

ORIGINAL ARTICLE

Selective recruitment of bacteria during embryogenesis of an earthworm

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Earthworms of the family Lumbricidae harbor specific and stable populations of *Acidovorax*-like bacteria within their excretory organs, the nephridia. The symbionts of *Eisenia foetida* are deposited into the egg capsules during mating and the nephridia of the juveniles are colonized before they hatch. The timing and mechanisms governing bacterial recruitment and colonization are unknown for the earthworm-*Acidovorax* association. This study examined the process of colonization of the symbiotic organ during development of the embryos within the egg capsules. Bacteria associated with the developing embryos were visualized using *in situ* hybridization to bacterial cells and laser scanning confocal microscopy. Bacterial cells were associated with earthworm embryos during the earliest stages of development—the ova through to hatching. Three-dimensional examination of stages of development revealed an embryonic duct that recruits the *Acidovorax*-like symbiont cells. As each segment matures, *Acidovorax*-like symbiotic bacteria are recruited into this duct, excluding most other bacterial types, and remain there for a period of days prior to migration into the nephridium. After colonization of the nephridial ampulla, the canal remains bacteria-free. In addition to the known *Acidovorax*-like bacteria, multiple types of bacteria interact with the embryos externally and internally during the full course of development, and ultimately fill the gut lumen near the end of development prior to hatching. Colonization of the correct tissues by specific bacteria during differentiation and maturation of the organs must involve selective host defenses and signaling between the two partners to prevent over growth of nascent tissues.

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Introduction

The Lumbricid earthworms (members of the family Lumbricidae), commonly found in North America and Europe, alter soil texture and chemical composition on a large scale due to the combined activity of both worm and bacteria (Darwin, 1889; Furlong *et al.*, 2002; Horn *et al.*, 2003; Ihssen *et al.*, 2003; Edwards, 2004; Egert *et al.*, 2004). The gut community essentially consists of ingested bacteria from the soil, which then shifts in composition in response to the earthworm gut conditions. The Lumbricid earthworms, also harbor a specific and stable population of extracellular Gram-negative bacteria within their excretory organs, nephridia (Knop, 1926; Schramm *et al.*, 2003). The nephridia are paired osmoregulatory/excretory organs on the internal body wall of each segment of the worm

(approximately 200 nephridia per worm) that pass fluid from the celom to the outside through a continuous winding tube that forms three major loops (Figure 1). *Acidovorax*-like bacteria colonize a pouch within the nephridia, the ampulla, at an estimated $3.33 \pm 1.87 \times 10^5$ colony forming units per nephridium in *Eisenia foetida* (Pinel *et al.*, in press). Although a function for these bacteria remains to be determined, evidence indicates this is a long-lived and stable association among the Lumbricidae with a cospeciation pattern between host and symbiont (Schramm *et al.*, 2003; Davidson, Lund, Stahl, James and Schramm unpublished data).

The earthworm-*Acidovorax* association has evolved mechanisms to ensure the transfer of the bacteria to the next generation. Colonization of a host by specific bacterial partners is most often initiated in the early life stages and influences the developmental program of the host (reviewed in McFall-Ngai and Ruby, 2000; McFall-Ngai, 2002). The common composting worm *E. foetida* transmits the nephridial bacteria directly into egg capsules where embryos develop (Davidson and Stahl, 2006; Figure 2). The eggs, sperm, albumin and bacteria are expelled through pores into a pre-capsule that forms

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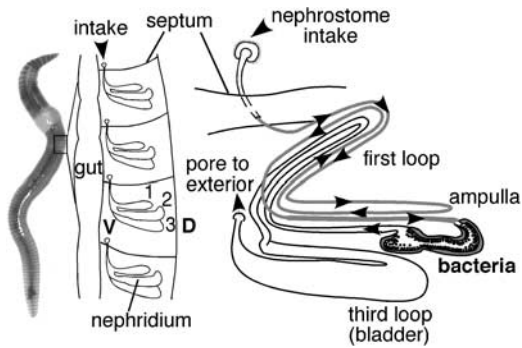


Figure 1 Anatomy of the earthworm nephridia. Left panel illustrates location of nephridia in the body wall. There are two nephridia in each segment (only one half of body is shown). Each takes in fluid from the celom of the worm through the nephrostome (top arrow heads). The fluid runs through three loops, including the ampulla in loop 2 containing symbiotic bacteria. Waste is expelled to the environment through a pore on the ventral side, nephridiopore. In the embryo, the bacteria ultimately enter through a pore that will become the nephridiopore.

