthood (L4 plus 2 or L4 plus 4, respectively), worms were washed as described above and transferred to plates seeded with a second (introduced) bacterial strain (either *E. coli* OP50-pGB5 or *S.* Typhimurium SL1344-pGB5). After 24 h, 10 worms were washed, homogenized by grinding, and plated on selective agars to quantify the two distinct (founder and introduced residual) bacterial populations.

To evaluate the competition to colonize *C. elegans*, lawns were composed of two bacterial species. Embryos were initially grown on NGM agar plates seeded with *E. coli* OP50 (Str^s). At day 3 of adulthood, worms were washed and transferred to new plates containing streptomycin and mixed lawns of *E. coli* OP50mgh (Str^r) and *S.* Typhimurium SL1344 (Str^r), with different ratios (0.1:10, 10:10, and 10:0.1) of the two bacterial strains. Worms were allowed to feed on the mixed lawns at 25°C for 6 h and then transferred back to plates seeded with *E. coli* OP50 (Str^s). After an additional 24 h, 10 worms for each condition were washed, homogenized by grinding, and plated on MacConkey agar supplemented with streptomycin (to eliminate the Str^s *E. coli* cells) to quantitate the two founding and competing Str^r bacterial populations.

Adaptation assay. *C. elegans* strain N2 was fed on mixed lawns of *E. coli* OP50mgh (Str^r) and *S.* Typhimurium SL1344 (Str^r) at a 10:10 ratio, allowed to feed for 6 h, and transferred back to plates seeded with *E. coli* OP50 (Str^s). After an additional 24 h, 10 worms were washed, homogenized by grinding, and plated on MacConkey agar supplemented with streptomycin. A single colony of *E. coli* was reisolated and expanded *in vitro* by subculturing. This *in vivo* passage was performed 10 times, and the sequentially *in vivo*-passaged *E. coli* cells were subsequently used for the creation of mixed (*Salmonella* and *E. coli*) bacterial lawns and for competition assays. In this way, *in vitro*- and *in vivo*-passaged *E. coli* cells could be compared in terms of *in vivo* fitness vis a vis *Salmonella* cells.

Fluorescence microscopy. Worms were washed and placed on a pad of 2% agarose in 5 μl of M9 buffer, with 30 mM sodium azide as an anesthetic. When the worms stopped moving, a coverslip was placed over the pad, and worms were examined by fluorescence microscopy using a Leica DMI 6000B inverted microscope.

Statistical analysis. All assays were repeated multiple times. Linear regression analysis was done using Sigma Plot V.10. Data were analyzed using two-sample *t* tests, assuming equal variances. A *P* value of <0.05 was considered significantly different from the control.

RESULTS

Host age and genotype determine the nature of persistent bacterial colonization of the *C. elegans* gut. In prior work, we characterized bacterial proliferation inside the *C. elegans* gut by quantifying viable bacteria and assessing variation in mutants with specific immune phenotypes (Portal-Celhay et al., submitted). As *C. elegans* ages, its intestinal bacterial load increases, and we found

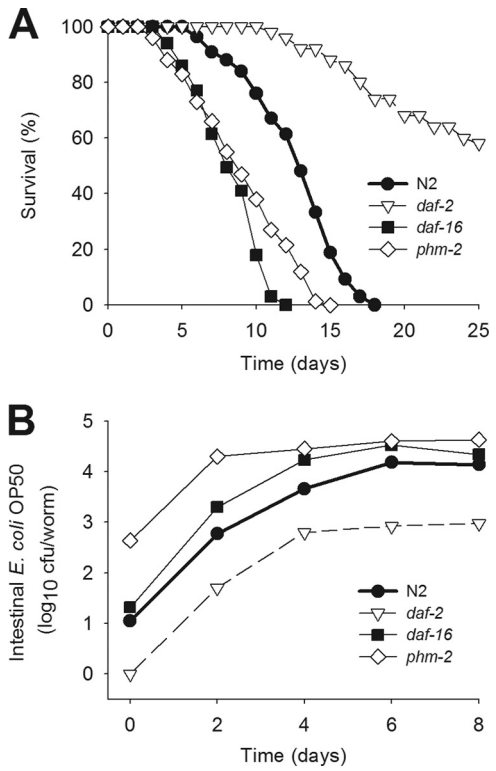

FIG 1 Survival and density of colonizing bacteria in the *C. elegans* gut. (A) Survival of the *C. elegans* N2 strain and *daf-2*, *daf-16*, and *phm-2* mutants when grown on lawns of *E. coli* OP50. (B) Load of *E. coli* OP50 within the gut of wild-type *C. elegans* N2 or in the *daf-2*, *daf-16*, and *phm-2* mutants for the first 8 days after the L4 stage.

an inverse relationship with life span. We now confirm that the *E. coli* OP50 intestinal colonization of the long-lived *daf-2* mutants is an order of magnitude lower than that of N2 worms (Fig. 1). There were significantly higher *E. coli* luminal densities in *daf-16* worms, and those in the pharynx-defective *phm-2* mutants were higher than in N2 worms, differences consistent with variation in worm longevity (Fig. 1A and B). Studies of the *phm-2* worms confirm the critical role (6) of an intact grinder in limiting introduction of viable bacteria into the *C. elegans* gut (Fig. 1B). Using the *daf-16*(*m26*) *daf-2*(*el370*) double mutant, we previously showed that the *daf-16* mutation suppresses the low levels of bacterial colonization in *daf-2* strains (Portal-Celhay et al., submitted). Notably, in each worm background we observed, we could enumerate viable bacterial colonization of the gut lumen persisting beyond a week at the host-specific level, permitting studies of the determinants of colonization.

The rise in gut bacterial counts that occurs during the early days of worm aging (Fig. 1B) could reflect new bacterial entry from the lawn, in excess of the grinder capacity, or alternatively, could reflect the persistence and proliferation of bacteria already present. We created model systems to evaluate these two possibilities. First, to determine whether individual bacteria are able to persist within the *C. elegans* gut, N2 worms were grown on lawns of founder bacteria (*E. coli* pSMC21) and transferred 48 h later to new plates seeded with *S.* Typhimurium pGB5 (introduced bacteria). In opposite experiments, worms were grown on lawns of founder *S.* Typhimurium pSMC21 and then transferred 48 h later to new plates seeded with (introduced) *E. coli* pGB5. We asked whether the founder bacteria present in the gut lumen could resist the introduction of bacteria from the new lawn that had circumvented the grinder. First, we found that no viable founder *E. coli* could be recovered from the *C. elegans* intestine 24 h after feeding with the introduced *Salmonella*; *Salmonella* completely replaced *E. coli* (Fig. 2A). In contrast, founder *Salmonella* was detected in the intestinal tracts of worms that had been transferred to lawns of introduced *E. coli* (Fig. 2B). Since the worms no longer were exposed to *Salmonella*, these observations indicated the resilience of the founder *Salmonella* cells in the intestinal niche, permitting persistence. As worms age, the founding bacterial populations show greater resilience in the face of introduced organisms (Fig. 3).

