ULTRASTRUCTURAL CHANGES IN EARTHWORM (*EISENIA FETIDA*) NEPHRIDIA RESULTING FROM SYMBIOTIC ASSOCIATION WITH BACTERIA (*ACIDOVORAX SP.*)

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Nina Jaffarzad, B.A.

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ABSTRACT

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Nina Jaffarzad, B.A.

Texas State University-San Marcos

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SUPERVISING PROFESSOR: JOSEPH R. KOKE

This study examined the specific association between earthworms, *Eisenia fetida*, and the host-specific bacteria in their nephridia, or excretory organs, in order to determine if bacterial colonization of the nephridia resulted in morphological changes in the eukaryotic nephridial cells in contact with the prokaryotic symbionts. It was hypothesized that the bacteria, *Acidovorax sp.,* may induce ultrastructural changes in the nephridia of *Eisenia fetida*, as has been discovered in other host-microbial systems. The nephridial tissue architecture of symbiont-containing worms was compared with that of worms that developed from eggs treated with antibiotics (asymbionts) by transmission electron microscopy. Presence or absence of bacteria was confirmed by fluorescence *in situ* hybridization. Results suggest the presence of *Acidovorax* is necessary for normal development of nephridial structures, in particular microvilli and/or stereocilia on the luminal surface of nephridial epithelial cells.

INTRODUCTION

Many bacterial lineages have evolved close associations with eukaryotic hosts, covering the gamut from invasive parasitism to obligate mutualism (Dale *et al*, 2002). Reviews of genetic and regulatory elements pertaining to pathogenic and symbiotic associations reveal no apparent general principles allowing distinction between the two (Hentschel *et al*, 2000). Understanding the mechanisms of prokaryotic-eukaryotic interactions could increase our knowledge of virulence mechanisms of bacterial pathogens (Goebel and Gross 2001) and provide insight as to how pathogenic interactions are subverted into mutually beneficial associations in symbiotic relationships. Given the proliferation of antibiotic resistance among human pathogens, this insight may have practical and theoretical value.

Bacterial pathogens associate intimately with host cells. Some remain attached to the surface of the cell, while others are internalized (Steele-Mortimer *et al*, 2000). A common pathological interaction involves the endotoxin, lipopolysaccaride (LPS). LPS is a component of the outer membrane of gram-negative bacteria and has direct local effects on cells, including an increased rate of cell division by enterocytes in the gastrointestinal tract. Enterocytes are first generation derivatives of intestinal crypt stem cells. LPS also has systemic effects including activation of immune cells (Cetin *et al.*, 2004). In wound healing situations, LPS inhibits epithelial regrowth thus promoting bacterial translocation across epithelia and further tissue injury (Cetin *et al.*, 2004).

Because of emerging infectious diseases, particularly in the last decade, and the proliferation of antibiotic resistance, much research has been done on virulence. This research has led to discovery of two common mechanisms by which bacteria can share pathogenic mechanisms, and, interfere with internal eukaryotic functions (Mecsas and Strauss, 1996) even while external to eukaryotic cells. The sharing mechanism is the presence of "pathogenicity islands" – regions of DNA containing genes of related functions that can be shared among bacterial species by lateral gene transfer (plasmids). The virulence mechanism results from the gene products of the pathogenicity islands, which include the proteins for type III secretion. Type III secretion allows proteins (also gene products of the islands) to be actively transported from the bacterial cytoplasm across both cell walls and to directly enter the cytoplasm of an attached eukaryotic cell. The bacterial proteins include factors that disrupt the actin cytoskeleton (Rosqvist et al., 1991; Cornelis, 2000).

Other bacteria may gain entry into eukaryotic cells. Once inside, bacterial pathogens may either remain within a vacuole or escape into the cytoplasm (Steele-Mortimer *et al* , 2000). Some of the bacteria that escape into the cytoplasm induce remarkable changes in the cytoskeleton. Interactions between pathogenic bacteria and the cytoskeleton are found with both *Shigella* and *Listeria*; these bacteria invade the eukaryotic cytoplasm, then move within the cytoplasm by a process involving continuous actin polymerization (Steele-Mortimer *et al* , 2000). This type of intercellular interaction is not limited to pathogenesis, but is also found in some symbiotic interactions, as in the case of the symbionts of the rice weevil, *Sitophilus spp.* (Dale *et al.,* 2002).

Understanding the interaction between host and symbiont can best be gained in monospecific relationships; that is where both the eukaryotic host and the symbiotic bacterium have coevolved a one to one relationship. Several such relationships have been extensively described in the literature; e.g., *Euprymna scolopes* and *Vibrio fischeri* (McFall-Ngai 1999). More recently, a relationship between *Acidovorax*-like symbionts and earthworm nephridia has been explored by investigators at the University of Washington in Seattle (Schramm *et al* , 2003).

The earthworm *Eisenia fetida*, a member of the class *Oligochaeta*, was chosen for this study. These organisms are epigeic redworms that influence the mixing and processing of organic matter through the soil. Their activities alter soil structure, nutrient availability, and microbial activities through different feeding strategies and burrowing habits (Edwards 1996). These earthworms have also been found to harbor host-specific bacteria in their nephridia (Schramm *et al.*, 2003)

The nephridia of the earthworms are paired excretory organs that occur internally on the body wall in each segment of the worm. The functions of the nephridia are to process coelomic fluid for excretion of waste and osmoregulation. Each nephridium consists of three loops and the enlarged region of the middle loop, or the ampulla, harbors the bacteria (see Figure 1). Research conducted by Schramm *et al* (2003) has shown that the bacteria within each ampulla consist of a high-density culture belonging to the *Acidovorax* genus. The *Acidovorax* are members of *Comamonadaceae* in the Beta subclass of the proteobacteria. These rod-shaped, gram-negative bacteria are thought by Schramm *et al* (2003) to assist the nephridia in waste transformation and elimination.