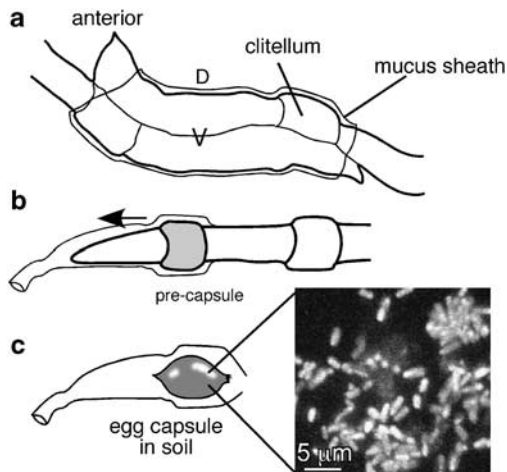


Figure 2 Earthworm mating and egg capsule formation. (a) Earthworms bind to each other on the ventral side, V, form a gelatinous mucoid covering and exchange egg and sperm. (b) The clitellum, a multilayered glandular structure, secretes the albumin and material necessary for the chitin shell formation to form the pre-capsule. This pre-capsule slides off the anterior of the worm picking up the eggs and sperm. (c) The capsule is deposited in the soil where the shell hardens. *Acidovorax* cells within the capsule were labeled by fluorescent *in situ* hybridization using the *Acidovorax*-specific oligonucleotide probe LSB145. D, dorsal; V, ventral.

on the exterior of the worm, then slides off the anterior to form the mature capsule with a sealed chitinous shell. The juveniles emerge fully colonized by their specific *Acidovorax*-like symbionts (Davidson and Stahl, 2006).

Because the formation of the capsule occurs externally, soil bacteria could be entrained along with bacteria originating from the nephridia. The presence of other bacteria within the capsules has been shown by culture-based studies of capsule contents and molecular analyses of 16S rRNA genes from egg albumin (Zachmann and Molina, 1993;

Davidson and Stahl, 2006). Although the egg capsule population is dominated by the *Acidovorax*-like symbiont (60–70% of the cells), the embryos of *E. foetida* develop within a dense population of the symbiont and other bacteria incorporated into the capsule during formation. Thus, the initial stage of this symbiotic association, although occurring within the protected environment of the capsule, presents a potential risk for the host. The animal must selectively recruit bacteria into the symbiotic organ while simultaneously avoiding invasion or overgrowth by bacteria during this vulnerable phase of development. The task of recruiting specific bacteria from a mixed population is a common challenge among symbiont associations involving extracellular symbiotic bacteria, but typically the juveniles must accomplish this after development and emergence from a protective, sterile environment, such as, egg shell.

In the study presented here, we identify the developmental timing and sequence of selective recruitment of symbiotic bacteria during embryogenesis and maturation of *E. foetida* within a mixed microbial community. Using a combination of fluorescent *in situ* hybridization (FISH) to bacterial cells in whole embryos visualized by laser scanning confocal microscopy, and electron microscopy these studies revealed a bacterial recruitment structure, and suggest a careful coordination of tissue differentiation with bacterial recruitment.

Methods

Laboratory rearing of *E. foetida*

Adult *E. foetida* (60 worms per 1l bedding; Yelm Earthworm and Castings Farm, Yelm, WA, USA) were maintained at 21 °C, in autoclaved hydrated (w/diH₂O) coir (mulched coconut husks, Coconut Palm Resources Inc., Hillsboro, OR, USA), 5g oatmeal and 10g coffee grounds. Bedding was changed approximately every 7–10 days.

Selection and fixation of *E. foetida* embryonic developmental stages

Newly deposited capsules (approximately 1 day old) were removed daily from bedding and examined by dissection microscope to confirm lack of developed embryos. The capsules were then maintained in Petri dishes on Whatman 1 filters moistened with distilled water, and examined to select apparently healthy embryos at stages day 5, 10–12, 14–15, 16, 22 and at hatching. Hatchlings emerged approximately 23 days after egg capsule formation (at 21 °C, this study). Early developmental stages up to 15 days were counterstained with Cell Tracker Green BODIPY (CT) prior to fixation by soaking egg capsules (ends cut) in a solution of 0.13 M NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, 0.5 mM phosphate buffer pH 7.3 and 5 µg ml⁻¹ CT for 15 min, then washed in buffer. Older embryos were sufficiently

autofluorescent for staining to be unnecessary. Embryos for *in situ* hybridizations, were fixed in egg capsules (ends cut) in buffered 4% paraformaldehyde (PFA 50 mM phosphate buffer, 0.15 M NaCl, pH 7.4) for 1.5 h at room temperature, washed in phosphate buffer and stored at -20°C in 70% ethanol until analysis. Embryos were left in the egg capsules until analysis by FISH to preserve the arrangement of the bacterial cells on the embryos.

