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Live and Dead GFP-Tagged Bacteria Showed Indistinguishable Fluorescence in *Caenorhabditis elegans* Gut[§]

Ju-Ya Hsiao[1], Chun-Yao Chen[2], Mei-Jun Yang[1], and Han-Chen Ho[1]*

[1]Department of Anatomy, [2]Department of Life Science, Tzu Chi University, Hualien 97004, Taiwan

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Caenorhabditis elegans **has been used for studying host-pathogen interactions since long, and many virulence genes of pathogens have been successfully identified. In several studies, fluorescent pathogens were fed to *C. elegans* and fluorescence observed in the gut was considered an indicator for bacterial colonization. However, the grinder in the pharynx of these nematodes supposedly crushes the bacterial cells, and the ground material is delivered to the intestine for nutrient absorption. Therefore, it remains unclear whether intact bacteria pass through the grinder and colonize in the intestine. Here we investigated whether the appearance of fluorescence is indicative of intact bacteria in the gut using both fluorescence microscopy and transmission electron microscopy. In wild-type N2 *C. elegans*, *Escherichia coli* DH5α, and *Vibrio vulnificus* 93U204, both of which express the green fluorescence protein, were found intact only proximal to the grinder, while crushed bacterial debris was found in the post-pharyngeal lumen. Nevertheless, the fluorescence was evident throughout the lumen of worm intestines irrespective of whether the bacteria were intact or not. We further investigated the interaction of the bacteria with *C. elegans phm-2* mutant, which has a dysfunctional grinder. Both strains of bacteria were found to be intact and accumulated in the pharynx and intestine owing to the defective grinder. The fluorescence intensity of intact bacteria in *phm-2* worms was indistinguishable from that of crushed bacterial debris in N2 worms. Therefore, appearance of fluorescence in the *C. elegans* intestine should not be directly interpreted as successful bacterial colonization in the intestine.**

Keywords: *Vibrio vulnificus*, *phm-2*, electron microscopy

Introduction

Caenorhabditis elegans is one of the simplest animal models and has been used for studying host-pathogen interactions for more than a decade (Tan *et al*., 1999a). There are several advantages of using *C. elegans* as a model, including a short generation time, simple growth conditions for propagation, fixed cell linage, and well-developed genetic and molecular tools for manipulation (Kurz and Ewbank, 2000). This hermaphroditic and free-living soil nematode is a good non-mammalian host model for studying human infectious diseases, since many human bacterial pathogens have been proved to be virulent to *C. elegans*. Infection models have been established for Gram-negative pathogens such as *Burkholderia cepacia* (Coenye *et al*., 2001; Köthe *et al*., 2003), *B. pseudomallei* (O'Quinn *et al*., 2001; Gan *et al*., 2002), *Listeria monocytogenes* (Thomsen *et al*., 2006), *Salmonella typhimurium* (Aballay *et al*., 2000; Labrousse *et al*., 2000), *Serratia marcescens* (Mallo *et al*., 2002; Kurz *et al*., 2003; Schulenburg and Ewbank, 2004), *Pseudomonas aeruginosa* (Darby *et al*., 1999; Tan *et al*., 1999a, 1999b; Tan and Ausubel, 2000; Ruiz-Diez *et al*., 2003; Zaborin *et al*., 2009), *Vibrio cholerae* (Vaitkevicius *et al*., 2006), *V. vulnificus* (Dhakal *et al*., 2006), and *Yersinia pestis* (Styer *et al*., 2005). Some Gram-positive bacteria (Garsin *et al*., 2001; Jansen *et al*., 2002; Bolm *et al*., 2004) and fungi (Mylonakis *et al*., 2002; Tang *et al*., 2005) are also capable of infecting *C. elegans*. This model has been applied for delineating interaction between animal host and pathogenic, commensal, or mutualistic microbes (Tan and Shapira, 2011). Different mechanisms of bacterial pathogenesis have been successfully studied using *C. elegans* model. For example, *P. aeruginosa* and *Burkholderia* species usually produce extracellular toxins to kill *C. elegans* (Tan and Ausubel, 2000; O'Quinn *et al*., 2001), *Microbacterium nematophilum* colonizes on the *C. elegans* cuticle around the rectum (Hodgkin *et al*., 2000); while *S. typhimurium* and *S. marcescens* colonize the *C. elegans* intestine (Aballay *et al*., 2000; Labrousse *et al*., 2000; Kurz *et al*., 2003).