We next asked whether the age of the worm at the time of the transfer affected the resilience of the colonizing populations. When worms were transferred to the new lawns after 96 h instead of after 48 h, overall colonization was at a higher level, as expected (Fig. 1). However, *E. coli* cells now were able to persist in the gut lumen despite the influx of *Salmonella* (Fig. 2C). At 96 h, *Salmonella* also persisted as a founder strain (Fig. 2D), as expected from the 48-h studies, and reached higher levels than did the *E. coli* founder strain in the reciprocal 96-h studies (Fig. 2C). Thus, the resilience of the founding bacterial population was determined by the specific combination of founder and introduced strains, in the context of host age.

Role of gut environment in persistence. We next examined how differing *C. elegans* gut environments affect founding bacterial persistence. First, we studied *daf-2* and *daf-16* worms, which have the essentially opposite phenotypes of restricting and enhancing bacterial colonization in the gut lumen, respectively (Fig. 1). As with wild-type worms, founder *E. coli* was not able to persist in the gut of the *daf-2* or the *daf-16* mutants when *Salmonella* was introduced at 48 h (Fig. 2A). In contrast, the founder *Salmonella* cells resisted *E. coli* introduction at the same time point (48 h) within both mutants (Fig. 2B). However, in the *daf-2* mutants, both founder and introduced strains were at significantly lower levels than in N2 worms. Transferring the mutant worms to new lawns at 96 h (instead of at 48 h) showed trends similar to those observed for N2 worms (Fig. 2C and D); however, founder *Salmonella* cells persisted at significantly higher levels in the *daf-16* mutants and at significantly lower levels in the *daf-2* mutants (Fig. 2D). In general, the *daf-2* environment was the most inhibitory one for founder *E. coli* or *Salmonella* in relation to the introduced strains.

We also asked whether the numbers of newly introduced competitors delivered to the gut affect the ability of founder bacterial cells to persist in the lumen. For these studies, we examined *phm-2* mutants in which defective grinders (7, 42) allow delivery of higher gut loads (Fig. 1B). With change of the lawn at 48 h, the gut load of the introduced strains was somewhat but not significantly higher than that of N2. With change at 96 h, the founder *Salmonella* persisted at significantly higher levels than those of the N2 worm (Fig. 2). In total, across the range of gut conditions present in the *daf-2*, *daf-16*, and *phm-2* mutants, parallel phenomena of strain specificity and worm age dependence in the gut colonization competition were observed. The worm genotypes studied affected overall bacterial load and, to a limited extent, altered the dynamics between the founder and introduced bacteria.

Worm age affects the resilience of the colonizing population. Since founder *Salmonella* organisms are highly resilient to dis-

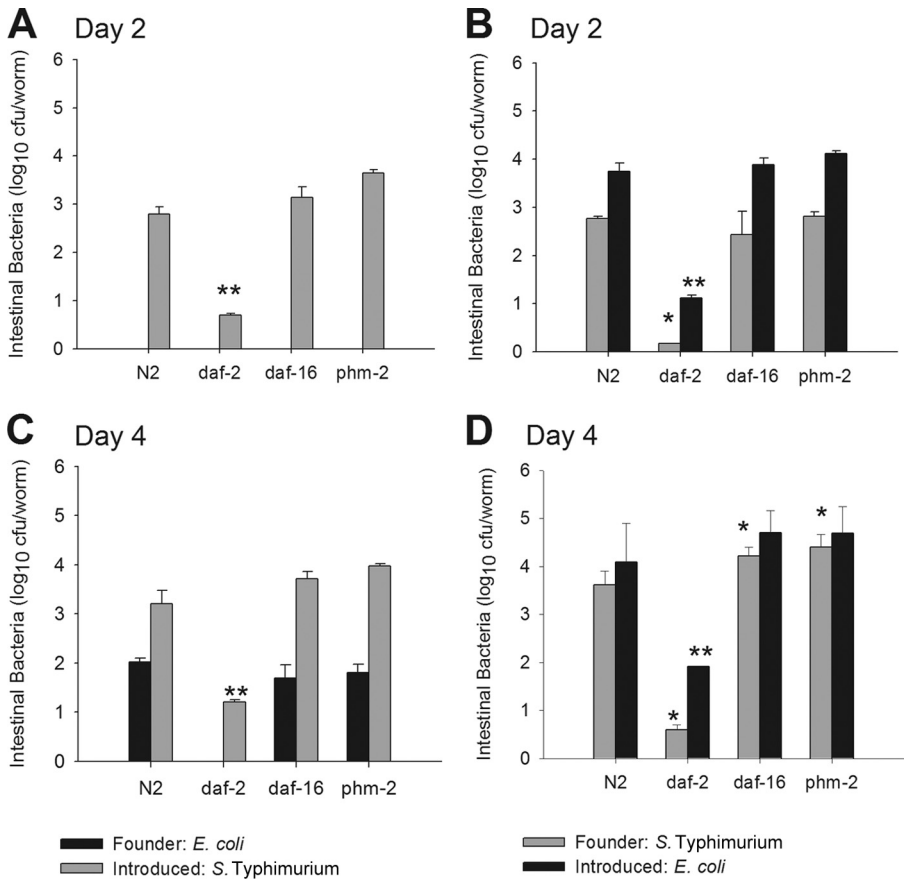

FIG 2 Resilience of bacterial gut colonization depends on the bacterial strain and on *C. elegans* age and genotype. Studies were done at 48 h (day 2) (A and B) or at 96 h (day 4) (C and D) following shift and competition assays, as described in Materials and Methods. *C. elegans* N2 or mutant strains were grown on lawns of the founder bacteria *E. coli* pSMC21 and then transferred to new plates seeded with *S.* Typhimurium pGB5 at 48 h (A) or at 96 h (C). Alternatively, *C. elegans* N2 or mutants were grown on lawns of the founder bacteria *S.* Typhimurium pSMC21 and then transferred to new plates seeded with *E. coli* pGB5 at 48 h (B) or at 96 h (D). Bacterial density was determined by plating the intestinal contents of lysed worms on selective media. Data represent means ± standard deviations (SD). Asterisks indicate a significant difference ($P < 0.05$) compared to the density of the founder strain recovered from N2 worms (*) or significant difference compared to the density of the introduced strain (**).