Quantification of bacteria in egg capsules by FISH

To prepare albumin for FISH and bacterial cell quantification, 3 μl of egg capsule (0–1 day old) contents were removed and homogenized in 300 μl of 4% buffered paraformaldehyde (sterile-filtered) and incubated for 1 h. The appropriate dilution to obtain sufficiently even bacterial cell spread was determined empirically. The contents were centrifuged to pellet the cells, resuspended in phosphate buffer saline for wash, pelleted again and resuspended in a final volume of 300 μl distilled water ($100\times$ dilution). This suspension (3 μl) was loaded onto multiwell slides (Cel-line 8-well 6 mm slides with ADCELL Bio-adhesive coating; Erie Scientific, Portsmouth, NH, USA), dried and stored at -20°C until use (within three weeks). FISH was performed as described below, with modifications for incubation of slides using the EUB 338 and LSB 145 probes. By confocal, 10 frames of $100\times 100\ \mu\text{m}$ square area each, were captured for each capsule sample, then cells in each frame were counted. It was necessary to dilute ($100\times$) and spread the cells for quantification due to the high density of cells within the albumin.

FISH

The locations of *Acidovorax*-like symbiotic bacterial cells, and other types of bacterial cells within embryos were analyzed by *in situ* hybridization with a series of fluor-labeled oligonucleotide probes (FISH), then viewed with a Zeiss Pascal laser scanning confocal microscope to obtain three-dimensional reconstructions. Three distinct fluoros were used in combination for simultaneous labeling of different bacterial types. These were Cy3, Cy5 and fluorescein. The set of bacterial probes for *in situ* hybridization was selected based on prior data from bacterial 16S rRNA gene clone libraries from egg capsule DNA that revealed a mixed bacterial community primarily of *Acidovorax*-like symbionts, Actinobacteria (two ribotypes), members of betaproteobacteria, gammaproteobacteria and members of other divisions that appear inconsistently in the libraries (Davidson and Stahl, in preparation). The LSB 145 (S-G-Acidov-0145-a-A-18; probe designations according to Wheeler Alm *et al.*, 1996) probe identifies a subset of the *Acidovorax* group that includes the earthworm bacterial symbiotic strains (Schweitzer *et al.*, 2001; Schramm *et al.*, 2003). An

Actinobacterial probe HGC 69a targets most Actinobacteria members (Roller *et al.*, 1994). EUB 338 (S-D-Bact-0338-a-A-18) probe identifies most members of the bacterial domain (Amann *et al.*, 1990). Probes specific for the 'betaproteobacteria' (Cy3- or FL-BET42a; L-D-Beta-1027-a-A-16) and 'gammaproteobacteria' (Cy3- or FL-GAM42a; L-D-Gamma-1027-a-A-16) were used together to control for specificity (Manz *et al.*, 1992), as well as identify gamma- and betaproteobacteria.

Fluorescent *in situ* hybridization was performed according to a published protocol under stringent conditions for the probes used (Pernthaler *et al.*, 2001). Intact embryos stored in 70% ethanol were removed from the egg capsules, brought to 30% ethanol, placed in hybridization buffer, changed once then incubated in hybridization buffer in 0.6 ml microfuge tubes with $5\ \text{ng}\ \mu\text{l}^{-1}$ probe at 46°C for two hours, then placed in wash buffer at 48°C for 30 min, with one change after 15 min. Whole embryos were mounted in Vecta Shield (Vector Labs Inc., Burlingame, CA, USA) on slides and probe-conferred fluorescence was imaged using appropriate wavelength excitation on a Zeiss LSM Pascal laser scanning confocal microscope (Carl Zeiss, Jena, Germany).

Electron microscopy

For electron microscopy, 12 and 14 day embryos were fixed in the capsules in 1.25% glutaraldehyde, 0.1 M sodium phosphate buffer, pH 7.3, overnight at 4°C , washed in buffer, removed from capsules and post fixed with 2% osmium tetroxide, in 0.05 M phosphate buffer 1.5 h, rinsed and stored in 0.1 M phosphate buffer, pH 7.3. Samples for TEM (transmission electron microscope) were en bloc stained with 2% uranyl acetate (Ted Pella, Redding, CA, USA), rinsed and dehydrated through a graded series of alcohols and propylene oxide then embedded with Spurr's resin. Sections for light and TEM were cut using a Reichert Ultracut E microtome. Sections were mounted on 150 mesh rhodium/copper grids, stained with uranyl acetate and lead citrate and examined using a JEM 1200EX II TEM (JEOL Ltd, Tokyo, Japan). Operating conditions for the instrument included an accelerating voltage of 80 kV, a 300 μm condenser aperture, a 50 μm objective aperture and a spot size setting of 3. Images were acquired and saved on Kodak 4489 electron microscope film with a 'below the viewing screen' film transfer system. For SEM (scanning electron microscope), specimens were washed in distilled water, en bloc stained with 2% uranyl acetate (Ted Pella), rinsed twice with distilled water, dehydrated through a graded series of ethanols then critical point dried with a Samdri-PVT-3B CPD (Tousimis Research, Rockville, MD, USA), mounted on aluminum stubs, sputter coated with gold-palladium (Polaron SEM Coating Unit, E 5100, Polaron Instruments Inc., Hatfield, PA, USA) and

examined with a JEOL JSM 6300F field emission scanning electron microscope (JEOL) at an accelerating voltage of 15 kV.