The interactions between some invertebrates and their bacteria are thought to be beneficial to both partners because the bacteria provide metabolites to the host, while the host provides a constant environment supplementing metabolic resources required for bacterial growth, such as suitable carbon and nitrogen compounds (Goebel and Gross 2001). Many modern bacterial symbioses seem to have evolved from a single bacterial species associating with an ancestral host (Krueger *et al*, 1996). Hosts may only harbor a single species of bacteria possibly because of competition among symbionts for resources and space or the increased evolutionary cost of recognizing, regulating, and transmitting more than one symbiont (Dubilier *et al.,* 1995). One host (mentioned above) that exhibits species-specific symbiosis with its bacteria is the bobtail squid, *Euprymna scolopes*. The bacteria, *Vibrio fischeri*, are recruited from the seawater by ciliary currents and collect in mucus (Nyholm *et al.,* 2000). The *V fischeri* cells then migrate into a pore and through a duct where they encounter oxidative stress in the form of free radicals produced by the host (Weis *et al*, 1996). The circulatory system of the host supplies the crypt environment with oxygen, which provides the substrate for host respiratory burst activity in response to microbes (McFall-Ngai 1999). This respiratory burst activity is not associated with energy production in mitochondria, but involves an increase in oxygen uptake. This increase may lead to the production of superoxides. Therefore, the symbionts can respond by lowering oxygen availability through the oxygen-requiring luminescence reaction (McFall-Ngai 1999).

During colonization of host tissue, bacteria may induce morphological changes in host epithelial cells involving rearrangements of cytoskeletal elements, increased densities of microvilli, and host cell swelling. This has been documented in *Euprymna*

scolopes during colonization of the light organ by luminescent, symbiotic *Vibrio fischeri* Once the juvenile squid are colonized by *V fischeri,* a number of morphological and biochemical changes such as dramatic epithelial cell swelling are triggered in the nascent light organ, leading to the development of the functional adult structure (Visick *et al*, 2000). Similar interactions are suggested, but remain to be established, for the *Acidovorax*-earthworm association.

There are currently no studies of the early colonization of the earthworm nephridia demonstrating morphological changes in host tissues induced by the symbiotic bacteria. TEM studies of adult nephridia have shown microvilli associated with bacterial cells in the ampulla, but have not documented possible developmental changes in the host cells or in the bacterial cells (Scott 1971; Villaro 1985). I hypothesized that *Acidovorax* may act as a morphogen, that is, be required for normal development of nephridial ultrastructure, as has been discovered in other host-microbial systems. The nephridial tissue architecture of symbiont-containing worms was compared by transmission electron microscopy with that of worms lacking bacteria. Presence or absence of bacteria in the nephridia prior to embedding and sectioning was established by fluorescence *in situ* hybridization (FISH).

MATERIALS AND METHODS

Specimens

Earthworms, *Eisenia fetida,* used in this study were obtained from Dr. Seana Davidson of the University of Washington in Seattle. Two groups of earthworms were obtained. One group was treated with antibiotics (kanamycin) to remove the bacteria from the nephridia and the other group was untreated. The earthworm egg capsules were treated with kanamycin antibiotics in the Stahl laboratory at the University of Washington. The egg capsules were collected, rinsed in diH_2O, and placed in clean petri dishes with a Whatman1 filter paper. Three milliliters (120 μg/ml) of antibiotic solution in diH_2O was added and changed daily for ten days. Control egg capsules were treated in a similar manner with diH_2O only. After treatment, the hatchling earthworms were kept in separate plastic containers with ventilated lids and allowed to grow to the adult stage. These worms were then sent in the separate bins to Texas State University-San Marcos. Autoclaved peat moss was used for the bedding and was kept moist by additions of sterile water. The bedding was changed every two weeks and the earthworms were fed autoclaved oatmeal and coffee grounds. Prior to dissection, the earthworms were anesthetized by placing them in a phosphate-buffered saline (PBS) solution (pH 7.4, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) saturated with carbon dioxide. Forceps and microscissors were used to dissect the earthworm along the dorsal side and remove the earthworm gut without disturbing the nephridia. The earthworm was

cut into small pieces with approximately six segments per piece. Segments were obtained from five colonized earthworms and five asymbiont earthworms. These segments were then prepared for either fluorescence *in situ* hybridization or transmission electron microscopy (Figure 1).

Fluorescence In Situ Hybridization

The hybridization was performed on the asymbiont as well as the symbiont-containing nephridia using probes designed to detect 16S rRNA from the *E. fetida* symbiont (see Table). The worm tissue was fixed with 4% paraformaldehyde in phosphate buffered saline (Electron Microscopy Sciences, Hatfield, PA) for 2 hours, and then washed with PBS three times for 15 minutes each. Hybridization solution containing 900 mM NaCl, 20 mM Tris-HCl (pH 8.0), double distilled water, 35% formamide and 0.01% sodium dodecyl sulfate (SDS) was prepared. Tissue samples were obtained from both the symbiont-containing earthworms as well as the asymbiont earthworms. The worm tissue was placed into eight wells of a 96 well plate. Twenty-five µl of the hybridization solution was pipetted into each of the wells, then 2.5 µliters of EUB338 (Amann *et al.*, 1990) and LSB145 (Schweitzer *et al.*, 2001) probe were added to the wells (Table). The probes were delivered in powder form, and were used at 50 ng/µl in TE (Tris-EDTA) pH 8.0. A 96 well plate was used to incubate the fixed earthworm segments, and it was wrapped and placed in a water bath for 2 hours at 46 °C. To stop hybridization, the tissue was washed with a solution that contained 20mM Tris-HCl, 80 mM NaCl, 5 mM EDTA, double distilled water and 0.01% sodium dodecylsulfate solution three times at 48 °C for 15 minutes each wash. The tissue was mounted in

