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Morphology and ultrastructure of the bacterial receptacle in *Steinernema* nematodes (Nematoda: Steinernematidae)

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ABSTRACT

Infective juveniles of entomopathogenic nematodes in the genus *Steinernema* harbor symbiotic bacteria, *Xenorhabdus* spp., in a discrete structure located in the anterior portion of the intestine known as the 'bacterial receptacle' (formerly known as the bacterial or intestinal vesicle). The receptacle itself is a structured environment in which the bacteria are spatially restricted. Inside this receptacle, bacterial symbionts are protected from the environment and grow to fill the receptacle. Until now, no comparative study across different *Steinernema* spp. has been undertaken to investigate if morphological variation in this structure exists at the interspecific level. In this study, we examined the bacterial receptacles of 25 *Steinernema* spp. representatives of the currently accepted five evolutionary clades. Our observations confirmed the bacterial receptacle is a modification of the two most anterior cells of the ventricular portion of the intestine. Size of the bacterial receptacle varied across the examined species. *Steinernema monticolum* (clade II) had the largest receptacle of all examined species (average: 46 × 17 μm) and *S. rarum* (no clade affiliation) was noted as the species with the smallest observed receptacle (average: 8 × 5 μm). At the morphological level, species can be grouped into two categories based on the presence or absence of vesicle within the receptacle. The receptacles of all examined species harbored an intravesicular structure (IVS) with variable morphology. All examined taxa members of the 'feltiae' (clade III) and 'intermedium' (clade II) clades were characterized by having a vesicle. This structure was also observed in *S. diaprepesi* (clade V), *S. riobrave* (clade IV) and *S. monticolum* (clade I).

1. Introduction

Entomopathogenic nematodes in *Steinernema* Travassos, 1927 (Rhabditida: Steinernematidae) and their bacterial symbionts *Xenorhabdus* Poinar and Thomas, 1979 (γ-Proteobacteria, Enterobacteriaceae) have a symbiotic partnership where both allies receive benefits from each other. The bacteria are carried in the intestine of the only free-living stage of the nematode, the third-stage infective juvenile (hereafter referred as IJ), in a structure known as the bacterial receptacle, originally named bacterial or intestinal vesicle by Bird and Akhurst (1983). The IJs live in the soil until they invade a susceptible insect host. Once in the host's hemocoel, the IJs release the bacteria into the insect's hemolymph. The bacteria proliferate in the insect cadaver, reaching high cell densities, at which point they produce diverse antimicrobial compounds that suppress the growth of antagonistic microorganisms (Akhurst, 1983; Forst and Clarke, 2002) creating an environment amenable to nematode reproduction and development. When nutrients are depleted, nematode progeny develop into the IJs that are then colonized by *Xenorhabdus* symbionts. The IJs then emerge into the soil ready to infect a new insect host.

Several studies suggest each *Steinernema* species has a specific natural association with only one *Xenorhabdus* species, though a single *Xenorhabdus* species may be associated with more than one nematode species (Boemare, 2002; Fischer Le Saux et al., 1998; Lee and Stock, 2010; Stock and Goodrich Blair, 2008). The bacterial receptacle is a modified structure of the anterior portion of the nematode's intestine that lies immediately beneath the esophago–intestinal junction and it is formed even in the absence of bacteria (Flores-Lara et al., 2007). Martens and Goodrich-Blair (2005) noted the presence of a nematode-derived subcellular structure called Intra Vesicular Structure (IVS) primarily studied this structure in *S. carpocapsae* (Weiser, 1955). This structure apparently plays a key role in the initiation of symbiont colonization (Martens and Goodrich-Blair, 2005). These authors also reported presence of a similar structure in two other *Steinernema* spp.: *S. karii* Waturu, Hunt and Reid, 1997 and *S. siamkayai* Stock, Somsook and Kaya, 1998. Until now, no comparative studies have been undertaken to examine the morphology of the bacterial

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receptacle and ancillary structures across *Steinernema* spp. In this study, 25 species representing five evolutionary clades depicted from the most recent molecular phylogenetic framework were considered to investigate whether or not morphological and ultrastructural variation of the bacterial receptacle's morphology and supplementary structures exist across different species.

2. Materials and methods

2.1. Nematode species considered in this study

Twenty-five *Steinernema* spp. representatives of five evolutionary clades, depicted in a recent multi-gene phylogenetic study by Lee and Stock (2010), were considered for this study. This molecular framework was used as a non-biased substrate to compare and address both morphological similarities and differences among species. Nematode strains' designation, geographic origin, clade affiliation, associated bacteria species, and original source of nematode/bacterium cultures are provided in Table 1. With the exception of *Steinernema scapterisci* and *S. scarabaei*, isolates in this study were established in the laboratory by *in vivo* rearing in last instar larvae of *Galleria mellonella* (Lepidoptera: Pyralidae). *Steinernema scapterisci* and *S. scarabaei* were reared in house crickets, *Acheta domestica* (Orthoptera: Acriididae) and scarab beetle larvae, *Cyclocephala* spp. (Coleoptera: Scarabaeidae), respectively.

In vivo rearing procedures followed Kaya and Stock (1997). Age of IJs examined in this study was standardized to 2-weeks old (i.e. storage age after initial emergence from the insect cadaver). Criteria for IJ age followed work done by Flores-Lara et al. (2007) who noted that receptacle shape and size changes over time (specifically, after a 6 weeks period of storage).