placement by introduced *E. coli*, whereas outcomes are more conditional on worm age for founder *E. coli* (Fig. 2), the use of founder *E. coli* provides further experimental opportunities to probe the selective pressures. To independently confirm the hypothesis that worm age determines the persistence of the founder bacteria, we conducted experiments in which *Salmonella* was introduced progressively later to worms with founder *E. coli* populations and then sampled the worms 24 h after each switch. As increasingly older *E. coli*-fed worms were exposed to the introduced *Salmonella* strain, there was progressively greater persistence of the founder *E. coli* strain (Fig. 4A), confirming and extending the 48-h and 96-h findings (Fig. 2). Next, to determine whether sampling only 24 h after the switch might reflect only transient events, we conducted experiments in which sampling of the worms after the switch ranged from 24 to 96 h. Over the course of the 96-h sampling after the switch (Fig. 2B), the founder bacteria continue to persist in essentially the same proportion to the introduced bacteria, despite continuing exposure to the latter. This experiment provides further evidence that founder bacterial cells can persist and multiply in the gut lumen and that the niches they establish can substantially resist a continuing influx of introduced and, thus, competing bacteria. In total, these data indicate that founder *Salmonella* is able to establish persistent gut colonization of N2 and mutant *C. elegans* that resist introduced *E. coli*, regardless of host age or worm genotype. However, the resilience of *E. coli* in the luminal environment has greater dependence on host age, whether in wild-type or mutant worms.

Competition *in vitro*. In the above-described studies, we examined the sequential exposure to potential bacterial colonizers, but next we questioned whether introducing the strains simultaneously would change the dynamics. To address this question, we performed experiments in which lawns were mixtures of *E. coli* and *Salmonella* in different proportions. First, we assessed whether the *E. coli* or *Salmonella* strains used had substantial *in vivo* growth advantage. We found that at 37°C, *E. coli* and *Salmonella* growth rates are almost identical (data not shown). However, at 25°C, the temperature at which the competition assays were conducted and in which the worms were incubated, *Salmonella* had a growth advantage (Fig. 5A). Similarly, and in an *in vitro* competition experiment, both wild-type and *Salmonella* Δ*spi-1* Δ*spi-2* outcompeted *E. coli* by a difference of 1 $\log_{10}$ (Fig. 5B and C). Taken together, these findings could in part explain the greater resilience of both founder and introduced *Salmonella* in the sequential coloniza-

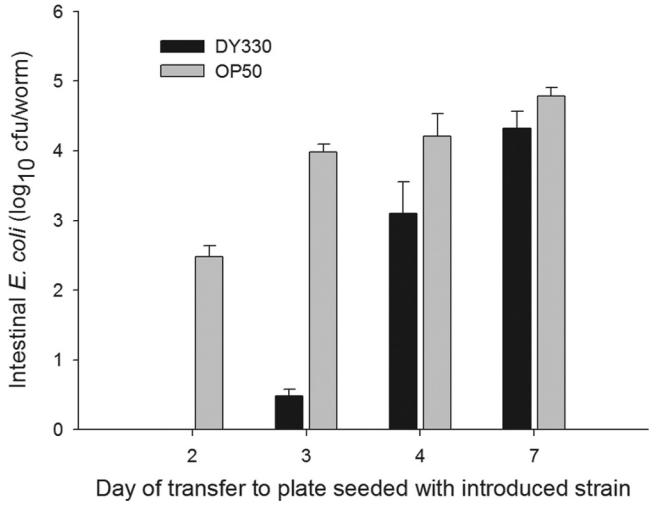

FIG 3 Effect of aging on *E. coli* founder strain persistence in *C. elegans*. *C. elegans* N2 worms were initially grown on lawns of founder *E. coli* DY330 (black bars) and then moved at different points in worm maturation (L4 stage plus 2, 3, 4, or 7 days) to plates with lawns of introduced *E. coli* OP50. Bars show intestinal densities of founder *E. coli* DY330 (black) and introduced *E. coli* OP50 (gray bars) recovered 24 h after shifting the worms to lawns of the introduced strain. The older the worms, the more the founder (DY330) strain was able to resist the newly introduced strains; with aging, the concentrations of the introduced (OP50) strain also increased but less dramatically. Data represent means ± SD.

tion studies (Fig. 2 and 4). Nevertheless, despite the growth differences, persistence in the gut lumen also could be demonstrated for *E. coli* (Fig. 2C and D).

Competition for gut colonization. Next, to examine the dynamics of *in vivo* competition, we created lawns with mixtures of *E. coli* and *Salmonella* cells to introduce the two strains simultaneously and then to determine the proportions of each that would be recovered from the worms. To calibrate the system, we first competed two variants of *S.* Typhimurium strain SL1344, differentiated by their plasmid-encoded antibiotic resistance phenotypes. As expected, the cells were recovered from the worms essentially in proportion to the input ratios; neither strain had any selective advantage (Fig. 6A). That the competition between the two strains was neutral provided an important validation of the assay for examining other strain combinations. Competition between two variants of *E. coli* was performed as well (Fig. 7); the unequal levels reflected colonization differences between the strains, possibly due to extra genetic cost.

When N2 worms were grown on the mixed lawns, the *Salmonella* cells substantially outcompeted *E. coli* even when *E. coli* was introduced at 100-fold-higher concentrations (Fig. 6B). We confirmed these findings by microscopy (Fig. 6C), visualizing *E. coli* OP50 expressing green fluorescent protein (GFP) and *S.* Typhimurium expressing red fluorescent protein (RFP) (DsRed) in worms fed on lawns with 100-fold *E. coli* excesses. Discrete clusters of green-fluorescing (*E. coli*) cells were observed predominately in the anterior gut (Fig. 6C, top), whereas red (*Salmonella*) fluorescence appeared uniformly throughout the gut lumen (Fig. 6C, middle). Overall, red dominates over green (Fig. 6C, bottom). Next, we asked whether the gut environment has a major role in the population dynamics by studying the *E. coli*/*Salmonella* competition in the opposing *daf-2* and *daf-16* mutants. Although the total bacterial levels in the *daf-2* and *daf-16* worms reflected their overall capacity for colonization (Fig. 2), *Salmonella* outcompeted *E. coli* to an extent paralleling that in the wild-type (N2) worms (Fig. 6D and E). Thus, in the setting of simultaneous introduction, bacterial species differences clearly affect the ability of the strains to persist in the worm lumen, whereas the studied host genotypes affect primarily the extent of the lumenal niche but not the competition dynamics.