depression slides in 90% glycerol with 1 mg/ml aqueous p-phenylenediamine for examination. Confocal microscopy was performed using an Olympus IX-70 fitted with a Bio-Rad MRC 1024 (Bio-Rad Laboratories Inc., Hercules, CA) confocal scanhead via the Keller port. Confocal settings were optimized for each fluor on labeled tissues in symbiont-containing worms, and those settings were used for acquisition of images from asymbiont worms. Image acquisition and initial processing were done using Bio-Rad LaserSharp software with final processing done using Adobe Photoshop CS (Adobe, Seattle, WA) on a Macintosh G5 computer (Apple Computer Inc., Cupertino, CA).

Transmission Electron Microscopy

Some earthworm segments, dissected as described above, were prepared for ultrastructural study. The worm tissue was fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA) and left in the fixative overnight at room temperature. The tissue was then washed in the same buffer 3 times for 15 minutes each, and then post-fixed in 1% OsO_4 in 0.05 M cacodylate buffer, pH 7.4. The worm segments were then processed routinely for transmission electron microscopy (TEM). Embedding was in Spurr's epoxy resin mixture (Spurr, 1969). After the polymerized blocks were removed from the oven and cooled, the blocks were trimmed appropriately to assure the ampullary region of the nephridia would be visible in the sections. The tissue was then sectioned at 70 nm using the Reichert Ultracut-S ultramicrotome. Sections were placed on 400 mesh copper grids and some sections were contrasted with a 2% uranyl acetate solution for approximately 2 hours. Micrographs were obtained using the JEOL 1200 EXII transmission electron microscope. Negatives were scanned at 1500 dpi on an Epson 2450 high-resolution scanner (Epson America,

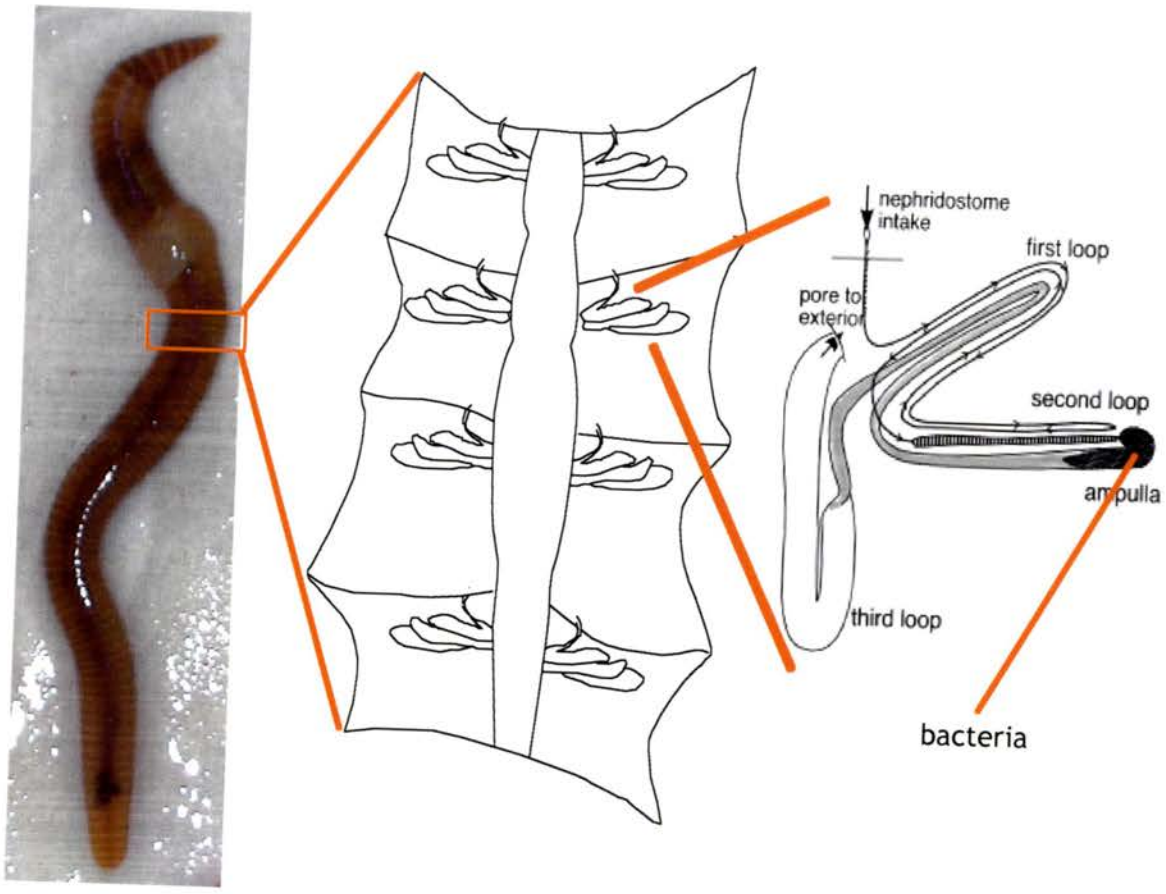
Long Beach, CA) using a Macintosh G5 computer. The resulting digital images were contrast enhanced using Adobe PhotoShop CS.