Results

Egg capsules for analysis were collected from laboratory beds of adult *E. foetida*, and approximately 23 days at 21 °C were required for development to hatching. Eight capsules (0–1 day old) were satisfactorily processed for quantification of bacterial cells, and an average, estimated $9 (\pm 4) \times 10^5$ cells μl^{-1} , was determined for the albumin at the beginning of development, a density similar to a dense laboratory bacterial broth culture (10^9 cells ml^{-1}). Approximately 70% were identified by FISH to be the *Acidovorax*-like nephridial symbionts. Change in this composition over the incubation period was not quantified in this study.

Earthworms develop from small eggs with little yolk and form a blastula with a very large blastocoel. A blastopore that initially occupies the entire ventral surface narrows to a slit that closes from posterior to anterior leaving a pore that persists as the mouth (Wilson, 1889). The mouth remains open and the embryo takes in albumin for nourishment throughout the rest of development. The posterior end remains closed until the completion of development just before hatching (Wilson, 1889). As a result, the embryo is exposed, both externally and internally, to the bacteria within the albumin during the full course of development from deposition of the ova to hatching. Around day 10, the first segments began to mature, and the embryo resembled a sphere with segmentation apparent

along the ventral midline. The differentiation of the segments continued sequentially from anterior to posterior as the embryos elongated. As each segment develops, the nephridia begin to form internally from mesoblast, and complete the connection to the exterior by an invagination of the ectoblast (Wilson, 1889). The exact timing of nephridial maturation has not been described for *E. foetida*. By day 17–18 embryos resembled the juvenile worms that emerge.

Probe-bound bacterial cells were visualized within the transparent embryos by laser scanning confocal microscopy. Bacterial cells were in contact with the embryos as early as day 5 after deposition of the egg capsule, after the embryo had formed a blastula with a pore (Figure 3). Occasionally, cells were detected intracellularly and on the surface (image not shown). By mid-developmental stages (day 10–12) the first segments, each comprised of three major lobes, had formed at the anterior end of the embryo. Consistently, bacteria were detected on the surface of the embryos positive with EUB 338, HGC 69a and LSB 145 probes (Supplementary Figure 1). *Acidovorax*-like bacteria, the known symbionts, were observed to initially gather in a dorso-lateral canal in the anterior lobe of the third segment (Figure 4b). As each segment matured, *Acidovorax* cells entered the canal through a dorsal pore and resided there for a period of time (approximately 2–3 days) prior to migration into the nephridia through an entrance on the ventral side of the embryo (Figures 4 and 5). The canals were approximately 15 μm in diameter and extended 200–400 μm depending on the segment. Although three distinct cell types were observed at the pore surface, predominantly the *Acidovorax*-like

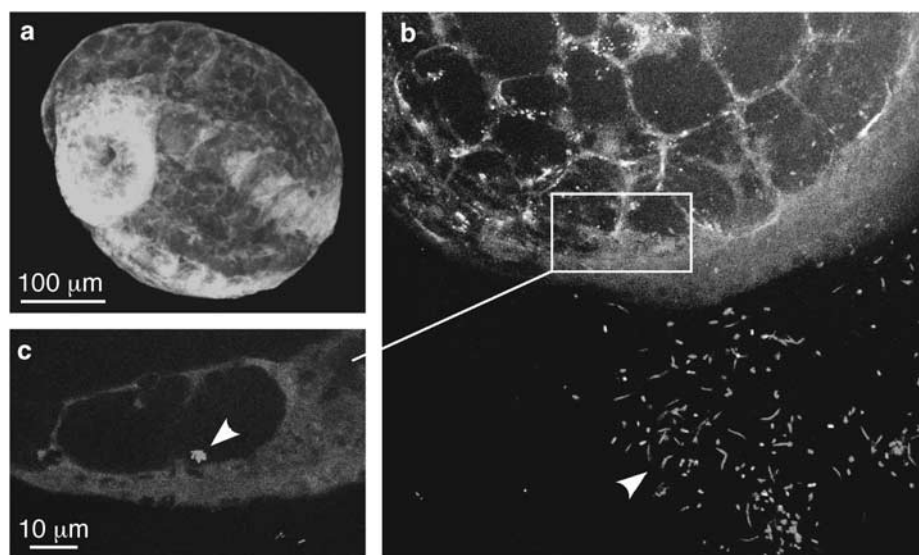


Figure 3 FISH LSCM of bacteria associated with early embryos in the egg capsule of *Eisenia foetida*. (a) Five day old embryo. (b) Optical stack of a portion of a blastula showing bacteria in albumin around embryo detected with the LSB 145 probe (yellow) and EUB 338 probe (red), box enlarged in (c), optical section through blastula revealing bacterial cells within the cells of the embryo. Arrows, bacterial cells. FISH, fluorescent *in situ* hybridization; LSCM, laser scanning confocal microscopy. See online version for color figure.