Role of *Salmonella* tissue interaction in colonization, competition with *E. coli*, and *C. elegans* life span. We next sought to develop a model to better understand why *Salmonella* is more efficient than *E. coli* at colonizing the *C. elegans* gut. Many of the genes that are involved in the interaction between *Salmonella* and its vertebrate hosts are located in the *Salmonella* pathogenicity

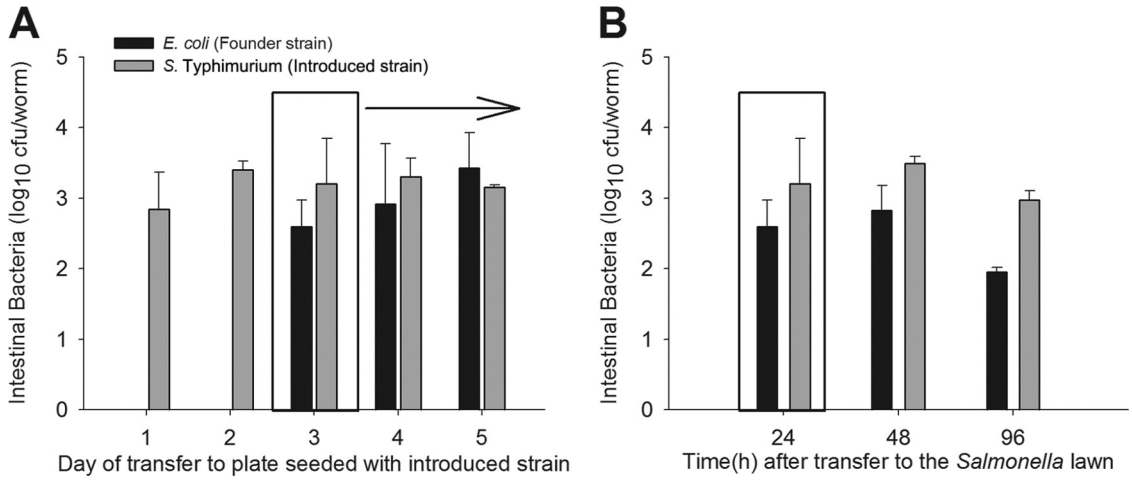

FIG 4 *E. coli* OP50 can resist the intrusion of *Salmonella* in the gut of older worms. (A) Gut densities of *E. coli* OP50 (black bars) and *S.* Typhimurium SL1344 (gray bars) recovered from *C. elegans* N2 after their growth on founder *E. coli* OP50 lawns and then after the substitution of *S.* Typhimurium lawns at several points in worm maturation (L4 stage plus 1, 2, 3, 4, or 5 days). Data represent means ± SD. (B) Intestinal bacteria recovered from *C. elegans* N2 after being switched from lawns of founder *E. coli* OP50 (black bars) to lawns of *S.* Typhimurium SL1344 (gray bars), which were transferred on day 3 (L4 plus 3), with worms then maintained on the introduced *S.* Typhimurium strain with sampling of the worms at 24, 48, and 96 h after the shift.

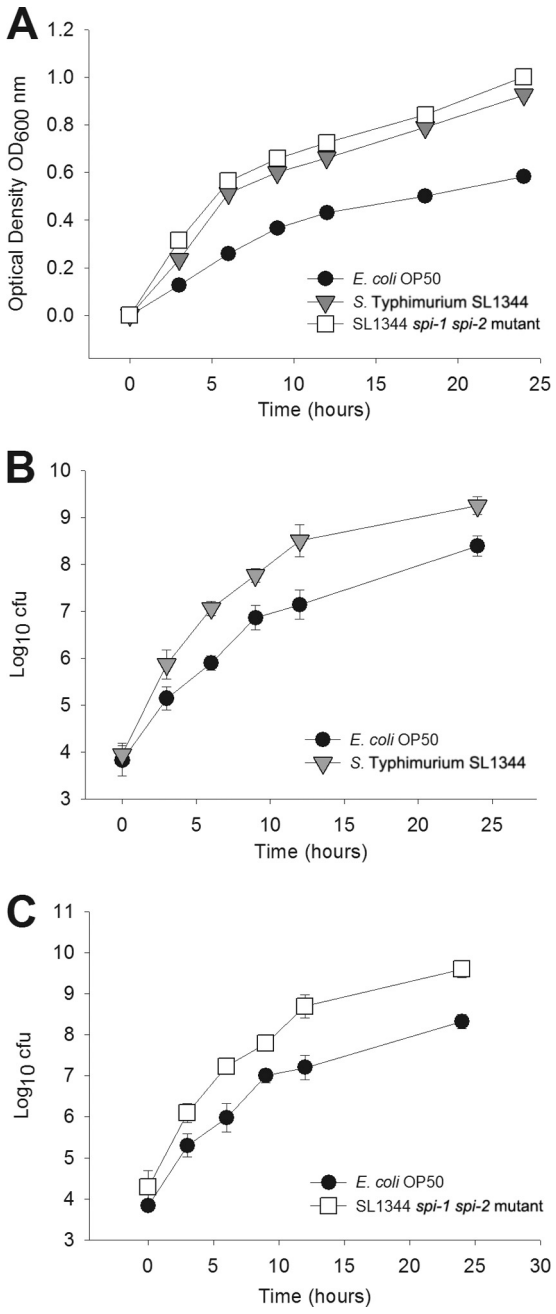

FIG 5 *In vitro* growth and competition of *E. coli* and *Salmonella*. (A) Growth of *E. coli*, *S.* Typhimurium SL1344, and the SL1344 *spi-1 spi-2* mutant on LB broth supplemented with 60 μg/ml streptomycin and incubated at 25°C; (B) competition between *E. coli* and *S.* Typhimurium SL1344 *in vitro* in broth culture; (C) competition between *E. coli* OP50 and the SL1344 *spi-1 spi-2* mutant, as described in panel B.

islands (SPI) (17). SPI-1 and SPI-2 each carry components of type III secretion systems (T3SSs), the former being critical for invasion and intestinal disease in mammals and the latter important for intracellular survival, systemic persistence, and disease (13).

An early step in the establishment of *S.* Typhimurium infection in the vertebrate host gastrointestinal tract involves attachment and/or invasion of intestinal epithelial cells. To determine the role of SPI-1 SPI-2-carried genes in *C. elegans* gut colonization, we grew wild-type worms on plates seeded with *S.* Typhimurium SL1344, isogenic *S.* Typhimurium SL1344 Δ*spi-1* Δ*spi-2*, or *E. coli*. As shown in life span assays for the N2 worms and as expected, *Salmonella* is more virulent (TD_{50} = 10.5 ± 0.5 days) than *E. coli* OP50 (TD_{50} = 13.8 ± 0.56; $P = 0.03$) (Fig. 8A). As expected, the *Salmonella* Δ*spi-1* Δ*spi-2* mutant was less virulent (TD_{50} = 13.1 ± 1.18 days) for *C. elegans* than isogenic wild-type *Salmonella*, confirming prior studies (4), and had virulence similar to *E. coli* OP50 (13.8 ± 0.56 days). However, the density of *Salmonella* Δ*spi-1* Δ*spi-2* in the *C. elegans* gut was consistently about 10-fold greater than that of *E. coli* OP50 through the first 8 days of adult life (Fig. 8B). The *Salmonella* Δ*spi-1* Δ*spi-2* mutant also colonized the *C. elegans* gut at higher levels than did wild-type *Salmonella*, despite its having a longer life span. Thus, bacterial density in the gut is not the sole determinant in life span, as shown by comparing the two *Salmonella* strains; host interaction is clearly critical.