Table. Probes used for FISH

Probe	**Specific for**	**Nucleotide sequence 5' – 3'**	**Dye**	**Supplier**
LSB 145	*Acidovorax*	CTTTCGCTCCGTTATCCC	Cy3	Molecular Probes (Eugene, OR)
EUB 338	Eubacteria	GCTGCCTCCCGTAGGAGT	FITC	Molecular Probes (Eugene, OR)

Figure 1

Dissection diagram of earthworm nephridia



Schramm *et al.*, 2003

RESULTS

Fluorescence In Situ Hybridizations (FISH)

Fluorescence in situ hybridization (FISH) was performed to confirm the presence and identity of the nephridial bacteria. Fluorescence from both the general eubacterial probe (EUB338) and the species-specific probe (LSB145) was found localized to the ampullary region of nephridia from worms presumed to be symbiont-containing with *Acidovorax* (Figure 2a). Neither probe appeared to bind to tissues of antibiotic treated worms (Figure 2b).

Transmission Electron Microscopy

The epithelial cells of the symbiont-containing nephridia appeared squamous with large, irregular nuclei (Figure 4a). The cytoplasm contained moderate amounts of rough endoplasmic reticulum. Mitochondria, with somewhat indistinct cristae, were present as well. In addition, fairly numerous round vesicles suggestive of lysosomes were observed, and some of the endoplasmic reticulum appeared swollen. The cytoplasm appeared somewhat edematous in comparison to the more dense cytoplasm seen in the asymbiont nephridia. Furthermore, the cells of asymbiotic nephridia in general were smaller, more compact appearing, more densely populated with organelles, and had smooth luminal surfaces completely lacking microvilli (Figure 4b).

The morphology of nephridia of symbiont worms was strikingly different from that of asymbiont worms, with the salient feature being the presence of large numbers of microvilli or stereocilia on the luminal surface of endothelial cells in the nephridia. The microvilli-like objects seem to occupy distinct zones in the ampulla and appeared to be of two types, a larger more darkly staining type approximately 80 nm in diameter and a smaller less distinct structure about 50 nm in diameter. The length of these structures was difficult to determine, as they appeared long and irregular, weaving in and out of the plane of the section. Profiles of bacterial-sized structures were present among the microvilli-like objects. These were of appropriate size for cross-sections of bacteria, but did appear typical of bacteria as visualized by TEM (Figure 3a).

A common feature observed in the lumen of nephridia was the presence of large osmophilic structures that appeared to be fat droplets. However, these differed in structure with the droplets in the symbiotic nephridia appearing homogenous, while those of the asymbiotic nephridia appeared subdivided with several myelin artifacts per droplet. All the fat droplets observed in the asymbiont worms showed the myelin artifact-like structures, while none were seen in the droplets in symbiont-containing nephridia. These osmophillic structures were approximately 2 μm in diameter and appeared to be spherical, and occupied a sizeable region in a typical cross-section of the ampulla (Figure 3b).

Figure 2

a. Confocal image of symbiont-containing earthworm showing bright fluorescence in ampullary region of nephridia. Projection of a single ampulla with presence of bacteria identified by FISH with probes EUB338 (labeled with FITC) and LSB145 (labeled with Cy3). Bar represents 50 μm.

b. Confocal image of asymbiont earthworm nephridium using the same probes and settings as for "a" exhibiting almost no fluorescence.

