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The *Xenorhabdus nematophila nilABC* Genes Confer the Ability of *Xenorhabdus* spp. To Colonize *Steinernema carpocapsae* Nematodes▽

Charles E. Cowles† and Heidi Goodrich-Blair*

Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706

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Members of the *Steinernema* genus of nematodes are colonized mutualistically by members of the *Xenorhabdus* genus of bacteria. In nature, *Steinernema carpocapsae* nematodes are always found in association with *Xenorhabdus nematophila* bacteria. Thus, this interaction, like many microbe-host associations, appears to be species specific. *X. nematophila* requires the *nilA*, *nilB*, and *nilC* genes to colonize *S. carpocapsae*. In this work, we showed that of all the *Xenorhabdus* species examined, only *X. nematophila* has the *nilA*, *nilB*, and *nilC* genes. By exposing *S. carpocapsae* to other *Xenorhabdus* spp., we established that only *X. nematophila* is able to colonize *S. carpocapsae*; therefore, the *S. carpocapsae-X. nematophila* interaction is species specific. Further, we showed that introduction of the *nilA*, *nilB*, and *nilC* genes into other *Xenorhabdus* species enables them to colonize the same *S. carpocapsae* host tissue that is normally colonized by *X. nematophila*. Finally, sequence analysis supported the idea that the *nil* genes were horizontally acquired. Our findings indicate that a single genetic locus determines host specificity in this bacteria-animal mutualism and that host range expansion can occur through the acquisition of a small genetic element.

Microbial associations with plant and animal hosts are ubiquitous in nature and tend to show specificity with regard to the taxonomy of each partner. For example, *Salmonella enterica* serotype Typhi (29), *Helicobacter pylori* (5), *Neisseria gonorrhoeae* (28), and *Haemophilus influenzae* (22) are pathogens with specificities for human or primate hosts, while *Rhizobium leguminosarum* forms mutualistic nodules only with Phaseolae legumes (30). The molecular basis underlying host range specificity is well understood in the mutualism between the *Rhizobiaceae* and their plant hosts: specificity is achieved by cross talk involving bacterial and plant signaling factors and receptors (15). In contrast, species-specific interactions between bacteria and animal hosts are not well understood at the molecular or genetic level. Although myriad traits have been proposed to contribute to host range specificity (16, 21, 36), none of these have been directly demonstrated to play a role in dictating the animal host range. The increasing global concern over new human pathogens emerging through host range expansion makes understanding the genetic basis of host range specificity in bacteria-animal associations essential (6, 27).

Insect parasitic nematodes of the genus *Steinernema* are mutualistically associated with bacteria of the *Xenorhabdus* genus. This association is a natural and tractable model for understanding the ecology, evolution, and molecular foundations of bacterial interactions with animal hosts. The soil-inhabiting infective stage of a *Steinernema* nematode is colonized by symbiotic *Xenorhabdus* bacteria, which it carries and releases into an insect host. *Xenorhabdus* bacteria provide activities that suppress insect immunity, kill the insect, and enzymatically degrade the cadaver to support nematode reproduction. When the insect cadaver is depleted, *Xenorhabdus* bacteria colonize progeny *Steinernema* nematodes, which emerge from the spent insect cadaver to hunt for new insect prey (14).

Field and phylogenetic studies indicate that specific pairs of *Xenorhabdus* and *Steinernema* species occur in nature (10, 11, 35). Furthermore, several studies have demonstrated that certain *Steinernema-Xenorhabdus* associations are exclusive in that noncognate pairs will not associate during experimental mixing (1, 31). Therefore, it is likely that *Xenorhabdus* bacteria have evolved specificity for their cognate *Steinernema* nematode hosts and vice versa. In nature, only *X. nematophila* has been found to be associated with *S. carpocapsae* nematodes, although a thorough investigation of the range of *Xenorhabdus* spp. that can colonize *S. carpocapsae* has not been reported.