V. vulnificus is a motile, curved bacillus and a human opportunistic pathogen, which presents in a variety of seafood and warm seawater. Fatal septicemia could be induced when contaminated shellfish are consumed raw. People with immuno-compromising conditions, such as chronic liver diseases and heavy alcohol consumption, are more susceptible to *V. vulnificus* infection (Strom and Paranjpye, 2000). In septic patients, the mortality rate could be over fifty percent (Hsueh *et al*., 2004). In addition, *V. vulnificus* also causes wound infections on exposure of open wounds to contaminated seawater. Many virulence factors of *V. vulnificus* have been studied *in vivo* and *in vitro* (Jones and Oliver, 2009). However, a mouse model infected via the oral route, which simulates the most usual infection route in human infection, was only minimally affected in a previous study (Hor *et al*., 2000). Therefore, an alternative oral infection model is needed. Maintaining *C. elegans* requires much less space

*For correspondence. E-mail: hcho@mail.tcu.edu.tw; Tel.: +886-3-856-5301; Fax: +886-3-856-4641
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and resources than traditional laboratory animals, and this model allows for large-scale screening manipulation, which is needed in studies identifying unknown virulence factors (Kurz and Ewbank, 2007).

C. elegans is a facile animal model for *V. vulnificus* infection and the virulence factors involved have been found to be essential to mammalian pathogenesis (Dhakal *et al.*, 2006). Nevertheless, as found in other bacteria that infect *C. elegans* by colonizing the intestine, the accumulation of fluorescence in the intestine is usually considered as an evidence of proliferation and colonization of the fluorescent pathogens fed to the worms. However, when *C. elegans* feeds on bacteria, the food is crushed by the cuticle-lining grinding apparatus, and only the ground material is delivered to the intestine for nutrient absorption. Therefore, whether intact bacteria could pass through the grinder and colonize in the intestine is unknown. The resolution of fluorescence microscope is not high enough to distinguish crushed bacterial debris from intact bacteria. In order to investigate whether the appearance of fluorescence is indicative of intact fluorescent bacteria in the gut, both fluorescence microscopy and transmission electron microscopy were applied in this study.

Materials and Methods

C. elegans strains

Wild-type Bristol N2 and *phm-2* mutant strain DA597 (Avery, 1993) were kindly provided by Prof. Yi-Chun Wu, National Taiwan University. The DA597 mutant has a dysfunctional grinder. All worms were maintained on nematode growth medium (NGM, containing 0.25% peptone, 0.3% NaCl, 1.7% Bacto-agar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mg cholesterol, 25 mM phosphate buffer) agar plates at 20°C and fed with *E. coli* OP50.

Bacterial strains, plasmids, and culture conditions

E. coli OP50 as laboratory food source of *C. elegans* was cultured in Luria-Bertani (LB) medium at 37°C. *V. vulnificus* 93U204, which is virulent to mouse, zebrafish, and tilapia (unpublished data), was isolated from the spleen of moribund tilapia and used in this study as a representative animal pathogen. A common laboratory *E. coli* strain DH5α was used in this study as a non-virulent control (Taylor *et al.*, 1993). Plasmid pGFPuv (Clontech Laboratories, Inc., USA), carrying the gene for a green fluorescent protein variant optimized for maximal fluorescence when excited by UV light, was electroporated into *V. vulnificus* 93U204 and *E. coli* DH5α. The pGFPuv-containing *V. vulnificus* 93U204 and *E. coli* DH5α bacteria were maintained in LB medium containing ampicillin (100 μg/ml) for selection. For *C. elegans* feeding and fluorescence observation, GFPuv-expressing *V. vulnificus* 93U204 and *E. coli* DH5α were cultured on modified NGM (1% peptone; additional 0.5% yeast extract was added). *E. coli* OP50 was used as a negative, non-fluorescence control.