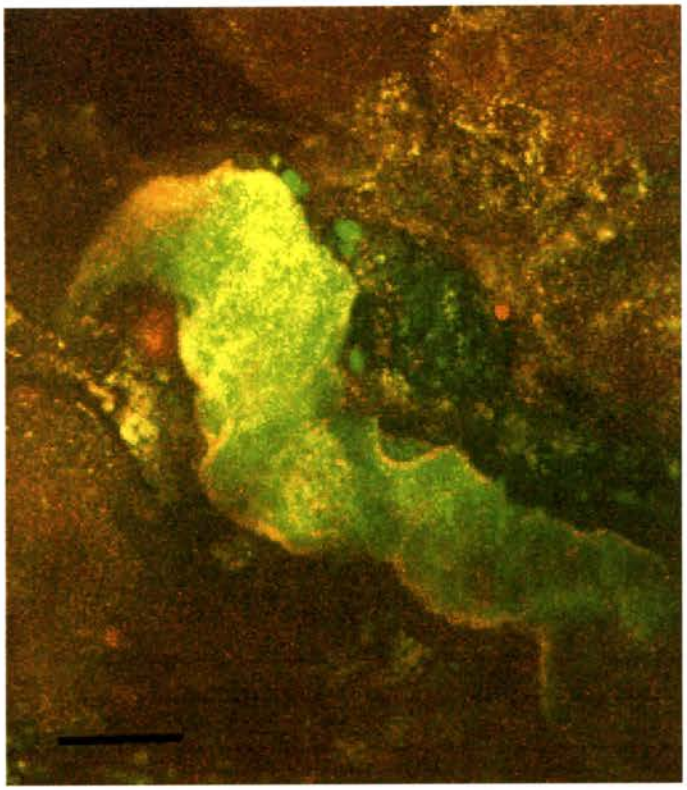
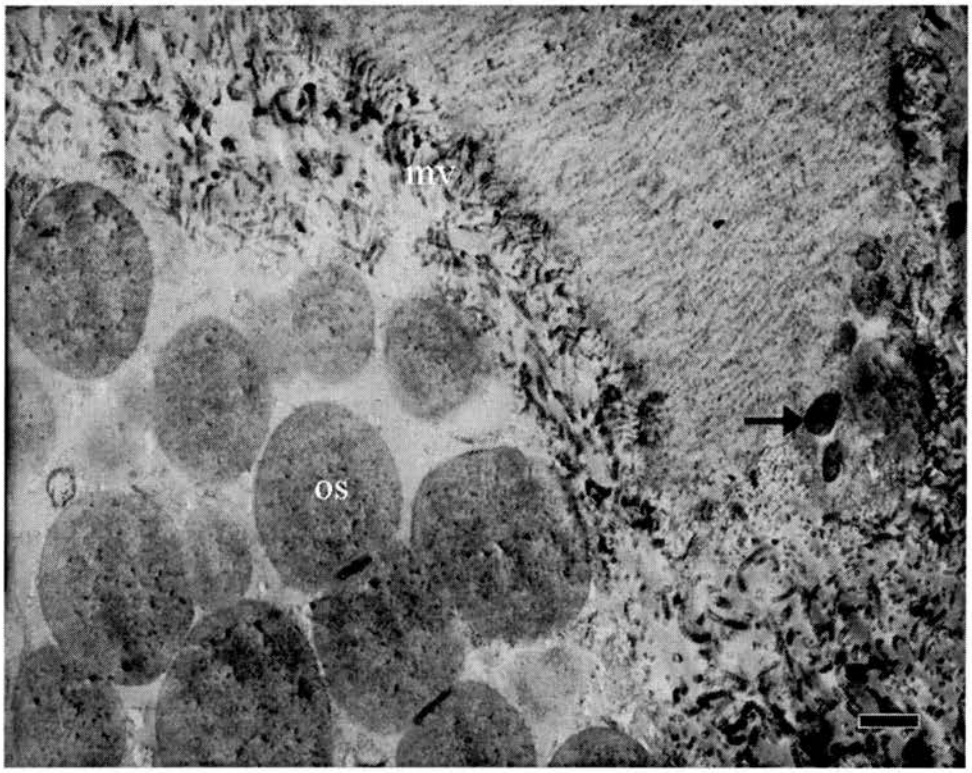


Figure 3

a. Transmission electron micrograph of a symbiont-containing nephridium. Most of the volume of the cells in this image consists of microvilli and objects that appear to be bacteria (arrow) can be seen deep among the microvilli (mv). The larger spherical osmophilic structures (os) were frequently found as illustrated here occupying a large cross-sectional area of the ampulla. Bar represents 500 nm.
b. Transmission electron micrograph of an asymbiont nephridium. The cells making up the inner lining are flattened and do not exhibit microvilli. In addition, the lumen (lu) appears to be septate with round osmophilic structures (os) similar in size to those observed in the symbiont-containing ampulla, but different in containing many myelin figures. Bar represents 500 nm.