To directly examine the dynamics of gut colonization, we performed competition studies using lawns with differing *E. coli* and *Salmonella* proportions (Fig. 8). In direct competition studies, the Δ*spi-1* Δ*spi-2* strain competed proportionally (or a little more so) in relation to the wild-type strain (Fig. 8C), consistent with the findings from studies in which monocolonization was done (Fig. 8B). Confirming the prior studies, wild-type *Salmonella* completely outcompeted *E. coli*, with essentially 4 $\log_{10}$ differentials, summating both the differences in lawn proportions and the observed colonization proportions (Fig. 8D). The Δ*spi-1* Δ*spi-2* strain also outcompeted *E. coli* (Fig. 8E), but the net differential, only 1 to 2 $\log_{10}$, was significantly less than that for wild-type *Salmonella* ($P < 0.05$). Since *in vitro* growth differences of *E. coli* with both strains are essentially the same (Fig. 5B and C), these experiments provide evidence that the differential colonization by *E. coli* and *Salmonella* cannot be explained solely on the basis of differential growth at 25°C but reflects host interaction.

Does worm passage select for better colonizers? Finally, we asked whether the *in vivo* milieu selects for better-adapted bacteria or whether the apparent resilience of persisters was merely stochastic and not inherited. To address this question, *E. coli* OP50 was passed 10 times through the *C. elegans* gut or, in control procedures, passed *in vitro* for the corresponding period. These passaged strains then were competed with *in vitro*-passed *Salmonella*. Both strains grew similarly *in vitro* at 25°C and less well than *Salmonella* (Fig. 9A). When competitions were performed using 3-day-old worms, the ability of *E. coli* to colonize and compete against *Salmonella* was greatly enhanced after the *in vivo* passages, with significantly higher levels ($P < 0.05$) of colonization than the *in vitro*-passed strain (Fig. 9B and C). Although *Salmonella* still outcompeted both of the *E. coli* strains, the data provide evidence that there had been *in vivo* adaptation. To further assess the contribution of aging to the colonization process, we carried out the same competition assay using older (5-day-old, or 120-h-old) adult worms. In the older worms, the *in vivo*-passed *E. coli* again outperformed the *in vitro*-passed strain (Fig. 9D and E). We also compared the colonization profiles in the older (5-day-old) and younger (3-day-old) adults to examine the effects of host age on the competition dynamics. Although *Salmonella* levels were essentially fixed under all conditions studied (Fig. 9B to E), the extent of *E. coli* colonization was dependent on its both *in vivo* passage history and host age. In total, these experiments indicate that *in vivo* selection for bacterial genotypes enables better colonization

