Salmonella typhimurium proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*

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Genetic analysis of host–pathogen interactions has been hampered by the lack of genetically tractable models of such interactions. We showed previously that the human opportunistic pathogen *Pseudomonas aeruginosa* kills *Caenorhabditis elegans,* that *P. aeruginosa* and *C. elegans* genes can be identified that affect this killing, and that most of these *P. aeruginosa* genes are also important for mammalian pathogenesis. Here, we show that *Salmonella typhimurium* as well as other *Salmonella enterica* serovars including *S. enteritidis* and *S. dublin* can also kill *C. elegans*. When *C. elegans* is placed on a lawn of *S. typhimurium*, the bacteria accumulate in the lumen of the worm intestine and the nematodes die over the course of several days. This killing requires contact with live bacterial cells. The worms die with similar kinetics when placed on a lawn of *S. typhimurium* for a relatively short time (3–5 hours) before transfer to a lawn of *E. coli*. After the transfer to *E. coli*, a high titer of *S. typhimurium* persists in the *C. elegans* intestinal lumen for the rest of the worms' life. Furthermore, feeding for 5 hours on a 1:1000 mixture of *S. typhimurium* and *E. coli* followed by transfer to 100% *E. coli*, also led to death after several days. This killing correlated with an increase in the titer of *S. typhimurium* in the *C. elegans* lumen, which reached 10,000 bacteria per worm. These data indicate that, in contrast to *P. aeruginosa*, a small inoculum of *S. typhimurium* can proliferate in the *C. elegans* intestine and establish a persistent infection. *S. typhimurium* mutated in the PhoP/PhoQ signal transduction system caused significantly less killing of *C. elegans.*

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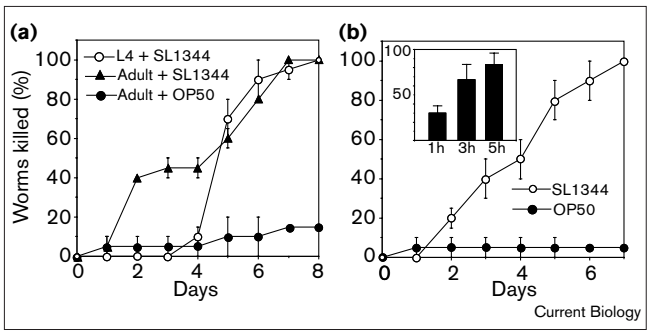
Results and discussion

Our laboratory and others have focused on the development of pathogenicity models using *C. elegans* as the host because of the extensive genetic and genomic resources available and because of the relative ease of identifying *C. elegans* mutants that exhibit altered susceptibility to pathogen attack [1–4]. One issue that has been raised with respect to the use of alternative model hosts such as *C. elegans* is that their utility may be limited to the study of broad host range opportunistic pathogens like *P. aeruginosa* [5]. To determine whether the highly specialized vertebrate pathogen *Salmonella typhimurium* can kill *C. elegans*, N2 worms were fed on lawns of various *Salmonella enterica* serovars at 25°C. As shown in Figure 1a, both larval stage 4 (L4) and 1-day-old adults died more quickly when fed on the commonly studied *S. typhimurium* strain SL1344 than when fed on *E. coli* strain OP50, the usual food of *C. elegans* in the laboratory. We concluded that SL1344 infects and kills *C. elegans*.

Figure 1



S. typhimurium kills *C. elegans.* **(a)** 10–20 L4 stage (open circles) or 1-day-old adult hermaphrodite (filled triangles and filled circles) worms fed either on *S. typhimurium* SL1344 (filled triangles and open circles) or on *E. coli* OP50 (filled circles). **(b)** *C. elegans* were fed on *S. typhimurium* SL1344 (filled circles) or *E. coli* OP50 (filled circles) for 5 h, then shifted to *E. coli* OP50 as described in Supplementary material. The insert shows the percentages of dead worms after transfer to OP50-containing plates after feeding for 1, 3 or 5 h on SL1344.

The time required for 50% of 1-day-old adult hermaphrodite nematodes to die (time to death 50; TD50) when feeding on an SL1344 lawn at 25°C was calculated in six independent experiments and determined to be 5.1 ± 0.7 days, whereas the time required for 50% of 1-day-old hermaphrodite nematodes to die when fed on an *E. coli* OP50 lawn was 9.9 ± 0.9 days. At 20°C, the TD50 was 17 days when feeding on SL1344 compared to 22 days for OP50.