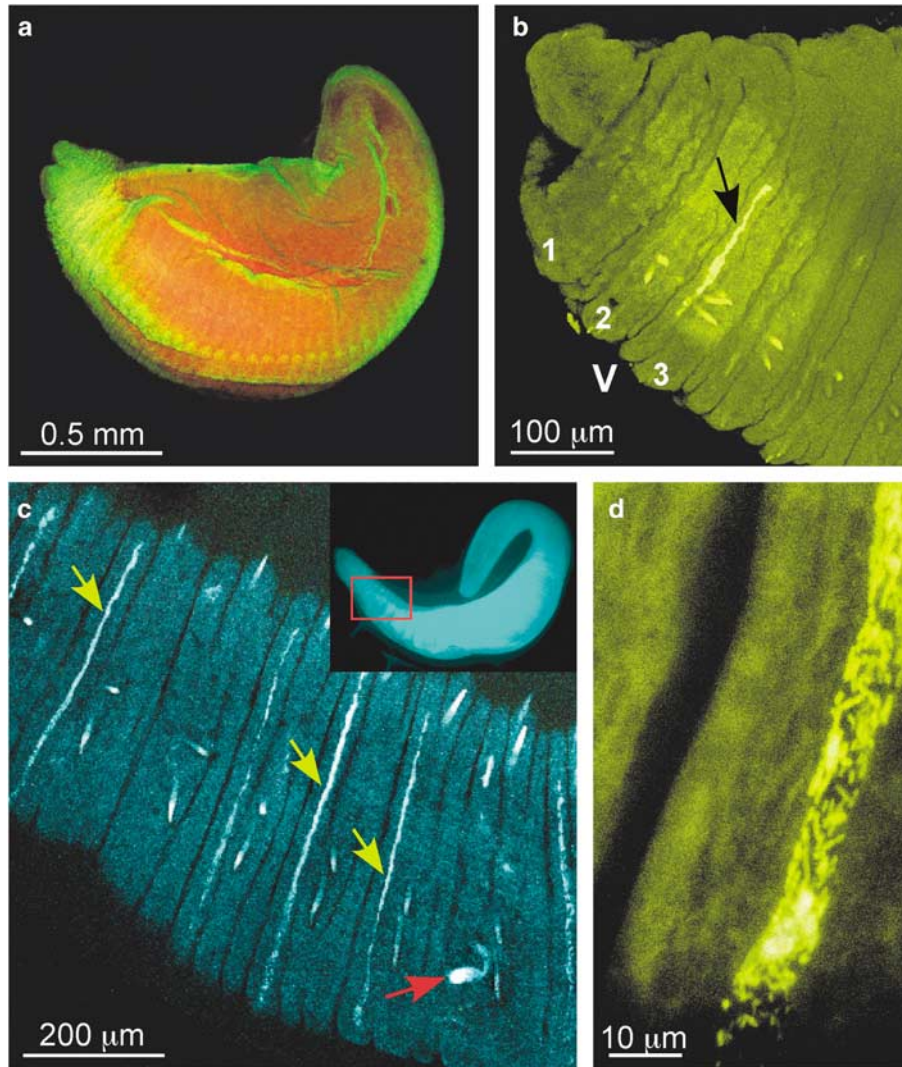


Figure 4 LSCM images of bacteria accumulating in canals of embryonic earthworms. (a) Ten to 12 day old embryo, dorsal side up. (b) Bacteria detected in segment three by FISH with an *Acidovorax*-specific probe LSB 145. V, ventral, arrow, location of bacteria in a canal of the third segment. (c) Fifteen day embryo demonstrating colonized canal in many segments along the length of the embryo (yellow arrows). Red arrow, colonized ampulla of a nephridium in segment with empty canal. (d) Enlargement to show bacteria within the canal. LSCM, laser scanning confocal microscopy.

symbiont cells entered and filled the canal (Figure 5; Supplementary Figure 1B and C). By day 14–16, one-third of the embryo's segments contained bacteria within canals, with a few segments having colonized nephridia visible through the body wall (Figure 4c). During this period, migrations into the nephridia occurred simultaneously on multiple segments (Figure 5A). By day 19–20, a day or two prior to hatching, the nephridia of the embryos were fully colonized, and the canals were free of bacteria.