The *X. nematophila nilA*, *nilB*, and *nilC* genes were previously identified in a signature-tagged mutagenesis screen that was designed to elucidate bacterial genes necessary for colonization of the *S. carpocapsae* nematode host (17). In this initial work, it was also revealed that the *nilABC* gene cluster is absent from two other *Xenorhabdus* species, *X. poinarii* and *X. beddingii*. In the present study, we assessed the role of the *nilA*, *nilB*, and *nilC* genes in species specificity.

MATERIALS AND METHODS

Strains, plasmids, media, growth conditions, and molecular biological methods. The bacterial strains and plasmid constructs used in this study are listed in Tables 1 and 2, respectively. Except where noted, the bacteria were grown in Luria-Bertani (LB) broth (25) at 30°C that for the *Xenorhabdus* spp. was either stored in the dark or supplemented with 0.1% sodium pyruvate (39). Antibiotics were used at the following concentrations: ampicillin (Ap), 30 μg/ml; chloramphenicol, 30 μg/ml; kanamycin (Km), 50 μg/ml; and erythromycin (Erm), 200 μg/ml.

Construction of the *nilA*, *nilB*, and *tn2* start codon mutations was done as previously described for the *nilC* start codon mutation (7). Briefly, the genes were mutated by amplifying pTn7/SR1 (SR1 stands for "symbiosis region 1," the original nomenclature used for the *nilABC* region [17]) plasmid DNA with the primers noted in Table 3 and Platinum *Pfx* (Invitrogen). The amplified plasmid

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin—Madison, 1550 Linden Dr., Madison, WI 53706. Phone: (608) 265-4537. Fax: (608) 262-9865. E-mail: hgblair@bact.wisc.edu.

† Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

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TABLE 1. *Xenorhabdus* strains used in this study

Strain[a]	Relevant characteristics	Source or reference
HGB007	Sequenced ATCC 19061 *X. nematophila* wild type	ATCC
HGB777	HGB007 parent, Δ(*nilA-nilC*)*7*-Km^r (XnΔSR1)	7
HGB1186	HGB777 parent, *att*Tn*7*::miniTn*7* from pEVS107	This study
HGB778	HGB777 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1	7
HGB1182	HGB777 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/AM1Z (*nilA21 M1Z*)	This study
HGB1183	HGB777 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/BM1Z (*nilB20 M1Z*)	This study
HGB779	HGB777 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/CM1Z (*nilC19 M1Z*)	7
HGB1184	HGB777 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/TnM1Z	This study
HGB340	HGB007 parent, chromosomal integration of pECM20	23
HGB003	ATCC 35271 *X. bovienii* unsequenced wild-type isolate	ATCC
HGB1055	*X. bovienii* sequenced wild-type isolate	Monsanto
HGB1166	HGB003 parent, *att*Tn*7*::miniTn*7* from pEVS107	This study
HGB1167	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1	This study
HGB1168	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/AM1Z	This study
HGB1169	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/BM1Z	This study
HGB1170	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/CM1Z	This study
HGB1171	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/TnM1Z	This study
HGB1180	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/rrs/GFP	This study
HGB1172	HGB003 parent, *att*Tn*7*::miniTn*7* from pTn*7*/SR1/rrs/GFP	This study
HGB086	ATCC 49121 *X. poinarii* wild-type isolate	ATCC
HGB1173	HGB086 parent, chromosomal integration of pTn*7*/rrs	This study
HGB1174	HGB086 parent, chromosomal integration of pTn*7*/SR1/rrs	This study
HGB1175	HGB086 parent, chromosomal integration of pTn*7*/SR1/AM1Z/rrs	This study
HGB1176	HGB086 parent, chromosomal integration of pTn*7*/SR1/BM1Z/rrs	This study
HGB1177	HGB086 parent, chromosomal integration of pTn*7*/SR1/CM1Z/rrs	This study
HGB1178	HGB086 parent, chromosomal integration of pTn*7*/SR1/TnM1Z/rrs	This study
HGB1181	HGB086 parent, chromosomal integration of pTn*7*/rrs/GFP	This study
HGB1179	HGB086 parent, chromosomal integration of pTn*7*/SR1/rrs/GFP	This study
HGB836	DSM 16338 *X. szentirmaii* wild-type isolate	20; A. Fodor
HGB1322	HGB836 parent, *att*Tn*7*::miniTn*7* from pEVS107	This study
HGB1323	HGB836 parent, *att*Tn*7*::miniTn*7* from Tn*7*/SR1	This study
HGB084	ATCC 49542 *X. beddingii* wild-type isolate	ATCC
HGB833	DSM 16342 *X. budapestensis* wild-type isolate	20; A. Fodor
HGB834	DSM 16337 *X. ehlersii* wild-type isolate	20; A. Fodor
HGB835	DSM 16336 *X. innexi* wild-type isolate	20; A. Fodor