As summarized in Table 1, other *S. enterica* serovars, including the two widely used wild-type *S. typhimurium*

strains 14028 (NCTC 12023 or ATCC 14028) and LT2 (SGSC1412) also killed *C. elegans*. In addition to *S. typhimurium*, at least two other *Salmonella* serovars, *S. enteritidis* strain 1047 and *S. dublin* strain 'Lane', also killed *C. elegans* at rates comparable to *S. typhimurium*.

As in *P. aeruginosa*-mediated killing of *C. elegans*, the motility of the worms and the rate of pharyngeal pumping gradually declined when feeding on *S. typhimurium* SL1344 until the nematodes became immobile and died. In addition, the lumen of the worms became distended. These effects first became evident 2 days after exposure to SL1344. Some of the worms also became laden with eggs, which hatched internally, suggesting that *S. typhimurium* may be affecting the egg-laying process. To evaluate the role of death due to internal hatching of progeny, we determined the rate of killing of the temperature sensitive sterile mutant *fer-1(hc1*ts) [6]. At 25°C, the temperature used for the infection assay, less than 2% of *fer-1*(*hc1*ts) worms produced progeny. The TD50 of *fer-1*(*hc1*ts) 1-day-old adult worms fed with *S. typhimurium* was 5.2 ± 0.6 days compared to 5.1 ± 0.7 for wild-type worms, indicating that the death due to internal hatching of progeny observed at the early stages of the killing process is not the major cause of death. The rate of killing of the *fer-1*(*hc1*ts) mutant during the first days of the infection was, however, slower than the killing of wild-type worms, and was similar to the profile obtained for L4 worms, suggesting that death due to internal hatching of progeny may contribute significantly to killing during the first few days of feeding on *S. typhimurium* (Figure 1a). In addition, some worms died containing eggs that never develop, exhibiting a so-called Emo phenotype (for endomitotic oocytes) and suggesting that *S. typhimurium* infection may alter some of the multiple events of hermaphrodite germ-line development.

Table 1

Killing of *C. elegans* by *Salmonella* strains.

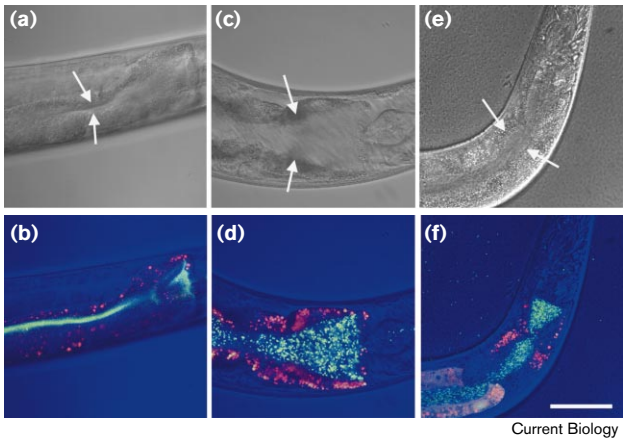
Strain	TD50*
E. coli OP50	9.9 ± 0.9
S. typhimurium SL1344	5.1 ± 0.7
S. typhimurium 14028	4.8 ± 0.9
S. typhimurium LT2	4.3 ± 0.2
S. dublin 'Lane'	3.7 ± 0.3
S. enteritidis 1047	5.3 ± 0.8
S. typhi 469	7.6 ± 0.7
S. paratyphi A 1094	7.9 ± 1.4
S. paratyphi B 1095	8.2 ± 0.5
S. paratyphi C 1096	9.0 ± 1.3

*The time for 50% of the nematodes to die (time to death 50, TD50) was calculated using the PRISM (version 2.00) computer program using the equation: Y = Bottom + (Top – Bottom)/(1 + 10^((LogEC50 – X)*HillSlope)), where X is the logarithm of days and Y is the average of dead worms. The data represent the means ± the standard deviation of at least two independent experiments.

As *S. typhimurium* pathogenesis in mammals is characterized by establishment of long-standing associations with their hosts [7], we were interested in determining whether SL1344 was capable of stably colonizing the *C. elegans* intestine. We therefore fed the worms with *S. typhimurium* for 5 hours and then transferred them to plates containing *E. coli* OP50. The results, shown in Figure 1b, indicate that 5 hours of exposure to *S. typhimurium* was sufficient to cause a similar rate of killing to the one obtained when *C. elegans* were in contact with *S. typhimurium* during the entire assay. As shown in the insert in Figure 1b, even an exposure of only 3 hours to *S. typhimurium* was sufficient to cause a significant amount of killing. Unlike *S. typhimurium*, *P. aeruginosa* fails to establish a long-lasting infection when worms are exposed to the bacteria for such short periods of time [3].