2.2. DIC microscopy and staining of the IJ intestine

A total of 25 IJs of each *Steinernema* spp. were measured and examined with differential interference contrast (DIC) optics in an Olympus BX51 microscope with a high resolution digital camera (ColorView III™) and digital imaging system (Olympus MicroSuite™). Examination of the bacterial receptacle was done *in situ* and also from extruded intestines according to Stock and Goodrich Blair (in press). Briefly, to extrude the IJs' intestine, 10 individuals of each species were placed onto the center of a drop of M9 buffer with neutral red stain (1 $\mu g\,ml^{-1}$) (Sigma–Aldrich, St. Louis, MO, USA). IJ body was then excised under a dissecting microscope at 50× magnification with a disposable scalpel blade (#10, Miltex®, Integra LifeSciences Plainsboro, NJ). Excision was made at the first 1/3 of IJ body to extrude the esophagus and the anterior portion of intestine. A cover slip was then applied and the slide was sealed with clear nail polish for microscopy examination using an Olympus BX51 microscope at 100× magnification.

Morphology and size (length and width) of the bacterial receptacle and ancillary structures was observed and recorded. Receptacle length (ReL) was measured at the longest point and parallel or sub-parallel to the nematode longitudinal body axis and receptacle width (ReW) was measured at the widest point and perpendicular to ReL according to Flores-Lara et al. (2007). Range, mean, and standard deviation were determined for these two characteristics (Table 2). One-way ANOVA was performed with all pairwise multiple comparison procedures (Holm-Sidak method) for Rel and ReW across taxa. Significant difference for all data analysis was accepted at $P < 0.05$.

Data were analyzed with SigmaStat v.4.0 (2008).

Supplementary structures observed included the intravesicular structure (IVS) and the vesicle. The 'vesicle' is herein defined as the structure inside the receptacle delimited by a protective layer or film (herein named 'envelope'), which contains the IVS alone or IVS and bacteria (Fig. 1) (Snyder et al., 2007).

2.3. Electron microscopy

Sixteen of the 25 *Steinernema* species considered in this study were subjected to transmission electron microscopy observations. Preparation of specimens followed method described by McClure and Stowel (1978). Briefly, IJs freshly emerged from White traps were rinsed in sterile distilled water three times and fixed overnight in glutaraldehyde (3% in 0.05 M sodium cacodylate buffer,

Table 1
Species information for 25 species of *Steinernema* nematodes with the list of their symbiotic bacteria.

Species	Culture code	Geographic origin	Symbiotic bacteria	Clade	Original source
S. affine (Bovien, 1937)	UK	UK	*X. bovienii*	I	E. San-Blas
S. arenarium (Artyukhovsky, 1967)	Type	Russia	*X. kozodoii*	V	E. Kozodoi
S. ashiunense Phan, Takemoto and Futai, 2006	A01	Japan	*X. sp.*	III	L. K. Phan
S. bicornutum Tallosi, Peters and Ehlers, 1995	Type	Yugoslavia	*X. budapestensis*	IV	B. Briscoe
S. carpocapsae (Weiser, 1955)	All	USA	*X. nematophila*	II	H. K. Kaya
S. costaricense Uribe-Lorío, Mora and Stock, 2007	CR9	Costa Rica	*X. szentirmaii*	NCA*	L. Uribe
S. diaprepesi Nguyen and Duncan, 2004	Type	Florida, USA	*X. doucetiae*	V	L. Duncan
S. feltiae (Filipjev, 1934)	Bodega Bay	California, USA	*X. bovienii*	III	S. P. Stock
S. glaseri (Filipjev, 1934)	NC	USA	*X. ponarii*	V	H. K. Kaya
S. hermaphroditum Stock, Griffin and Chaerani, 2004	T87 and T74	Malaysia	*X. griffiniae*	V	C. Griffin
S. intermedium (Poinar, 1985)	Type	USA	*X. bovienii*	I	H. K. Kaya
S. jollieti Spiridonov, Krasomil-Osterfeld and Moens, 2004	Monsanto	Missouri, USA	*X. bovienii*	III	H. Goodrich-Blair
S. kraussei (Steiner, 1923)	Quebec	Quebec, Canada	*X. bovienii*	III	G. Belair
S. longicaudum Shen and Wang, 1992	B2	China	*X. ehlersii*	V	B. Briscoe
S. monticolum Stock, Choo and Kaya, 1997	Mt. Jiri	Gyungnam, Korea	*X. hominickii*	II	H. Y. Choo
S. oregonense Liu and Berry, 1996	OS-10	Oregon, USA	*X. bovienii*	III	H. K. Kaya
S. puntauvense Uribe-Lorío, Mora and Stock, 2007	Li 6	Costa Rica	*X. bovienii*	III	L. Uribe
S. rarum (de Doucet, 1986)	7C and E	Texas, USA	*X. szentirmaii*	NCA*	D. Shapiro-Ilan
S. riobrave Cabanillas, Poinar and Raulston, 1994	TX	Texas, USA	*X. cabanillasii*	IV	E. Cabanillas
S. sangi Phan, Nguyen and Moens, 2001	TX1	Vietnam	*X. vietnamensis*	V	L. K. Phan
S. scarabaei Stock and Koppenhoefer, 2003	AMK001	New Jersey, USA	*X. koppenhoeferii*	V	A. Koppenhöfer
S. scapterisci Nguyen and Smart, 1990	FL	Florida, USA	*X. innexi*	II	Becker Underwood
S. siamkayai Stock, Somsook and Reid, 1998	T9	Thailand	*X. stockiae*	II	V. Somsook
S. websteri Cutler and Stock, 2003	Unknown	Peru	*X. nematophila*	NCA*	J. Alcazar
S. weiseri Mráček, Sturhan and Reid, 2001	Turkey	Turkey	*X. bovienii*	III	S. Hazir

* NCA = no clade affiliation.