[a] HGB stock number of *Xenorhabdus* strain.

was subsequently digested with DpnI to cut parental methylated DNA and was then transformed into *Escherichia coli* S17-1(λ*pir*). Plasmids were conjugated from *E. coli* S17-1(λ*pir*) into *Xenorhabdus* spp. through triparental conjugations with a pUX-BF13 helper plasmid (2) as previously described (7). The correct insertion of Tn*7* constructs into the *att*Tn*7* site of the *X. nematophila* chromosome was confirmed by using PCR with AttTn7EXT and ErmAnch1 primers (Table 3). The transposition of Tn*7* was unsuccessful in *X. poinarii*; thus, a 1,477-bp EcoRI fragment of *X. poinarii* chromosomal DNA (carrying a 16S rRNA gene [*rrs*] region; GenBank accession no. D78010) was first cloned into the EcoRI site of pBluescript (Stratagene) and then directionally subcloned into the

TABLE 2. Plasmids used in this study

Plasmid	Relevant properties[a]	Source or reference
pECM9	Source of P_{aphA}-*gfp* amplicon; Ap^r	23
pECM20	*mob*RP4, *ori*R6K integrates into *X. nematophila* chromosome to express *gfp*; Cm^r	23
pEVS107	*ori*R6K, *mob*RP4, mobilizable suicide miniTn*7*-Erm delivery vector; Erm^r Km^r	34
pUX-BF13	Mobilizable Tn*7* transposition helper plasmid that expresses Tn*7* transposase in *trans*; Ap^r	2
pTn*7*/SR1	pEVS107 parent, delivers miniTn*7*-Erm/*nil*	7
pTn*7*/SR1/AM1Z	pTn*7*/SR1 parent with *nilA21* ATG to TAG point mutation	This study
pTn*7*/SR1/BM1Z	pTn*7*/SR1 parent with *nilB20* ATG to TAG point mutation	This study
pTn*7*/SR1/CM1Z	pTn*7*/SR1 parent with *nilC19* ATG to TAG point mutation	7
pTn*7*/SR1/TnM1Z	pTn*7*/SR1 parent with *tn2* ATG to TAG point mutation	This study
pTn*7*/rrs	pEVS107 parent, integrates into *X. poinarii rrs*	This study
pTn*7*/SR1/rrs	pTn*7*/SR1 parent, integrates into *X. poinarii rrs*	This study
pTn*7*/SR1/AM1Z/rrs	pTn*7*/SR1/AM1Z parent, integrates into *X. poinarii rrs*	This study
pTn*7*/SR1/BM1Z/rrs	pTn*7*/SR1/BM1Z parent, integrates into *X. poinarii rrs*	This study
pTn*7*/SR1/CM1Z/rrs	pTn*7*/SR1/CM1Z parent, integrates into *X. poinarii rrs*	This study
pTn*7*/SR1/TnM1Z/rrs	pTn*7*/SR1/TnM1Z parent, integrates into *X. poinarii rrs*	This study
pTn*7*/rrs/GFP	pTn*7*/rrs parent, integrates into *Xenorhabdus rrs*, constitutively expresses GFP	This study
pTn*7*/SR1/rrs/GFP	pTn*7*/SR1/rrs parent, integrates into *Xenorhabdus rrs*, constitutively expresses GFP	This study

[a] Cm, chloramphenicol.