mv
os

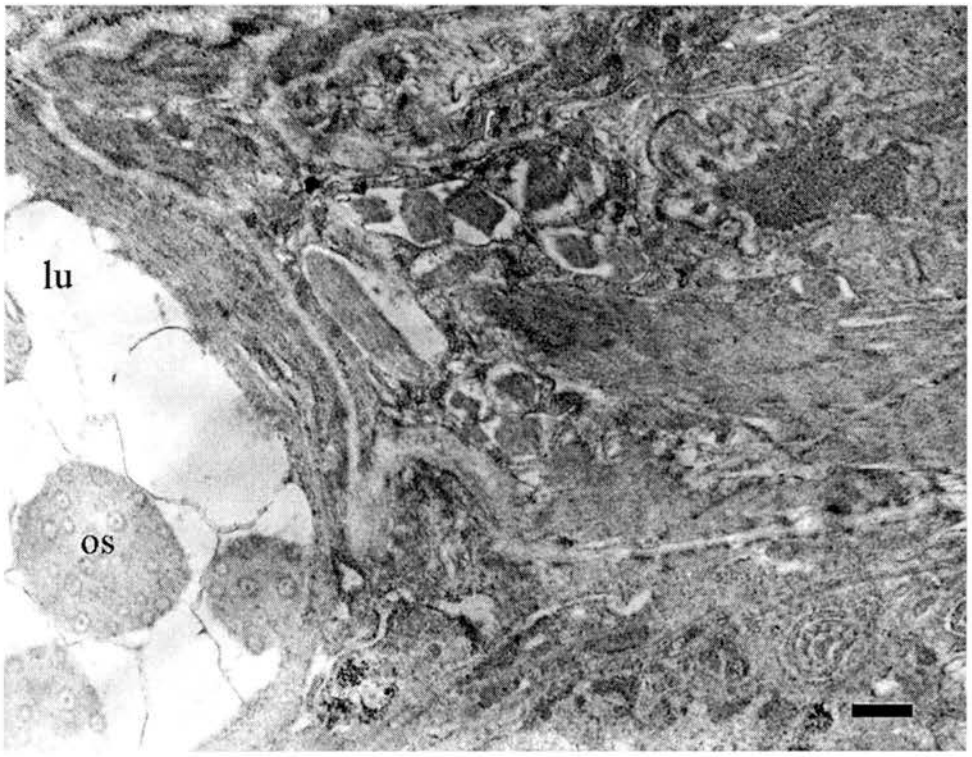
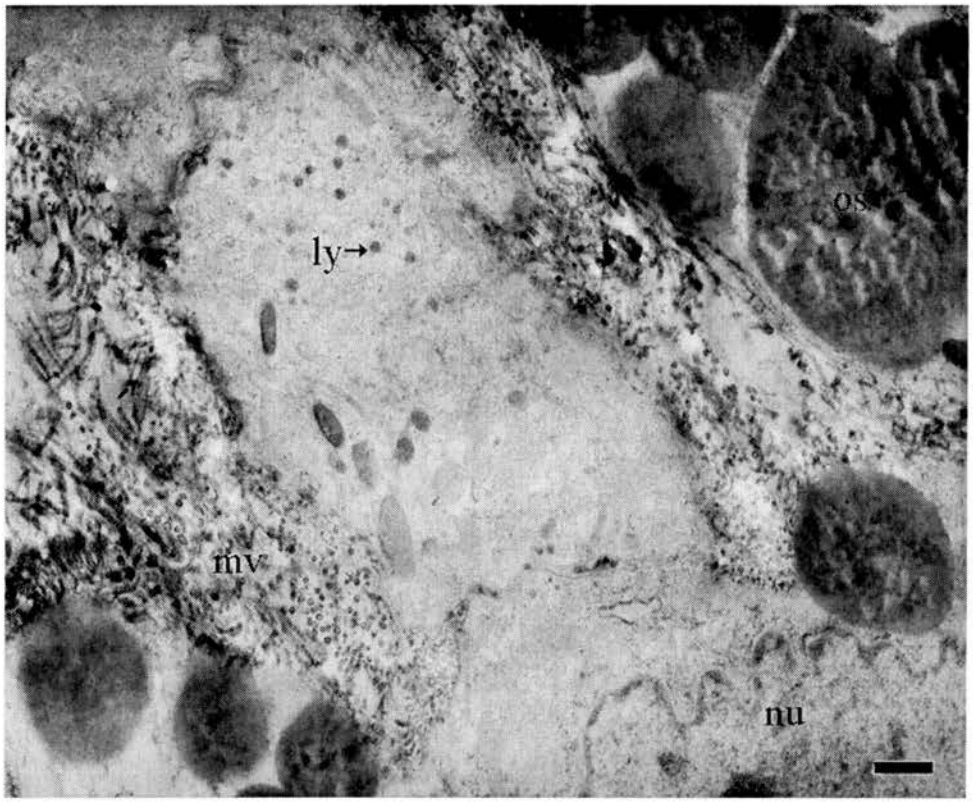
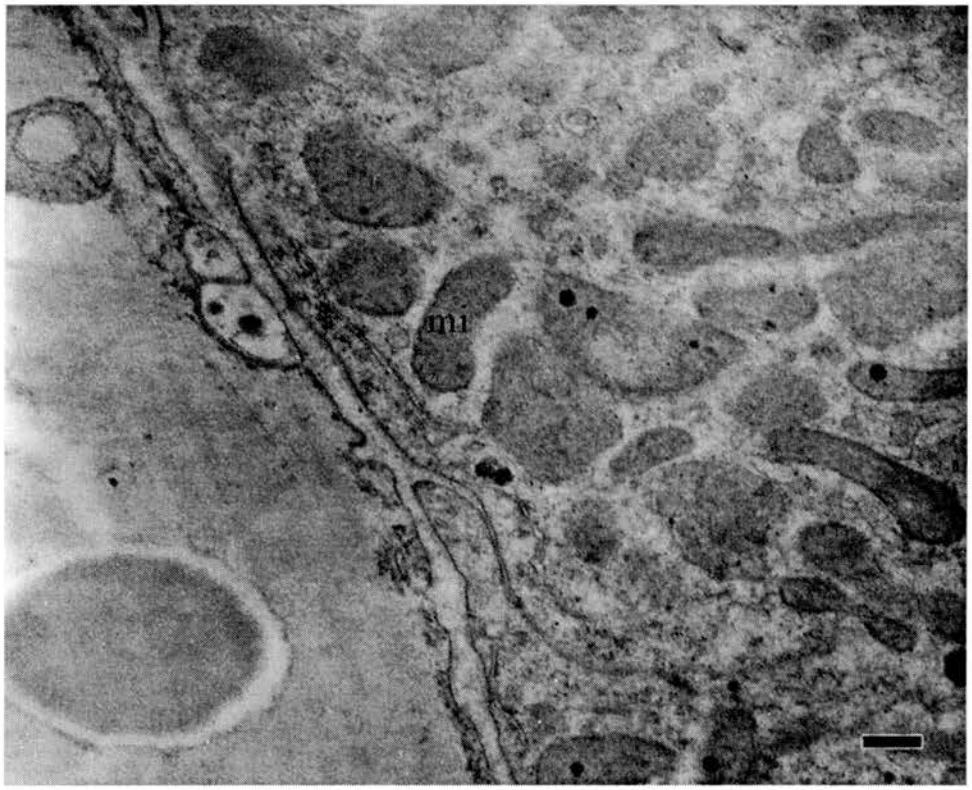

lu
os

Figure 4

a. Transmission electron micrograph of a symbiont-containing nephridium. The endothelial cells appear squamous with large, irregular nuclei (nu). The cytoplasm contains rough endoplasmic reticulum, mitochondria (mi), and lysosomes (ly). There are numerous microvilli as well as large, circular osmophilic structures (os) measuring approximately 2-5 μm in diameter. Bar represents 500 nm.

b. Transmission electron micrograph of an asymbiont nephridium. The endothelial cells appear much more flattened and exhibit no swelling. The cytoplasm is denser and there are no visible bacteria or microvilli; however, numerous mitochondria (mi) are present. Bar represents 500 nm.