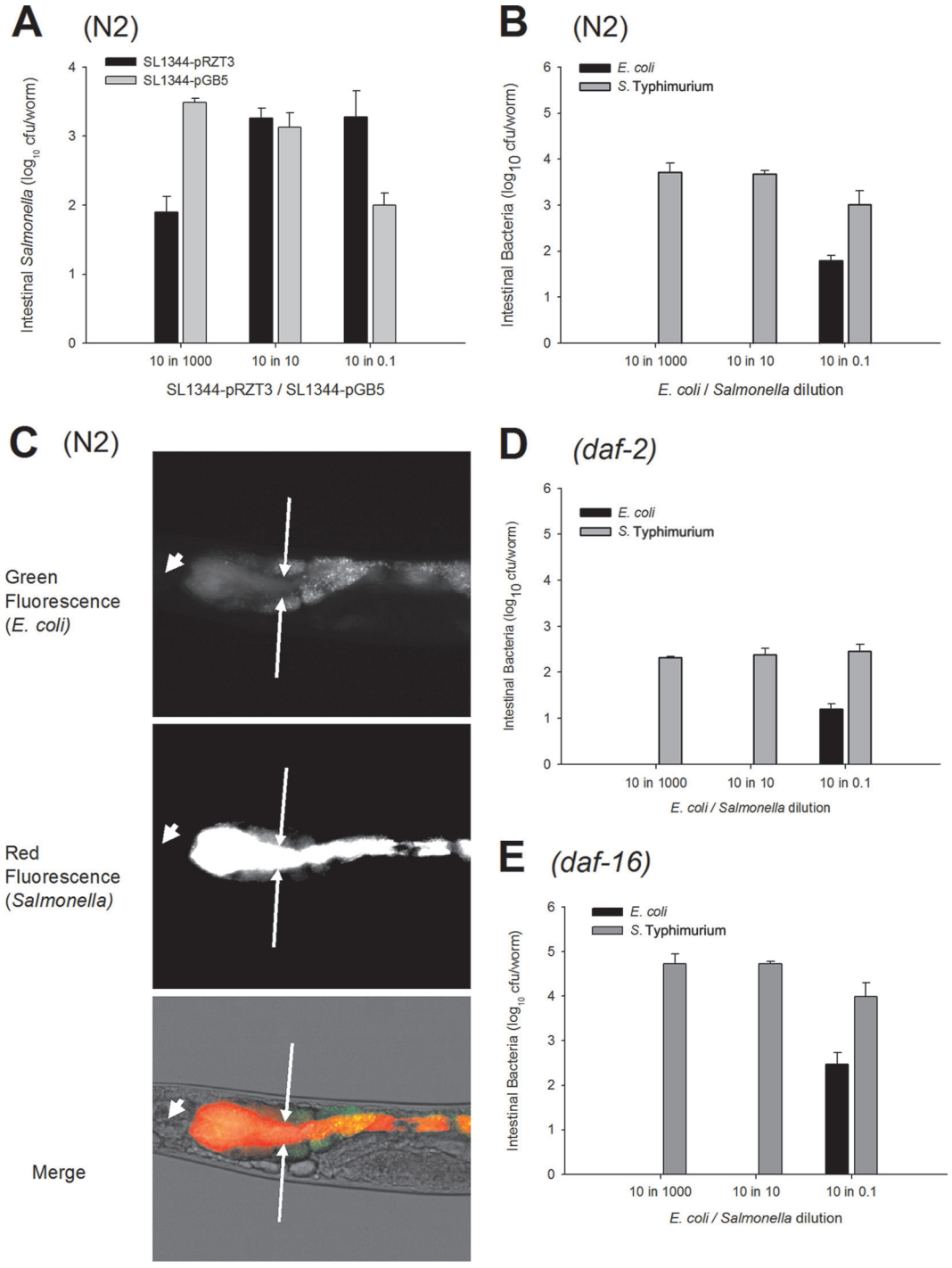

FIG 6 Simultaneous competition between *E. coli* and *S.* Typhimurium for gut colonization of *C. elegans* N2 and mutants. (A) Gut densities at 24 h after exposure of N2 worms to *S.* Typhimurium SL1344-pRZT3 expressing DsRed (dark bars) and *S.* Typhimurium SL1344-pGB5 expressing GFP (gray bars) grown for 6 ho on mixed lawns at ratios of 10:1,000, 10:10, and 10:0.1. Data represent means ± SD. (B) Lawns with differing ratios (10:1,000, 10:10, 10:0.1) of *E. coli* OP50 and *S.* Typhimurium SL1344 were prepared, and worms fed on these lawns for 6 h. Levels of *E. coli* (black bars) and *S.* Typhimurium (gray bars) in the gut were measured 24 h later in wild-type *C. elegans* N2, as described above. (C) Fluorescence microscopy of *C. elegans* N2 after feeding for 12 h on mixed lawns of *E. coli* OP50-pGB5 and *S.* Typhimurium SL1344-pRZT3 at a ratio of 10:0.1 (100-fold *E. coli* excess). Arrows demarcate the gut lumen. Arrowheads indicate the pharyngeal grinder. Magnification, ×40. The green fluorescence channel shows *E. coli* inside the gut lumen and gut autofluorescence, and the red channel shows *Salmonella* fluorescence. The merged image shows bacterial colocalization (yellow). (D) Same as panel B in *daf-2* mutants. (E) Same as panel B in *daf-16* mutants.

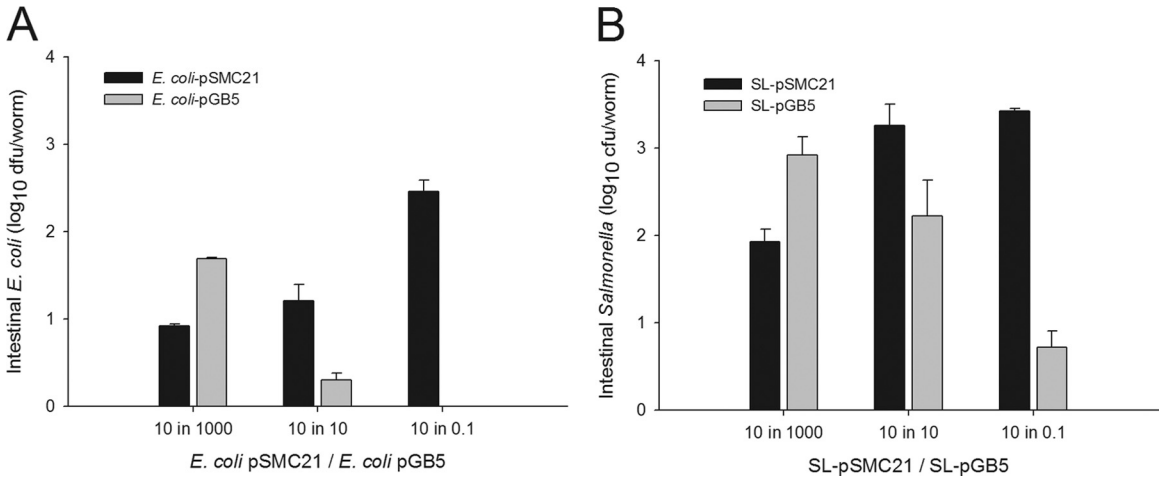

FIG 7 Simultaneous competition between two variants of *E. coli* (A) and two variants of *S.* Typhimurium (B) for gut colonization of *C. elegans* N2. (A) Gut densities at 24 h after exposure of N2 worms to *E. coli* pSMC21 expressing Amp and Km resistance (black bars) and *E. coli* pGB5 expressing Amp and Tet resistance (gray bars) grown for 6 h on mixed lawns at ratios of 10:1,000, 10:10, and 10:0.1. Data represent means ± SD. (B) *S.* Typhimurium pSMC21 and pGB5 on lawns, as explained for panel A.

of worms and that advancing worm age independently and disproportionately favors *E. coli* colonization.

DISCUSSION

Simplified and genetically tractable models of gut ecosystems are needed to understand how both hosts and bacteria actively collaborate to shape the overall intestinal milieu (27, 28). Earlier studies have shown that bacteria accumulate in the *C. elegans* intestine as they age (23) and that *S.* Typhimurium proliferates and establishes a persistent infection in the worm intestine (3). In prior work, we confirmed these findings and found a strong negative correlation between bacterial numbers and life span (Portal-Celhay et al., submitted). We also showed that the bacterial load reaches a strain-specific and host genome-specific plateau that extends until the worm's demise. We attributed this finding to a cohort effect, in which the fraction of worms examined in late worm adulthood constitutes a subpopulation that survived because they maintain the ability to control bacterial proliferation. Alternatively, late in life, the bacterial populations develop specific syntrophic equilibria that are resilient to changes in host milieu. But did we really observe bacterial proliferation, or are the bacteria accumulating in the intestine without dividing? In this study, we established the utility of the *C. elegans* gut as a model system to study intestinal bacterial colonization and proliferation and identified factors that contribute to microbial persistence. Furthermore, we provide evidence of active competition for colonization niches in the *C. elegans* gut, as determined by host age, bacterial factors, and *in vivo* selection.

In our experimental system, we found that there is resilience (defined as the ability to recover quickly from change) of naturally introduced *Salmonella* cells in the gut niche of young adult worms (L4 plus 48 h), permitting their persistence. In contrast, *E. coli* as a founder strain was entirely outcompeted by introduced *Salmonella*. Since *Salmonella* can disrupt the pharyngeal grinder (3), better resilience could simply be due to a higher number of cells delivered to the gut. However, in the *phm-2* mutants, *Salmonella* persisted despite *E. coli* introduction (6), and *E. coli* founders were unable to persist after *Salmonella* introduction, indicating that grinder function is not a primary determinant of the competition. That founder *E. coli* also was not able to persist in the *daf-2* or *daf-16* mutants indicates that gut colonization resilience is bacterial strain specific, whereas the host genotypes studied predominantly determined the total number of bacterial cells that can colonize the gut.

The same experiments using older adult worms (L4 plus 96 h) showed that *E. coli* now better persists, which shows that worm age is a determinant of the resilience of founder bacteria. These results could be explained by three alternative but not exclusive mechanisms. One possibility is that the gut milieu of older worms is more permissive for bacterial cells in general, and the colonization experiments (Fig. 1 and 2) provide some support, yet colonization generally plateaus by about day 4. A second possibility is that over time within the *C. elegans* gut, there is selection for *E. coli* cells, which are better adapted to the gut niche in terms of avoiding killing by antimicrobial defenses, garnering nutrients, or resisting being flushed from the gut. A third possible mechanism is that *E. coli* is able to persist in the *C. elegans* gut once cellular and structural damage of the host intestine has been produced by other bacteria, such as *Salmonella.* Comparison of *Salmonella* competition with *in vitro*-passed and *in vivo*-passed *E. coli* (Fig. 9) in worms of different ages provided clear evidence for *in vivo* selection. Thus, this model system can now be used to further identify the selective forces and the relevant phenotypes (and genotypes) of the selected bacterial cells. Future work should include sequencing of the bacteria after several rounds of *in vivo* passaging.