To test whether the premature death of the nematodes in the transfer experiment described above correlates with persistence of *S. typhimurium* in the *C. elegans* intestine, we constructed a *S. typhimurium* strain expressing the *Aequorea victoria* green fluorescent protein (GFP). Figure 2c and d show that after 72 hours of continuous feeding on *S. typhimurium* SL1344, the intestinal lumen was distended and full of intact bacteria. As reported previously, similar results were obtained when the worms were fed with GFP-expressing *P. aeruginosa* for 24 hours (Figure 2e,f). In contrast, no intact bacteria were observed when the worms were fed with *E. coli* DH5α–GFP for 72 hours and the lumen was not distended (Figure 2b). When the worms were fed with *S. typhimurium* SL1344 for 5 hours, however, and then transferred to *E. coli* DH5α–GFP, after

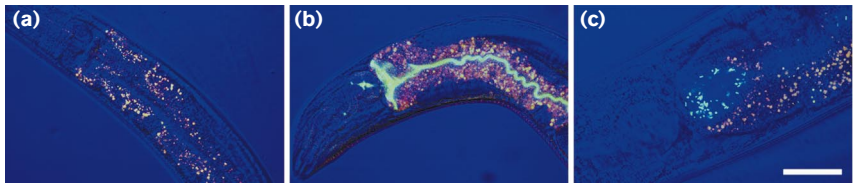
Figure 2



Confocal images showing bacterial colonization of the *C. elegans* intestine. Young adult hermaphrodite worms were fed on **(a,b)** *E. coli* DH5α–GFP for 72 h, **(c,d)** *S. typhimurium* SL1344–GFP for 72 h, or **(e,f)** *P. aeruginosa* PA14–GFP for 24 h. (a,c,e) In these transmission images the intestinal margins are indicated with arrows. (b,d,f) These merged images show bacterial fluorescence (green channel) and the gut autofluorescence (red channel). The scale bar represents 50 μm.

Figure 3

C. elegans colonizes the worm intestine. Young adult worms were fed on **(a)** *E. coli* DH5α–GFP or **(b,c)** *S. typhimurium* SL1344–GFP for 5 h and then transferred to *E. coli* OP50 for (a,b) 24 h or (c) 96 h. The merged images show bacterial fluorescence (green channel) and gut autofluorescence (red channel). The scale bar represents 50 μm.



72 hours the lumen became distended and full of DH5α–GFP (data not shown). This indicated that *Salmonella*-infected worms were impaired in grinding, digesting, and/or defecating *E. coli*.

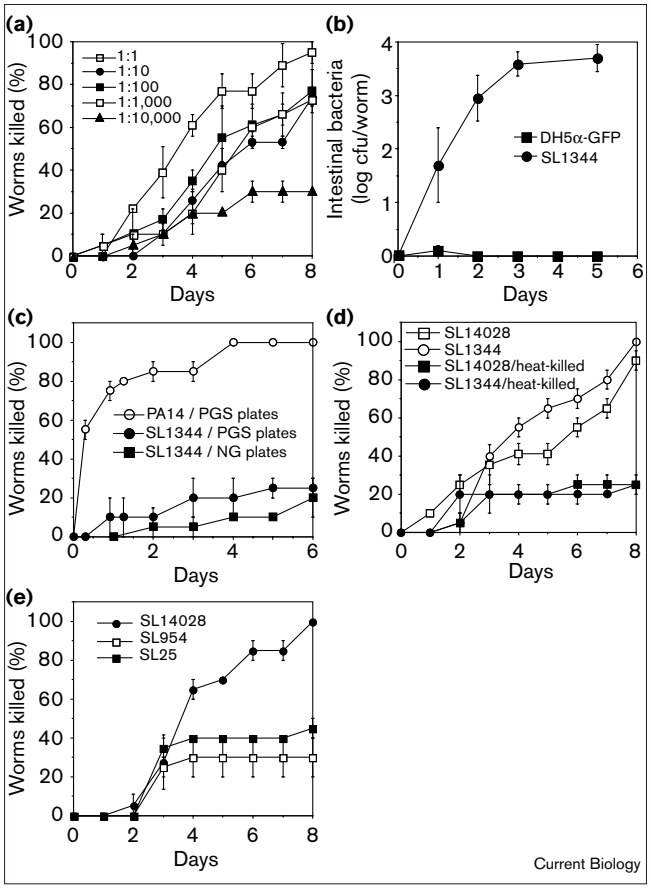
An early step in the establishment of a *S. typhimurium* infection in the gastrointestinal tract of a vertebrate host involves attachment to intestinal epithelial cells [8]. We postulated that if a similar step occurred in *C. elegans*, the infection should persist even if the worms were removed from the *S. typhimurium* source. We tested for persistence of *S. typhimurium* in the *C. elegans* intestine by feeding worms with *S. typhimurium* SL1344–GFP. Figure 3b shows that a high level of green fluorescence was observed in the *C. elegans* intestinal lumen 24 hours after transfer from *S. typhimurium* SL1344–GFP to *E. coli*, whereas Figure 3a shows that no green fluorescence could be detected 24 hours after transfer from *E. coli* DH5α–GFP to non-labeled *E. coli* OP50. Figure 3c shows that even 96 hours after transfer to *E. coli*, GFP-labeled bacteria could still be found in the distended intestine. As shown in Figure 3b, GFP-expressing bacteria were also found in the pharyngeal grinder, suggesting colonization of the pharynx. We cannot, however, rule out the possibility of regurgitation caused by manipulation of the worms while processing them for microscopy.