os
ly→
mv
nu


mi

DISCUSSION

The results of this study strongly suggests that the presence or absence of the symbiotic bacterium *Acidovorax sp.* may be related to differences in ultrastructure in the nephridia of *Eisenia fetida.* This finding in turn suggests that the presence of the bacterial symbiont is required for normal development of nephridial architecture and function. In comparison to the asymbiotic nephridia, the endothelial cells of the symbiont-containing nephridia exhibited numerous microvilli as well as increased cell size. The microvilli-like objects present in the symbiont-containing nephridial endothelial cells were approximately 80 nm in diameter, and had core structures that looked similar to published images of microvilli (Scott *et al.*, 1971). However, these structures were also of appropriate size and morphology to be considered stereocilia; structures that may be relatives of microvilli found in the organ of Corti and other sensory structures (Jastrow, 1998). These structures were determined not to be motile cilia because the typical "9+2" arrangement of microtubules was not present. However, this cannot be considered conclusive evidence, as microtubules (and microfilaments and intermediate filaments) were not observed in sections of either the symbiont-containing or asymbiont worm.

Stereocilia also lack the 9+2 microtubular core, and it is also of interest to note that kanamycin (used to "cure" the symbiotic "infection" in the worms described as

asymbionts here) causes the loss of stereocilia from the cochlea when used for prolonged periods of time (Hashino et al., 1990). This is discussed further, below.

Previous studies have shown that certain strains of bacteria are essential for development of the host symbiotic organ. This has been elucidated in the mutualistic association between the squid *Euprymna scolopes* and the bacterium *Vibrio fischeri*. The symbiotic bacteria induce changes in the shape and size of the epithelial cells of the light organ that contains them, and also tissue level (histological) changes in the overall morphology of the light organ. By comparing symbiont-containing squids with asymbiont squids at similar stages of development, it was shown that infection with *V fischeri* induces regression of the ciliated surface epithelium as well as inducing further differentiation in the layer of epithelial cells that directly interact with the bacteria, including reorganization of the submembrane cytoskeleton and elaboration of microvilli (Montgomery and McFall-Ngai 1994). Furthermore, asymbiont juveniles lacked several cellular structures that were present in the symbiont-containing squid, specifically cells bearing microvilli (Montgomery and McFall-Ngai 1994). Similar results were found in this study of *Eisenia fetida*, as microvilli-like structures failed to appear in nephridia of worms lacking their nephridial symbiont.

The bacteria found in nephridia of *Eisenia fetida* are *Acidovorax*-like. This has been shown by PCR and FISH performed by Schramm *et al.* (2003). They found that the bacterial cells formed a biofilm-like structure on the ampulla walls, whereas the lumen of the ampulla remained open. FISH results reported here are consistent with the description of Schramm *et al*, (2003) and were used to confirm the presence or absence of bacteria in worms subjected to ultrastructural analysis. Densely packed bacteria were

detected in the ampulla region of the nephridia with the eubacterial probe, EUB338, and the highly specific probe, LSB145, for the symbiont. *Eisenia fetida* that had been treated with antibiotics showed no fluorescence in the ampullary region when viewed on the confocal microscope. Furthermore, TEM revealed that bacteria were common in the ampulla region of the symbiont-containing nephridia, and the presence of bacteria in the epithelium was strongly associated with the presence of microvilli-like structures. Another difference between symbiont-containing and asymbiont nephridia were the spherical osmophilic structures. While these objects were similar in size, the osmophilic structures of the asymbiont nephridia contained what appeared to be myelin artifacts. Myelin artifacts are thought to be related to the degree of unsaturation in the fatty acids of phospholipids (Hayat, 1986). The appearance of these artifacts only in the lipid droplets of the asymbiotic nephridia suggests a difference in the lipid content, further suggesting that processing of nephridial lipids may be one of the functions of the symbionts.

Interactions between nonpathogenic bacteria and their hosts play critical roles in both partners (Hooper *et al.*, 1998), for example in the gastrointestinal tracts of mammals. The intestinal microbiota provides essential products to the host such as vitamins K and B_{12}, and a line of defense against invasion by pathogenic organisms (Abrams *et al.*, 1966; Hooper *et al.*, 1998). Furthermore, other studies have shown that certain gastrointestinal microorganisms affect the gastrointestinal microbial flora and influence the host not only morphologically but also physiologically, especially the epithelial cells of the alimentary tract (Kawai *et al* , 1977). Jeong *et al.*, (2000) found differences between the quantity of microvilli-bearing cells in lymphoid tissues in the nasal epithelium of SPF (specific pathogen-free) rats and conventional rats. The conventional rats had increased numbers

of non-ciliated cells with microvilli compared to the pathogen-free rats and germ-free rats. The authors attributed the difference to the influence of the rat immune response on the lymphoid tissues of the nasal epithelium, however, since similar findings have been made in squid and earthworm, perhaps the production of microvilli is a common animal cell response to the presence of bacteria. In a related, earlier study, researchers found ultrastructural differences between conventional and gnotobiotic (exposed to specific microorganisms, in this case *Cryptosporidium parvum* oocysts) piglets on the surface of their intestinal mucosa. The microvilli of absorptive cells of conventional piglets were more robust and longer than those of other absorptive cells without parasites (Vitovec and Koudela, 1992). These findings may be related to the changes that occur in the nephridia *Eisenia fetida* that have been treated with antibiotics.