Microscopic observation of *C. elegans*

Bristol N2 and *phm-2* mutant strain DA597 were fed with GFPuv-expressing *V. vulnificus* 93U204 and *E. coli* DH5α; *E. coli* OP50 was fed to the *C. elegans* as control. An overnight LB broth culture (20 μl) of the test bacterial strain was spread on a 3.5-cm-diameter plate of modified NGM agar containing ampicillin (100 μg/ml) and incubated overnight at 37°C. Each plate was seeded with 10 adult worms for egg-laying and the worms were removed on the second day. Eggs were allowed to develop to adulthood and fed the test bacteria from the day they hatched. After feeding for 4 days, the worms were fixed with 4% paraformaldehyde and examined under differential interference contrast (DIC) microscope and fluorescence microscope (Olympus IX 71, Olympus, Japan) equipped with an Olympus DP70 digital camera. All experiments were carried out at 20°C.

Transmission electron microscopy

Worms fed with test bacteria for 4 days as described above were prefixed in 4% paraformaldehyde and 2.5% glutaraldehyde/0.1 M cacodylate buffer containing 1% tannic acid at 4°C overnight and again in 2.5% glutaraldehyde/0.1 M cacodylate buffer at 4°C for 1 h the next day. The specimens were washed in 5% sucrose/0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide/0.1 M cacodylate buffer for 2 h at room temperature. After fixation, the worms were *en bloc* stained with 2% uranyl acetate, dehydrated in a graded series of ethanol and twice in 100% acetone. Specimens were infiltrated with Spurr's resin overnight and embedded in fresh Spurr's resin the next day. Serial ultrathin sections of approximately 80 nm were cut with a diamond knife on a Leica Ultracut R ultramicrotome (Leica, Switzerland) and examined using a Hitachi H-7500 transmission electron microscope (TEM, Hitachi, Japan) at 80 kV. In each experiment, at least 9 worms were sectioned, and at least 10 ultrathin sections were examined for each worm.

C. elegans killing assays

An overnight LB broth culture (20 μl) of the test bacterial strain was spread on a 3.5-cm-diameter NGM agar plate and incubated overnight at 37°C. The killing assays were conducted at 20°C, with a total of 240 worms used. In each assay, 20 L4 larvae or adult worms were added per plate, and each assay was carried out in triplicate. Live worms were scored everyday and transferred to a new plate containing the test bacteria until all worms were dead. A worm was considered dead when it no longer responded to touch. Nematode survival differences were tested for significance by use of the log-rank test (software PAST, version 2.16, http://folk.uio.no/ohammer/past/). $P<0.05$ was considered significant.

Results

Intact bacteria hardly passed through the grinder of wild-type worms

The grinder is located in the terminal bulb of *C. elegans* pharynx. In wild-type N2 worms, this cuticle specialization ground up the intact bacteria ingested from the mouth, and only bacterial debris was found to pass through the pharyngeal-intestinal valve and enter the intestine (Fig. 1). In

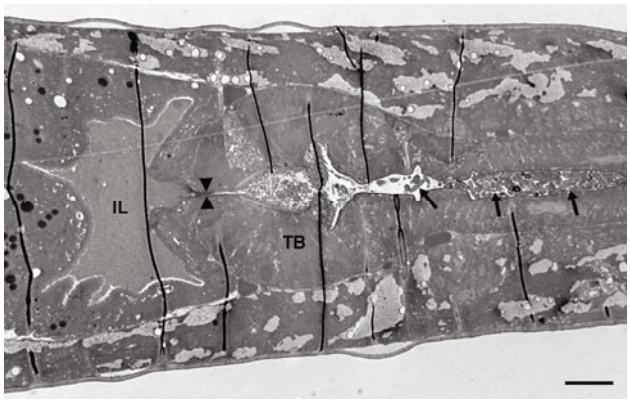

Fig. 1. Transmission electron micrograph of N2 *C. elegans* fed with *E. coli* OP50. Intact bacteria were found trapped in the pharynx (arrows), and those ground into pieces by the grinder were found in the terminal bulb (TB). Only bacterial debris passed through the pharyngeal-intestinal valve (arrowheads) and entered the intestinal lumen (IL). Scale bar=5 μm.

order to investigate whether fluorescence observed in the *C. elegans* intestine represents intact fluorescent bacteria colonization, we examined the fate of two GFPuv-expressing bacterial strains, the non-virulent *E. coli* DH5α and virulent *V. vulnificus* 93U204, in *C. elegans* wild-type and mutant strains. Green fluorescence was observed throughout the lumen of the entire alimentary tract of wild-type N2 worms for both *E. coli* DH5α and *V. vulnificus* 93U204 (Figs. 2A–2D). The proximal intestinal lumen and the hindgut appeared distended and fluoresced intensely. However, under TEM, no intact bacteria were found in the distended lumen of the 18 wild-type worms been sectioned. Intact bacteria were present only proximal to the grinder, while only crushed bacterial debris was present in the intestinal lumen (Figs. 2E and 2F).