In the case of mammalian gastro-intestinal infections, environmental factors such as gastric pH, the presence of normal flora, the presence of food in the stomach, and the overall physiological state of the host are important during the early stages of an infection [8]. In humans, it has been reported that 10^5–10^{10} *Salmonella* cells are required to initiate an infection [9] though the minimum size of the inoculum is affected by the factors described above. To determine whether *S. typhimurium* is actually infectious and can proliferate inside *C. elegans*, we fed *C. elegans* mixtures of *E. coli* and *S. typhimurium* in which the *E. coli* was present in excess. The results are shown in Figure 4a and show that 1:10, 1:100 and 1:1000 mixtures of *S. typhimurium* and *E. coli* killed *C. elegans* at a rate only slightly slower than that observed on 100% *S. typhimurium*. However, at a ratio of 1:10,000, the rate of killing was significantly reduced. These results suggested that even at low initial doses, *S. typhimurium* can proliferate in the *C. elegans* intestine until it reaches a titer high enough to kill the worms. The experiment shown in Figure 4b confirmed this interpretation. A 0.1% mixture of *S. typhimurium* SL1344/GFP in *E. coli* OP50 was fed to *C. elegans* for 5 hours, after which the worms were transferred to *E. coli* OP50 plates. Every 24 hours, 10 worms were disrupted and the number of *S. typhimurium* or *E. coli* associated with the worms was determined by diluting and plating on ampicillin plates (to select for the GFP plasmid). As shown in Figure 4b, essentially no *E. coli*–GFP could be recovered from worms after the transfer, whereas SL1344–GFP proliferated to a relatively high titer (~10^4 cells per worm). Similar results were obtained when MacConkey agar was used to distinguish *E. coli* from *Salmonella* on the basis of colony color, ruling out the possibility that the ampicillin-resistance plasmid was being transferred to *E. coli*.

We showed previously that when *P. aeruginosa* is grown on a rich high-osmolarity medium, it secretes diffusible low molecular weight toxins, including phenazines, which kill *C. elegans* relatively quickly [1]. We refer to this type of killing as 'fast killing'. Heat-killed *P. aeruginosa* grown under fast-killing conditions also kill *C. elegans*. Moreover, when *P. aeruginosa* is grown on a 0.45 μm filter on fast-killing agar medium, *C. elegans* is killed in the absence of bacteria by the *P. aeruginosa* toxins released into the agar. To determine whether *S. typhimurium*-mediated killing is caused by diffusible toxins, *S. typhimurium* SL1344 was grown for 12 hours on 0.45 μm filters on either modified NG agar medium or on a high-osmolarity agar medium (PGS), the filters containing the bacteria were removed, heat-inactivated *E. coli* was spread on the plates to prevent starvation, and L4 hermaphrodite worms were placed on the agar. Figure 4c shows that there was no premature death of the worms either under the growth conditions used in this work or under the fast-killing conditions used for *P. aeruginosa*. Moreover, Figure 4d shows that two different heat-killed *S. typhimurium* strains (SL1344 and 14028) failed to kill *C. elegans*. These results indicate that direct association between viable *S. typhimurium* and *C. elegans* is necessary for killing.