In addition to the *Acidovorax*-like symbionts, two other cell types were observed associated with the surface of the embryo by FISH and TEM (Figure 5B), including an actinobacteria-probe-positive small rod, and a long thin cell positive with the eubacterial probe only. The two other cell types also entered the canal, but were in the minority and did not appear to

migrate into the nephridia. After migration, the actinobacteria-probe-positive cells were observed to remain as a small aggregate in the pore entrance of the canal (Supplementary Figure 2). Throughout development dense populations of bacteria were observed within the embryo gut lumen by FISH, comprising multiple morphologies and cell types positive for each of the probes tested and appeared packed within the pre-hatchling gut at maturity of the embryo (Figures 6a–d). Although the different fractions of this population were not quantified, by FISH the *Acidovorax*-like cells dominated during the earlier stages of development, and by late development *Acidovorax* cells were in the minority in the gut lumen (Figure 6d).

The structure of the bacterial recruitment canal was detailed by SEM and TEM. Bacteria were

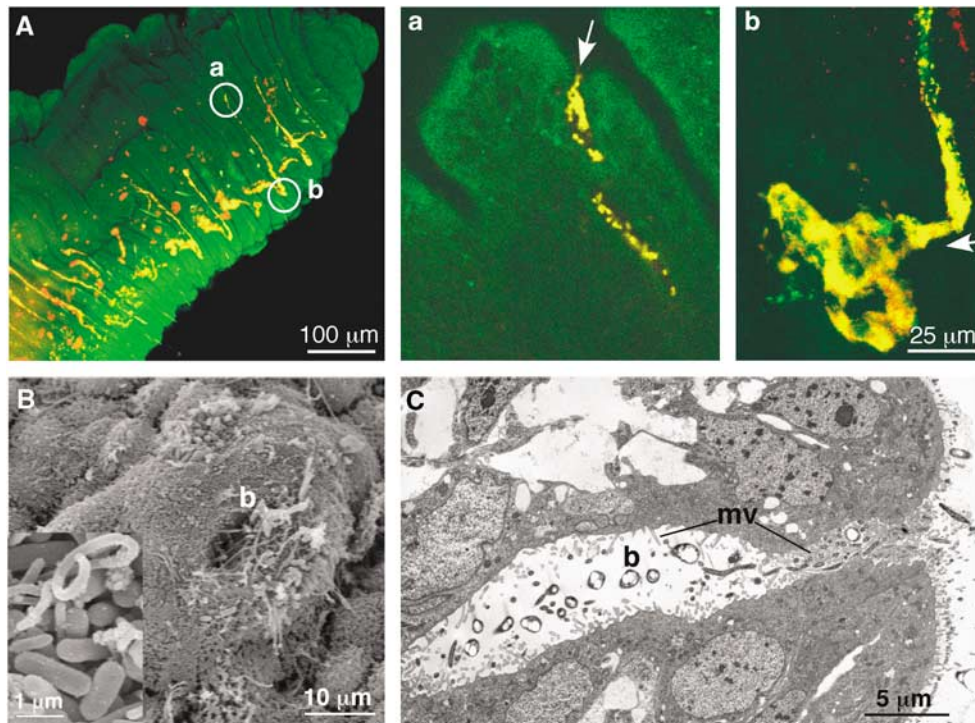


Figure 5 Details of the colonization canal. (A) Bacterial migration in several segments of a 14 day embryo, (a) site of entrance into canal on dorsal side, (b) site of migration into nephridium on ventral side, yellow cells, *Acidovorax* probe, red cells, eubacterial probe. (B) SEM of entrance pore with bacteria adhering to the surface, inset, enlargement of bacterial cells on the surface of the pore. (C) TEM cross-section of entrance to canal demonstrating microvilli (mv) along channel and bacteria (b) within the channel (additional images available in Supplementary Figure 3). SEM, scanning electron microscope; TEM, transmission electron microscope.

observed collecting around and entering a pore both by FISH and SEM (Figure 5B). TEM revealed extensive microvillar membranes immediately interior to the pore and microvilli sparsely lining the canal that runs just under the epithelial cells (Figure 5C). Cilia were not found, suggesting that bacteria are not drawn in by a ciliary current at this location. *Acidovorax*-like symbiont cells were observed occasionally adhered to the interior surface of the canal, but did not form a lining.