Intact bacteria fluoresced indistinguishably from bacterial debris

To understand whether intact bacteria exhibit different fluorescence pattern or intensity inside the nematode intestine, *C. elegans phm-2* mutant strain was used. The *phm-2* mutant has a dysfunctional grinder because of defects in the pharyngeal contractile muscles. As expected, when *phm-2* mutants fed with *E. coli* DH5α as non-virulent control, intact *E. coli* DH5α in the intestinal lumen was easily observed under TEM due to the defective grinder. However, the distribution pattern of fluorescence and distention of the worm intestine were similar to that found in the N2 *C. elegans* (Fig. 3). Similarly, after *phm-2* mutants fed with *V. vulnificus* 93U204 for 4 days, intact *V. vulnificus* 93U204 instead of debris were found accumulated in the intestinal lumen (Fig. 3F). The fluorescence was still found throughout the alimentary tract, and accumulated mostly in post-pharyngeal lumen and rectum. The morphologically intact bacteria did not fluoresce differently from bacterial debris (Figs. 2, 3, and Supplementary data Fig. S1).

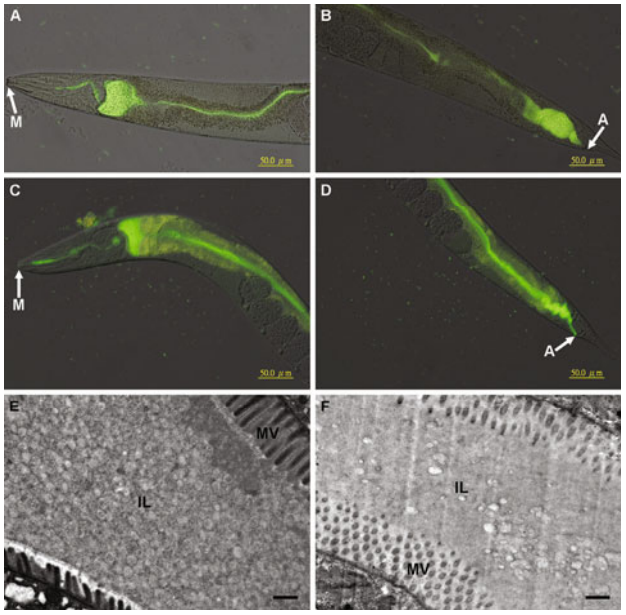

Fig. 2. Micrographs of N2 *C. elegans* fed with fluorescent *E. coli* DH5α (A, B, E) and *V. vulnificus* 93U204 (C, D, F) for 4 days. Merged DIC/fluorescent images (A–D) of the representative nematodes showed that the green fluorescence was indistinguishably present throughout the lumen of the entire alimentary tract of all worms fed with fluorescent bacteria. (E, F) Transmission electron micrographs revealed that both *E. coli* DH5α and *V. vulnificus* 93U204 became debris in the intestinal lumen (IL). M, mouth; A, anus; MV, microvilli. Scale bars: 50 μm in A–D; 500 nm in E, F.