That *Salmonella* was able to establish persistent gut colonization independent of the order of introduction or age of the host, whereas *E. coli* resilience depended on host age, establishes the principle of species-specific variation in adaptation for *C. elegans* gut colonization (18). Further *E. coli/Salmonella* dilutions and a time course to show proliferation of small inoculums of *Salmonella* would have been useful to better characterize the resilience of the *Salmonella* populations. However, that *Salmonella* grows faster *in vitro* than *E. coli* at the temperature (25°C) at which the competition assays are done complicates the analysis. However, the differing dynamics of the two *Salmonella* strains in relation to

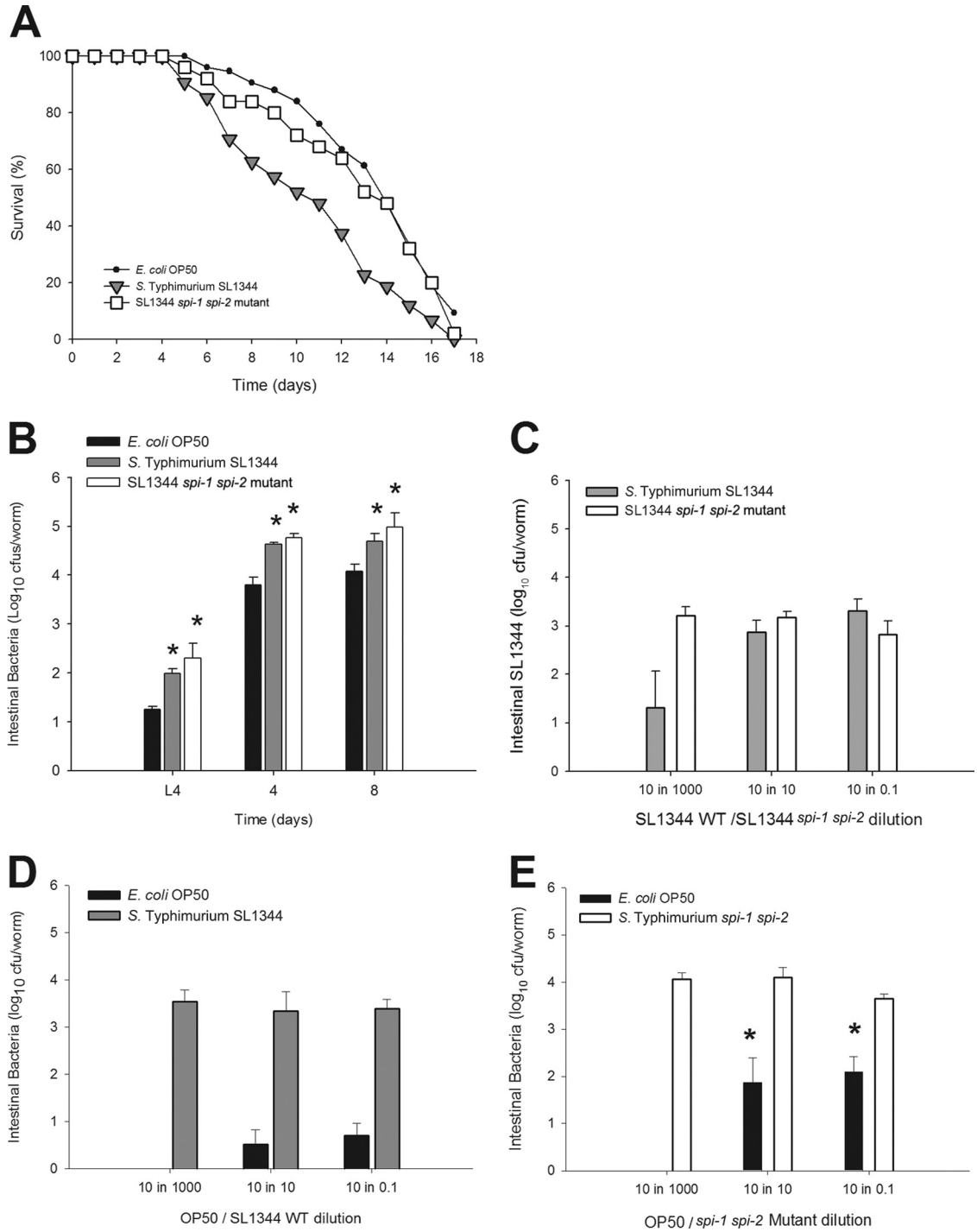

FIG 8 Role of the SPI-1/-2 islands in *Salmonella* colonization, resilience, and *C. elegans* longevity. (A) Life span assays of *C. elegans* raised on lawns of *E. coli* OP50 (black circles), wild-type *S.* Typhimurium SL1344 (gray diamonds), or *S.* Typhimurium SL1344 Δ*spi-1* Δ*spi-2* (white squares). (B) For worms grown on lawns of *E. coli* OP50 (dark bars), *S.* Typhimurium SL1344 (gray bars), or SL1344 Δ*spi-1* Δ*spi-2* (white bars), their gut densities in *C. elegans* N2 at L4 stage (L4 plus 0), day 4 (L4 plus 4), or day 8 (L4 plus 8) of their adult life span. Data represent means ± SD. An asterisk indicates a significant difference ($P < 0.05$) compared to growth on *E. coli* OP50. (C) Gut density of the *S.* Typhimurium SL1344 wild type (WT) (gray bars) and *S.* Typhimurium SL1344 Δ*spi-1* Δ*spi-2* (white bars), as described in panel D. (D) Gut density of *E. coli* OP50 (black bars) and *S.* Typhimurium SL1344 (gray bars) within wild-type *C. elegans* N2 grown on mixed lawns with ratios of 10:1,000, 10:10, and 10:0.1 of *E. coli* to *Salmonella.* Data represent means ± SD. (E) Gut density of *E. coli* OP50 (black bars) and *S.* Typhimurium SL1344 Δ*spi-1* Δ*spi-2* (white bars), as described in panel B.