To evaluate whether the *C. elegans* model will be useful for studying and identifying *Salmonella* virulence factors

Figure 4



(a–d) *C. elegans* killing correlates with proliferation of *S. typhimurium* and is not mediated by a diffusible toxin. (a) Dilutions of *S. typhimurium* in *E. coli* were prepared on NG plates and young adult worms were immediately placed on the plates. (b) Young adult worms were placed on plates containing a 1:1000 mixture of *S. typhimurium* SL1344–GFP and *E. coli* DH5α (filled circles) or on plates containing only *E. coli* DH5α–GFP (filled squares). After 5 h the worms were washed in M9 buffer and transferred to *E. coli* OP50 (non-GFP) plates. Every 24 h, 10 worms were transferred to M9 buffer containing 1% Triton X-100, the worms were mechanically disrupted, and the number of ampicillin-resistant bacteria determined. (c) *P. aeruginosa* PA14 (open circles) was grown on 0.45 μm filters placed on PGS plates and SL1344 was grown on 0.45 μm filters placed either on PGP (filled circles) or NG plates (filled squares). Following growth of the bacteria, filters were removed, the plates were exposed to UV light for 5 min to kill contaminating bacteria, heat-killed OP50 was added as a food source, and worms were added. (d) Twenty worms feeding on heat-killed (filled circles) or live (open circles) *S. typhimurium* SL1344 or heat-killed (filled squares) or live (open squares) *S. typhimurium* 14028. **(e)** The PhoP/PhoQ system is required for *C. elegans* killing. One-day-old adult hermaphrodite worms were fed on the *S. typhimurium* strain 14028 (SL14028) (closed circles), or on SL14028 with a point mutation in *phoQ* (SL25) (closed squares) or with a *phoP/phoQ/purB* deletion (SL954) (open squares).

relevant to human disease, we analyzed the role of the *S. typhimurium* PhoP/PhoQ signal transduction system, a major regulator of virulence-related genes in vertebrates [10]. We observed that both *S. typhimurium* with a *phoP/phoQ/purB* deletion [11] and *S. typhimurium* with a point mutation in the *phoQ* gene that results in unregulated expression of PhoP-activated genes [12] caused significantly less killing of *C. elegans* (Figure 4e). Further work will be required to fully elucidate the role of the PhoP/PhoQ regulon in *C. elegans* killing.

Note added in proof

In the accompanying article, Labrousse *et al.* [13] also show that *S. typhimurium* kills *C. elegans*. Moreover, they show that *S. typhimurium* mutants that affect acid tolerance, an important aspect of *Salmonella* virulence, are less virulent in the *C. elegans* model.

Supplementary material

Supplementary material including additional methodological details is available at http://current-biology.com/supmat/supmatin.htm.

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References

1. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM: **Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model.** *Cell* 1999, **96:**47-56.
2. Darby C, Cosma CL, Thomas JH, Manoil C: **Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*.** *Proc Natl Acad Sci USA* 1999, **96:**15202-15207.
3. Tan MW, Mahajan-Miklos S, Ausubel FM: **Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis.** *Proc Natl Acad Sci USA* 1999, **96:**715-720.
4. Tan MW, Rahme LG, Sternberg J, Tompkins RG, Ausubel FM: ***Pseudomonas aeruginosa* killing of *Caenorhabditis elegans* used to identify *P. aeruginosa* virulence factors.** *Proc Natl Acad Sci USA* 1999, **96:**2408-2413.
5. Finlay BB: **Bacterial disease in diverse hosts.** *Cell* 1999, **96:**315-318.
6. Ward S, Miwa J: **Characterization of temperature-sensitive, fertilization-defective mutants of the nematode *Caenorhabditis elegans*.** *Genetics* 1978, **88:**285-303.
7. Galan JE, Bliska JB: **Cross-talk between bacterial pathogens and their host cells.** *Annu Rev Cell Dev Biol* 1996, **12:**221-255.
8. Darwin KH, Miller VL: **Molecular basis of the interaction of *Salmonella* with the intestinal mucosa.** *Clin Microbiol Rev* 1999, **12:**4025-4028.
9. Blaser MJ, Newman LS: **A review of human salmonellosis: I. infective dose.** *Rev Infect Dis* 1982, **4:**1096-1106.
10. Garcia VE, Soncini FC, Groisman EA: **The role of the PhoP/PhoQ regulon in *Salmonella* virulence.** *Res Microbiol* 1994, **145:**473-480.
11. Angelakopoulos H, Hohmann EL: **Pilot study of phoP/phoQ-deleted *Salmonella enterica* serovar typhimurium expressing *Helicobacter pylori* urease in adult volunteers.** *Infect Immun* 2000, **68:**2135-2141.
12. Gunn JS, Hohmann EL, Miller SI: **Transcriptional regulation of *Salmonella* virulence: a PhoQ periplasmic domain mutation results in increased net phosphotransfer to PhoP.** *J Bacteriol* 1996, **178:**6369-6373.
13. Labrousse A, Chauvet S, Couillault C, Kurz CL, Ewbank JJ: ***Caenorhabditis elegans* is a model host for *Salmonella typhimurium*.** *Curr Biol* 2000, **10:**1543-1545.