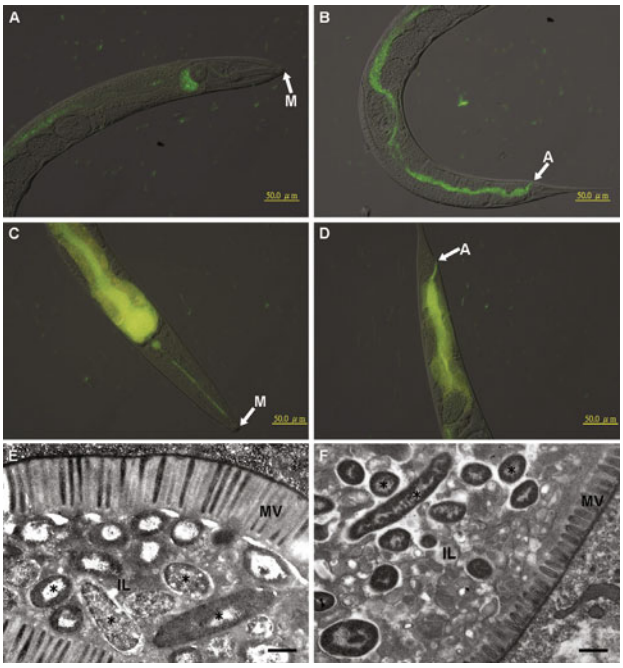

Fig. 3. Micrographs of *phm-2* mutant *C. elegans* fed with fluorescent *E. coli* DH5α (A, B, E) and *V. vulnificus* 93U204 (C, D, F) for 4 days. Merged DIC/fluorescent images (A–D) of the representative *phm-2* nematodes also showed that the green fluorescence was present throughout the lumen of the alimentary tract of all mutant worms. Under TEM, *E. coli* DH5α (E) and *V. vulnificus* 93U204 (F) were found intact (*) and accumulated in the intestinal lumen (IL) due to the dysfunctional grinder. M, mouth; A, anus; MV, microvilli. Scale bars: 50 μm in A–D; 500 nm in E, F.

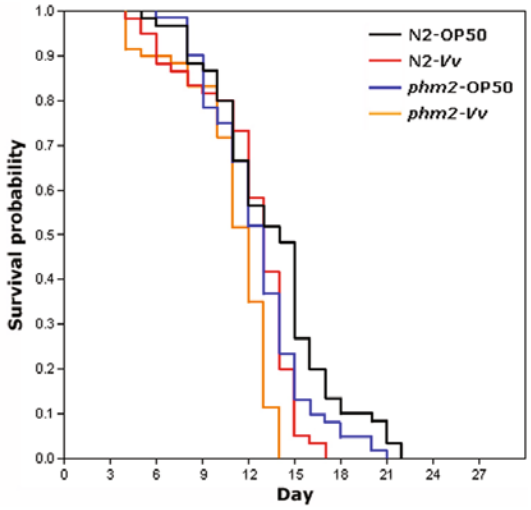

Fig. 4. Survival of *C. elegans* fed with *E. coli* OP50 and *V. vulnificus* 93U204. On NGM plate, the lifespan of wild-type N2 fed with *V. vulnificus* 93U204 (red line) was significantly shorter than N2 fed with *E. coli* OP50 (black line, P=0.0034). All worms were dead by day 16 when fed with *V. vulnificus* 93U204, while worms fed with *E. coli* OP50 lived till day 22. The survival period of *phm-2* mutants grown on *V. vulnificus* 93U204 was further reduced to 14 days (orange line), significantly shorter than *phm-2* worms fed with *E. coli* OP50 (blue line, P=0.0007). Log-rank test was used to analyze the survival differences, and P<0.05 was considered significant.

Virulence of *V. vulnificus* on *C. elegans*

In order to verify the virulence of *V. vulnificus* 93U204 on *C. elegans*, a survival test was conducted. In case of both N2 and *phm-2* worms, those that were fed *V. vulnificus* 93U204 had a shorter lifespan than those that were fed *E. coli* OP50 (control) (Fig. 4). *V. vulnificus* treatment significantly reduced survival rate in N2 worms (P=0.0034, log-rank test, versus *E. coli*), and in *phm-2* worms (P=0.0007, log-rank test, versus *E. coli*), indicating that pathogen treatment did lead to change in *C. elegans* survival. When comparisons were made by N2 and *phm-2* worms that were fed with the same test bacterial strain in order to assess the effect of bacterial viability on *C. elegans* survival, a significantly reduced lifespan was seen in *phm-2* worms (P=0.00009, versus N2, log-rank test, worms were fed *V. vulnificus*; P=0.0378, versus N2, log-rank test, worms were fed *E. coli*). These data suggest that colonization of intact bacteria in the gut reduces the lifespan of *C. elegans*, and this phenomenon was more significant if worms were fed pathogen *V. vulnificus* 93U204.