TABLE 3. Oligonucleotides used in this study

Oligonucleotide	5′ to 3′ sequence[a]	Purpose
AttTn7Ext	TGTTGGTTCACATCC	Tn*7* insert amplification
ErmAnch1	TACTTATGAGCAAGTATTGTC	Tn*7* insert amplification
AMtoZfornew	GACAAAATGACTCTAGATTTCTAATTATTATCCTAATCTGC	Mutagenesis
AMtoZrevnew	GCAGATTAGGATAATAATTAGAAATCTAGAGTCATTTTGTC	Mutagenesis
BMtoZfor	TGGATTTGATTTTTTTCTAAGAGTTTTCATTGGTAATG	Mutagenesis
BMtoZrev	TACCAATGAAAACTCTTAGAAAAAAATCAAATCCATCG	Mutagenesis
TnMtoZfor	TTTATGGATGTATTCGCTCTACCATTTAGTTTACGCCGC	Mutagenesis
TnMtoZrev	GCGTAAACTAAATGGTAGAGCGAATACATCCATAAAAGC	Mutagenesis
pKmGFPEcoFor	GAATTCGTTGTGTCTCAAAATCTCTG	*gfp* amplification
pKmGFPEcoRev	GAATTCGGATATAGTTCCTCCTTTCAGC	*gfp* amplification

[a] Underlined regions indicate ATG to TAG null mutations.

EcoRI/PvuII sites of all miniTn*7* plasmid constructs by digestion with EcoRI and EcoRV (Table 2), thereby allowing integration into the chromosome with homologous recombination.

To create *X. poinarii* and *X. bovienii* strains expressing green fluorescent protein (GFP), miniTn*7* derivatives were engineered to constitutively express GFP by amplifying the P_{aphA}-GFP fragment from plasmid pECM9, using primers pKmGFPEcoFor and pKmGFPEcoRev, and cloning the EcoRI-digested PCR product into the similarly cut plasmids pTn*7*/rrs and pTn*7*/SR1/rrs to yield pTn*7*/rrs/GFP and pTn*7*/SR1/rrs/GFP, respectively. These plasmids were integrated into the *X. poinarii* chromosome by homologous recombination or integrated into the *att*Tn*7* site of the *X. bovienii* chromosome by Tn*7*-mediated transposition, as described above. *X. nematophila* strains were engineered to express GFP by using chromosomal integration of plasmid pECM20 as previously described (23).

The complete genome sequences of *X. nematophila* and *X. bovienii* (Jollieti) are available at http://www.xenorhabdus.org. The presence or absence of the *nil* locus in *Xenorhabdus* strains was determined by Southern hybridization, using pTn*7*/SR1 as a probe labeled with the ECF random prime labeling and amplification system (Amersham Pharmacia, Pittsburgh, PA) as previously described (17), and by BLASTn analysis of the *X. bovienii* (Jollieti) complete genome sequence.