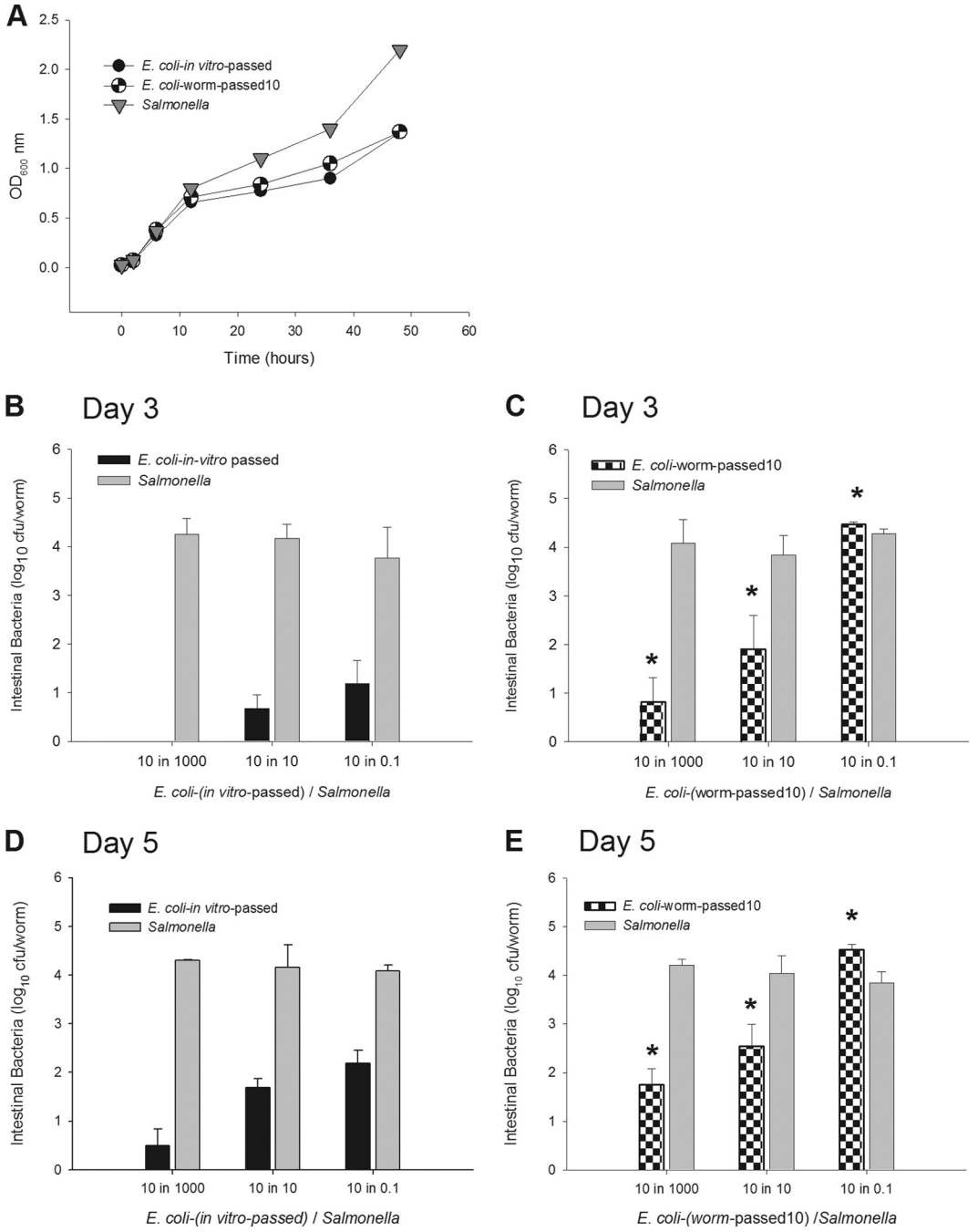

FIG 9 Selection for bacteria with improved colonization. (A) Growth of *E. coli* and *Salmonella* cells *in vitro* at 25°C, as shown in Fig. 4A. Cells include *E. coli in vivo* passed for 10 generations of worms (black-and-white circles), *E. coli* with parallel *in vitro* passage (black circles), and wild-type *S.* Typhimurium SL1344 (gray triangles). Lawns with differing ratios (10:1,000, 10:10, 10:0.1) of *in vitro*-passed *E. coli* OP50 and *in vitro*-passed *S.* Typhimurium SL1344 were prepared, and 3-day-old worms (B) or 5-day-old worms (D) fed on these for 6 h. Gut levels of *E. coli* (black bars) and *S.* Typhimurium (gray bars) were measured 24 h later in wild-type *C. elegans* N2. An asterisk indicates a significant difference ($P < 0.05$) compared to panel B. *E. coli* OP50 was sequentially passed through the worm gut 10 times (see Materials and Methods). Lawns with differing ratios (10:1,000, 10:10, 10:0.1) of worm-passed *E. coli* OP50 and *in vitro*-passed *S.* Typhimurium SL1344 were prepared, and 3-day-old worms (C) or 5-day-old worms (E) fed on these for 6 h. Gut levels of *E. coli* (black-and-white bars) and *S.* Typhimurium (gray bars) were measured 24 h later in wild-type *C. elegans* N2. An asterisk indicates a significant difference ($P < 0.05$) compared to panel B. OD, optical density.

E. coli indicate that growth differences are not sufficient to explain the differing findings.

Why is *Salmonella* better at colonizing the *C. elegans* gut than *E. coli*? One possibility is that *Salmonella*'s advantage reflects its more profound interaction with host intestinal cells, garnering an extra resource stream. However, the less virulent Δ*spi-1* Δ*spi-2* mutant strain colonized as well or better than the wild-type strain (Fig. 6D). It is not clear whether *Salmonella* adheres or invades the *C. elegans* gut epithelial cells (3, 4, 33, 42). Nevertheless, the Δ*spi-1* Δ*spi-2* mutant did not compete as effectively as did the wild type

against *E. coli*, making exploration of the role of the SPI-carried genes worthwhile. Another possibility could be that *E. coli* cannot easily survive in an environment in which an active host response, elicited by wild-type *Salmonella*, is present. Competition against the *Salmonella* Δ*spi-1* Δ*spi-2* mutant, which elicits less intense host responses than does the wild type (34), should give *E. coli* the opportunity to fare better, which could explain the experimental observation (Fig. 8). Although the Δ*spi-1* Δ*spi-2* strain competes well against its wild-type competitor, it is inferior to the wild type in competing with *E. coli* and markedly less pathogenic for worms than the wild type (Fig. 8A). Thus, the SPI-1/-2 islands play roles in both intraluminal colonization and in host interactions that affect longevity, and in this case, immunosenescence may result in decreased ability to cope with factors produced by colonizers, in addition to the inability to clear ingested bacteria. Consistent with this hypothesis are the observations that nontoxic low-interaction bacteria (e.g., *Enterococcus faecium*) may accumulate to high densities in the intestinal environment but with small effects on longevity (24).

The results of these studies provide evidence for bacterial persistence in the *C. elegans* gut lumen, with ongoing selection dictating interspecies and intrastrain competition for survival. Use of the well-defined *C. elegans* model to examine bacterial competition within the intestinal tract of animals should enhance a mechanistic understanding of host-microbe interactions underlying commensalism in more complex animals, including humans.

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