Discussion

C. elegans has been an important model organism for studying host-pathogen interactions. Many breakthrough findings proved *C. elegans* as a powerful tool (Tan and Shapira, 2011). Nevertheless, most studies evaluated bacterial pathogens under light or fluorescence microscopes, which are usually not able to differentiate whole bacteria from crushed bacterial debris inside the nematode gut. Therefore, in this study, we investigated bacterial status under electron microscope and correlated the results with fluorescent images. It is surprising to find that both whole fluorescent bacteria and crushed debris fluoresced indistinguishably in the worm's gut. Therefore, it is dangerous and could be misleading for studies that feed fluorescent bacteria to *C. elegans* to conclude colonization of bacteria in the intestine just by the appearance of fluorescence in the gut under fluorescence microscope. Unless researchers could design a fluorescent bacterial construct that only fluoresces when bacteria are intact, appearance of fluorescence in the worm intestine is not equivalent to intact bacteria colonizing in the intestine. Otherwise, a short fasting period to see changes in fluorescence or examination under TEM may be needed in order to confirm the intestinal colonization of the tested pathogen.

Distention of the intestine, whether the whole alimentary tract or only the rectum and anal region, has also been considered to indicate active proliferation of bacteria in those regions (Dhakal *et al.*, 2006; Spanier *et al.*, 2010). Under TEM, we showed that the distended regions could be packed with bacterial debris instead of whole, living bacteria. Therefore, we speculate that the distended gut results from failure of the gut function (for example, due to the influence of bacterial toxin), and is not necessarily the result of bacteria proliferation. Nevertheless, bacterial packing in the pharynx and gut was shown to increase with age (Garigan *et al.*, 2002). Since we only observed 4-day-old worms, it would be interesting to see whether older worms behave differently from younger ones.

In our study, we only managed to section a total of 36 worms to correlate the fluorescent images with TEM findings. The number of worms observed might not be sufficient due to the limitations of our method. Besides, we could have missed the parts of wild-type worms with live intact bacteria in their guts for we only took representative images instead of sectioning through the whole worm. Recently, by removing surface bacteria and incubating worm lysates on agar plates, Portal-Celhay and Blaser (Portal-Celhay and Blaser, 2012) provided strong evidence that bacteria, pathogenic or not, do have the chance to escape from the functional grinder of wild-type *C. elegans* and colonize within their guts. Therefore, in our study, we are not arguing that intact bacteria have no means to pass through the functional grinder of *C. elegans* and enter the gut. What we are concerned with more is that fluorescence alone is not a good indicator to represent intact bacteria, as shown in this study.

The *V. vulnificus* 93U204 used in this study is virulent to tilapia and zebrafish. We have also demonstrated that virulence of *V. vulnificus* 93U204 to mice is equivalent to human pathogenic isolate YJ016 (unpublished data). In this study, *V. vulnificus* 93U204 reduced the lifespan of *C. elegans* significantly, no matter the bacteria were crushed or remained intact, suggesting *V. vulnificus* 93U204 debris alone might be virulent enough to *C. elegans*. In addition, accumulation of intact *V. vulnificus* 93U204 in the intestine of *phm-2* worms seemed to exacerbate infection, indicating that colonization of *V. vulnificus* 93U204 in the gut is more virulent to *C. elegans*.

Despite possessing a dysfunctional grinder, the *phm-2* mutants behaved similar to the wild-type N2 worms in our study, except that the bacteria remained intact inside the

intestinal lumen of the *phm-2* mutants. Infection of wild-type *C. elegans* may require the pathogen to paralyze the grinder, or wait until the small numbers of pathogen that escape grinder destruction to successfully colonize the intestine. Therefore, this model is limited in studying highly virulent pathogens. However, the grinder-defective *phm-2* mutant may be more suitable for studying opportunistic bacterial pathogens. The *phm-2* model has been successfully used in studying *B. cepacia* (Köthe *et al*., 2003) and should have much broader application.

In this study, we provided ultrastructural evidence to show that the appearance of fluorescence in the *C. elegans* intestinal lumen does not directly imply intact fluorescent bacteria colonization in those areas. Researchers using *C. elegans* as a model to study host-pathogen interactions should be cautious in clarifying bacterial colonization and interpretation.

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