Microscopy and nematode colonization assays. All microscopic observations of nematodes and in vitro nematode colonization assays were performed as previously described (7, 23). Briefly, lawns of the bacterial strains to be tested were inoculated onto lawns of lipid agar (37), sterile *S. carpocapsae* eggs were added, and plates were incubated at room temperature. After approximately 2 weeks, infective juvenile stage *S. carpocapsae* nematodes were harvested from White's water traps (38), surface sterilized with 0.5% sodium hypochlorite for 3 min, and washed with sterile water. The number of surface-sterilized nematodes was then adjusted to 10,000 nematodes in 1 ml of water by comparison to known standards. One milliliter of LB broth was then added, and the surface-sterilized nematodes were sonicated for 1 min in a sonicating water bath to release the colonizing bacteria. The number of CFU/ml was calculated by plating dilutions of the sonicates onto LB plus 0.1% pyruvate. In vivo nematode colonization assays were performed by injecting *Galleria mellonella* greater wax moth larvae (five replicates of three larvae per replicate; Vanderhorst Wholesale) with 12.5 μl of a nematode and bacteria mixture per larva. The mixture contained ~2,500 sterile first- and second-instar juvenile *S. carpocapsae* nematodes resuspended in an overnight culture of the test strain. The emergent nematodes were determined to be apoxenic or monoxenic by performing colonization assays as described above and by plating sonicates on LB agar plates containing 0.1% pyruvate and 50 μg/ml ampicillin. The bacterial colonies were verified to be *Xenorhabdus* spp. by testing for a lack of catalase activity and by examining colony pigmentation: *X. nematophila* colonies are cream-colored, *X. bovienii* colonies are yellow, and *X. poinarii* colonies are rust colored.

Fractionation and NilC immunoblotting. Strains carrying the *nil* locus (HGB007, HGB1167, and HGB1174) were washed in phosphate-buffered saline and normalized to an optical density of 10.0. Twofold dilutions of the boiled lysates were separated by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (10 μl of gel per lane), transferred to nitrocellulose, and immunodetected with a 1:5,000 dilution of anti-NilC antibody (7) followed by a 1:8,000 dilution of anti-rabbit- horseradish peroxidase-conjugated secondary antibody (Amersham, Pittsburgh, PA). Detection was performed with ECL luminescent substrate (Amersham, Pittsburgh, PA). Similarly, the NilC levels in strains carrying *nilA* and *nilB* start codon point mutations (HGB1182, HGB1183, HGB1168, HGB1169, HGB1175, and HGB1176) were compared to those of strains of the same species carrying the native *nil* locus, using twofold dilutions.

Strains carrying the *nil* locus (HGB007, HGB1167, and HGB1174) were washed in phosphate-buffered saline, normalized to an optical density of 10.0, and lysed by French pressure lysis. One milliliter of each cell-free lysate was fractionated into the soluble and insoluble fractions by ultracentrifugation. The insoluble fraction was resuspended in 1 ml of 1% sodium dodecyl sulfate to maintain its original ratio relative to the soluble fraction proteins in the lysate.

G-plus-C content sequence analysis. The G-plus-C content of the circular *X. nematophila* genome sequence (http://www.xenorhabdus.org) was analyzed at every position with a sliding window of 3,463 nucleotides (nt), the size of the entire *nil* locus, or 1,783 nt, which corresponds to the size of the *nilAB* portion of the *nil* locus, using MATLAB version R2007a (The MathWorks, Natick, MA). Of 4,521,243 sequences, 216,994 had a G-plus-C content that was less than that of the *nil* locus and 57,504 sequences had a G-plus-C content that was less than that of *nilAB*. To avoid correlations due to overlapping, we also sampled 1,000 random probes of 1,783 or 3,463 nt from the genome and generated two data sets of 100 independent samplings each. The means and standard deviations of these two data sets were nearly identical to those of the *X. nematophila* genome sampled with a sliding window at every position.