The ultrastructural changes in the nephridia of *E. fetida* seem to be associated with their symbiotic bacteria, *Acidovorax*. However, even though the kanamycin treatment was to the egg capsule prior to emergence of the earthworm (when they normally become "infected"), and evidence from Schramm *et al.* (2003) indicates the antibiotic does not penetrate the egg capsule, the possibility remains that antibiotic-induced changes in nephridial structures of the embryonic worms may have occurred. This possibility must be taken into consideration because several studies have shown chronic applications of aminoglycosides, including kanamycin, typically produce irreversible degeneration of sensory hair cells toward the base of the cochlea, which results in permanent hearing loss. Hashino *et al* (1990) described hair cell damage and recovery following chronic administrations of kanamycin in the avian cochlea using SEM. The control cochlea had a mosaic pattern of hair cells tightly packed on the basilar

papilla and the length of the stereociliary bundles increased from the basal to apical end of the cochlea. However, in the kanamycin-treated cochlea, all the stereociliary bundles and supporting cell surfaces that formed the boundaries between neighboring hair cells had disappeared. Lenoir *et al.*, (1999) reported that the inner hair cells of rat cochlea showed signs of apoptosis in response to amikacin treatment and most of them were missing by one week after the end of the aminoglycoside treatment . A few of the inner hair cells survived; however, they were devoid of normal stereocilia (Lenoir *et al.*, 1999).

The mechanism of injury to stereocilia and hair cells seems to be due to aminoglycoside effects on microtubules. In a study by Leonova and Raphael (1997), the first visible sign of aminoglycoside-induced damage in the organ of Corti of adult Guinea pigs involved microtubules in the cytoplasm of the hair cells. The main focus of their study was the reformation of junctional complexes during scar formation following hair cell death. Results from other studies indicate that aminoglycoside antibiotics affect the developing chick inner ear in the same manner that they affect the developing mammalian organ of Corti (Fermin and Igarashi, 1983; Cruz *et al* , 1987). Researchers found that a small increase in the number of dense osmophilic inclusions in the cytoplasm of hair cells constituted the main indicator of intoxication in kanamycin-injected chicks (Fermin and Igarashi, 1983). These inclusions, believed to be lysosomes, have repeatedly been shown to be a characteristic sign of hair cell intoxication (Fermin and Igarashi, 1983). Cruz *et al.* (1987) also found that after significant hair cell loss in the cochlea, there was a partial recovery of hair cell numbers, which may be due to the differentiation of supporting cells into more specialized hair cells to replace damaged ones.

To date, no studies have linked kanamycin or other aminoglycosides to loss of microvilli. Li *et al.* (2000) demonstrated tachyplesin (an aminoglycoside from horseshoe crabs) had effects on the overall morphology of human gastric carcinoma cells, but did not cause loss of microvilli. If the nephridial structures are indeed microvilli, then it is unlikely that the difference between the symbiont and asymbiont is due to kanamycin-induced loss. Other changes, particularly those observed in the lipid droplets, also seem unlikely to be a result of antibiotic treatment. Nevertheless, if the nephridial structures are stereocilia, then the possibility that aminoglycosides directly induced their regression must be further investigated.

It should also be taken into consideration that bacteria have developed mechanisms to resist antibiotics such as kanamycin, which could lead to ineffectiveness of the antibiotics. Aminoglycoside-resistant bacteria often emerge as a result of acquiring plasmid-borne genes encoding aminoglycoside-modifying enzymes (Shaw *et al.*, 1993). The main mechanisms which may affect all aminoglycosides involve either a decreased uptake of the drug in bacteria or the bacterial expression of enzymes which modify the antibiotic and thereby inactivate it (Mingeot-Leclercq *et al.*, 1999). There are three different mechanisms by which aminoglycoside antibiotics are known to be inactivated: acetylation of amino groups, phosphorylation of hydroxyl groups, or adenylylation of hydroxyl groups (Benveniste and Davies, 1973). Potential contamination and/or potential antibiotic resistance may have resulted in a chance of microbial contamination because the *E fetida* were not maintained under barrier conditions to prevent bacterial contamination. Although FISH probes did not detect bacterial contamination, a more sensitive technique such as PCR may have detected

contamination. However, since the bacteria are normally acquired from soil upon the hatching of the earthworm (the interior of the egg is bacteria-free), the question of incomplete "clearing" of bacteria is not relevant. Although the worms were raised in autoclaved soil, the possibility of infection at a later date (after hatching) remains. Yet, there was no evidence of bacteria by FISH or electron microscopy in worm nephridia in worms reared from treated egg capsules in this or previous studies (Schramm *et al.*, 2003).

In conclusion, I have shown there are ultrastructural differences between nephridia of symbiont-containing earthworms and nephridia of earthworms treated with antibiotics. These differences suggest the presence of the symbiont is necessary for the normal development of nephridial structures. The specific differences are most likely associated with the absence of the bacteria, but the possibility of antibiotic effects cannot be eliminated. Future studies should be directed at positive identification of the microvilli-like structures to settle the question regarding antibiotic effects – are the structures present in the worm embryos and lost as a result of antibiotic "curing" of the symbiosis, or do the structures not develop until a signal is received from the infecting *Acidovorax*? The preponderance of the evidence here supports the latter possibility, but the former cannot yet be excluded.

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VITA

Nina Jaffarzad was born in Lafayette, Louisiana, on October 27, 1980, the daughter of Farah Jaffarzad and Behrooz Jaffarzad. After completing her work at Mayde Creek High School, Houston, Texas, in 1998, she entered the University of Texas at Austin. She received the degree of Bachelor of Arts in Psychology from the University of Texas in May 2002. In August 2002, she entered the Biology program of the Graduate College of Texas State University-San Marcos.

Permanent Address: 711 Daria Ct.

Houston, TX 77079

This thesis was typed by Nina Jaffarzad.