Discussion

This study described three main phases of colonization of *E. foetida* embryos that proceeds from anterior to posterior as the embryo develops; initial recruitment into the canal, a waiting period and final migration into the nephridium. The observed sequence of recruitment beginning with the anterior segments matches the described pattern of nephridial development and is likely related to the level of differentiation and maturity of the tissues in each segment. Nephridia develop internally beginning at the anterior segments and continuing posteriorly. Near the completion of nephridial development, occurring as each segment matures in the latter half of development, the nephridiopore opens to the exterior (Wilson, 1889). Thus, the model for

colonization of the earthworm embryo is a sequential acquisition that begins when a canal matures in a segment and releases an attractant inducing selective migration of the *Acidovorax*-like bacteria. The symbionts, along with a few interlopers, migrate into the canal (200–400 μm) extending under the epithelium to the location of the future nephridiopore on the ventral side (Figure 7). The bacteria persist in this canal until the nephridial duct develops sufficiently to open and allows migration into the nephridium, completing the maturation of this organ. Subsequent bacterial invasion into the canal does not occur owing to changes elicited by the transient exposure to bacteria or colonization of the nephridial tissue. This canal may be a transient developmental structure. The molecular details of these changes, mechanisms of selection for *Acidovorax*-like symbionts and host-bacterial communication are important topics for further study.

This pattern of recruitment is similar to that observed for colonization of the bacterial light-organ of the bobtail squid after it hatches into seawater (reviewed in Nyholm and McFall-Ngai, 2004), but occurs during embryogenesis in the worm. In the squid-*Vibrio* association, the juvenile nascent light organ creates a current to draw bacteria from the seawater. *Vibrio fischeri* are selectively collected from the mixed bacterial population in the sea water and accumulate by binding specifically to the

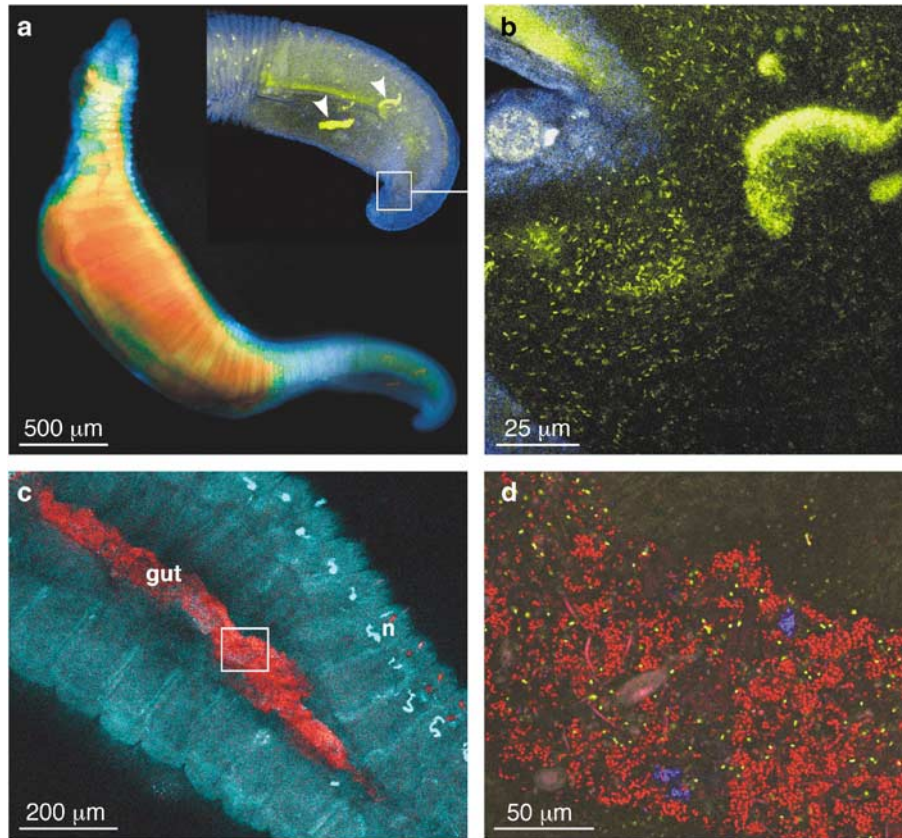


Figure 6 Bacteria within the embryonic gut lumen. (a) Day 15 embryo with incomplete gut tract. Inset, LSCM image stack of posterior end, yellow, bacterial cells binding EUB 338 probe, arrows, large bacterial aggregates, box, area magnified in panel b. (b) Optical section of a portion of the posterior end revealing large bacterial aggregates and free cells. (c, d) Day 22 embryo, n, nephridia, box, corresponds to area in panel d. (d) Bacterial cells in gut, red, gammaproteobacteria, blue, betaproteobacteria and yellow, *Acidovorax*. LSCM, laser scanning confocal microscopy.

mucus of the symbiotic organ with a few contaminant cells (Nyholm *et al.*, 2000; Nyholm and McFall-Ngai, 2003). After a waiting period (2–3 h), *V. fischeri* migrate into the nascent light organ through ciliated ducts that possess an oxidatively stressful environment to exclude other bacterial types (Small and McFall-Ngai, 1999; Davidson *et al.*, 2004). Motility and resistance to oxidative stress are required for this migration (Graf *et al.*, 1994; Visick and Ruby, 1998). Once the correct tissue is colonized, the ducts change to prevent further entrance, and the ciliated field that creates the current undergoes apoptosis and regresses (Foster *et al.*, 2000). Similar and distinct mechanisms are likely involved in the recruitment of *Acidovorax* into the earthworm embryo, including chemotaxis and specific adherence.