RESULTS

The *X. nematophila*-*S. carpocapsae* interaction is species specific. To explore the basis of host range specificity in a *Xenorhabdus*-*Steinernema* interaction, we focused on the nematode *S. carpocapsae*, as the relationship between it and its bacterial symbiont, *X. nematophila*, is well studied at the molecular level (13). Because nonbiological factors such as geographic isolation of hosts and/or microbes can give the appearance of microbe-host specificity where it does not exist, we sought to experimentally establish that this interaction is, in fact, species specific. We mixed *S. carpocapsae* nematodes with eight different *Xenorhabdus* species [*X. nematophila*, *X. beddingii*, *X. bovienii* (ATCC 49109), *X. bovienii* (Jollieti), *X. budapestensis*, *X. ehlersii*, *X. innexi*, *X. poinarii*, and *X. szentirmaii*], including two *X. bovienii* isolates from distinct nematode species (4, 11, 20, 33). Except for *X. szentirmaii* and *X. bovienii* (Jollieti), which did not support nematode development and reproduction, all species tested supported *S. carpocapsae* development into adults that produced progeny. Progeny nematodes derived from each strain were assessed for the presence of bacteria in the colonization site by sonication and dilution plating (see Materials and Methods; limit of detection = 0.0002 CFU/nematode). Only the native symbiont, *X. nematophila*, colonized *S. carpocapsae* nematodes; progeny nematodes that developed from lawns of the other species did not have any bacteria associated with them. Thus, the *X. nematophila*-*S.*

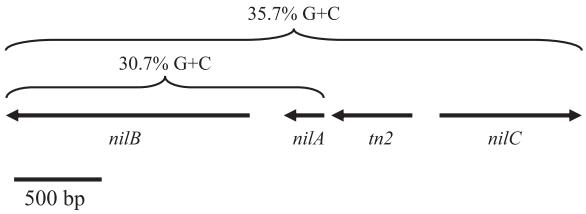

FIG. 1. Map of the *X. nematophila nil* locus. Each line arrow indicates the orientation and length of the gene named below it. The percentages of G-plus-C content of the portions indicated by brackets are shown.

carpocapsae interaction is species specific and is likely maintained in nature by the exclusive ability of *X. nematophila* to colonize *S. carpocapsae* nematodes.

***X. nematophila nilABC* colonization genes are absent from other *Xenorhabdus* species.** We previously identified a 3.5-kb region of the *X. nematophila* chromosome that encodes four genes: *nilA*, *nilB*, *nilC*, and *tn2* (collectively referred to as the *nil* locus) (Fig. 1) (17). Deletion of the *nil* locus from the *X. nematophila* chromosome results in the inability of the bacterium to colonize *S. carpocapsae* nematodes but in no other known phenotypic changes (7). The *nilA*, *nilB*, and *nilC* genes are predicted or known to encode membrane proteins that may function in adherence (7, 17). Through Southern blot analysis using the *nil* locus as a probe, we found that of all the *Xenorhabdus* species tested, only *X. nematophila* harbors the *nil* locus (data not shown). This observation is supported by the lack of any sequences similar to the *nil* locus in the complete genome sequence of *X. bovienii* (Jollieti) (http://www.xenorhabdus.org).

The *X. nematophila nil* locus enables colonization of *S. carpocapsae* by *Xenorhabdus* species. The critical role of the *nil* locus in *X. nematophila* colonization of *S. carpocapsae* nematodes (7, 8, 17), coupled with its absence in other *Xenorhabdus* species, suggests that it may encode proteins that function as *S. carpocapsae* host range specificity determinants. To test this, we introduced the *nil* locus onto the chromosomes of *X. bovienii* strain ATCC 49109, *X. poinarii* strain ATCC 49121, and *X. szentirmaii* DSM16338 (representing three distinct *Xenorhabdus* clades [35]) to create *X. bovienii* Tn*7-nil*, *X. poinarii* Tn*7-nil*, and *X. szentirmaii* Tn*7-nil*, respectively (see Materials and Methods). *X. szentirmaii* Tn*7-nil* did not support nematode development, indicating that the *nil* locus is not involved in this aspect of host range specificity (data not shown). However, *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* colonized *S. carpocapsae* nematodes, while the corresponding control strains lacking the *nil* locus did not (Fig. 2 and Table 4). Therefore, the *nil* locus is necessary and sufficient for *Xenorhabdus* bacterial colonization of the *S. carpocapsae* nematode, indicating that it contributes to defining the host specificity of *X. nematophila*.