In contrast to the nephridial canal, by the end of development the interior lumen of the embryo contained a mixed population of bacteria composed primarily of cell types in the gamma- and beta-proteobacteria, suggesting a lack of specific selection in this location. It is highly likely that members of the capsule bacterial community, or at least subsets of this population, serve varied functions in the life history of the earthworm. A subset of

the bacteria within the gut lumen may continue to reside as gut bacteria in the later juvenile stages. Given the competitive nature of the soil community, the egg capsules may require defenses against fungal and bacterial overgrowth and predation. The capsule's shell does offer separation from the soil bacterial community, but the level of protection is unknown. There are many examples from soil and marine invertebrates of egg and larva-associated bacteria providing chemical protection against microorganisms (Gil-Turnes *et al.*, 1989; Gil-Turnes and Fenical, 1992; Lindquist, 2002; Kaltenpoth *et al.*, 2005). We hypothesize that bacteria associated with earthworm capsules provide chemical defenses.

Early development of *E. foetida* must involve expression of mechanisms to mediate interactions with bacteria during early differentiation before host tissues are fully formed. Although recruitment of specific extracellular bacteria from mixed populations is a common challenge for juvenile stages of animals, this usually occurs after differentiation and maturation of the embryo. Maternally-transmitted intracellular bacteria in the reproductive cells, and subsequently in the developing embryos of insects represents a different situation (Douglas, 1989; O'Neill *et al.*, 1997; Baumann, 2005). In this case,

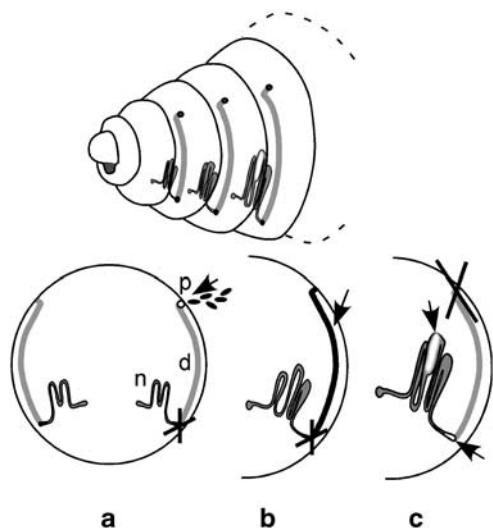


Figure 7 Stages of bacterial migration during colonization of embryonic nephridia. Top illustrates location of the recruitment canal on three segments of the anterior embryo with dorsal pores, and ventral nephridial entrances. Bottom sequence shows step-wise process of colonization, which begins in mid development around day 10 and proceeds over a period of days, as the segments mature from anterior to posterior, to completion by day 18. Each step begins anew as a segment becomes competent. (a) Beginning day 10 with third segment, bacteria enter pore, nephridial development not complete; (b) bacteria persist in canal leading to nephridia, but do not enter; (c) beginning around day 14 with anterior-most segments and continuing until completion, symbiotic bacteria migrate into nephridia, canal leading to exterior remains bacteria-free. Arrows indicate locations of bacteria, crosses signify inhibition of bacterial access.

the symbiotic bacteria have highly reduced genomes and are obligate intracellular inhabitants of the host. The developing embryonic tissues are still not exposed to a mixed external bacterial population. The interaction of multiple types of bacteria with the embryo, within the surface folds and the lumen of the future gut is an unusual, or at least undocumented, early developmental history.

The cellular mechanisms conferring protection of embryos during the early stages of division and differentiation are not well-characterized, apart from evidence of antibacterial protein expression in extra embryonic elements of insects, but not in the embryo itself (Bettencourt *et al.*, 2000; Gorman *et al.*, 2004). The only other documented example of extracellular mixed bacterial communities associated with developing embryos is in marine viviparous sponges, in which bacteria from the adult sponge breach a protective follicle and are incorporated into the developing embryo (Ereskovsky and Bouryesnault, 2002; Ereskovsky *et al.*, 2005). In contrast, the earthworm embryos are not surrounded by a protective follicle. Earthworm embryonic recruitment of symbionts offers an opportunity to study the expression of immune defenses, and other cellular features, involved in recruitment of bacteria during the earliest phases of development.

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