Although *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* colonize *S. carpocapsae* nematodes to levels that are far greater than those of their counterparts that do not have the *nil* gene (Fig. 2; Table 4), they do not colonize to the same level as *X. nematophila*. To determine if this was due to improper expression or localization of the *nil* gene products, we monitored the expression of NilC protein by immunoblotting. *X. bovienii* Tn*7-nil* cells express NilC at a level equal to that expressed by *X. nematophila*, whereas *X. poinarii* Tn*7-nil* cells express approx-

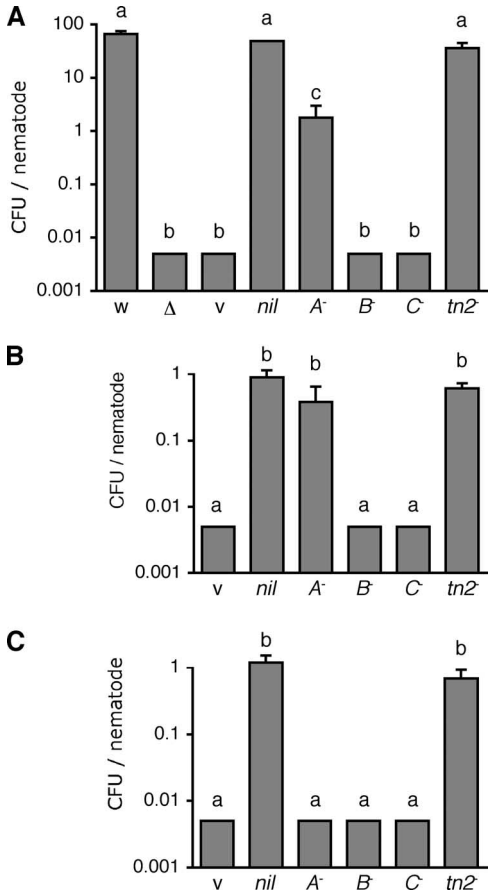

FIG. 2. The *nilA*, *nilB*, and *nilC* genes are required by *X. nematophila* (A), *X. bovienii* (B), and *X. poinarii* (C) for *S. carpocapsae* nematode colonization. For trials where no bacteria were detected, the colonization level is shown as the limit of detection, 0.005 CFU/nematode. Results are shown as means ± standard deviations ($n = 4$ or 5 replicates per trial). The bars represent the colonization levels of wild-type *X. nematophila* (w), *X. nematophila* with the *nil* region deleted (Δ), and *Xenorhabdus* spp. with an integrated Tn*7* that is empty (v), carries the *nil* region with no mutations (*nil*), or has start-to-stop codon mutations in *nilA* (A^-), *nilB* (B^-), or *nilC* (C^-). The different letters above the bars indicate values that are significantly different ($P < 0.001$) from one another by analysis of variance and Tukey's test with a 95% confidence interval.

imately twofold less than that expressed by *X. nematophila* (Fig. 3A). In both *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil*, NilC was localized to the membrane fraction, as it is in *X. nematophila* (7) (Fig. 3B). NilC was not detected in *X. bovienii* or *X. poinarii* carrying a vector control, nor were its levels affected by point mutations in *nilA* or *nilB* (data not shown). Therefore, the difference in absolute colonization levels is not likely due to differences in the expression or localization of NilC. The colonization levels of different *Xenorhabdus* species vary considerably across different *Xenorhabdus-Steinernema* species pairs. For example, a recent study reported average colonization levels of 43.8, 1.0, and 0.7 CFU/nematode (determined by crushing 500 nematodes) for *X. nematophila*, *X. cabanillasii*, and *X. japonica*, respectively, in their cognate nematode hosts (32). Therefore, the colonization levels of *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* in *S. carpocapsae* may

TABLE 4. *S. carpocapsae* nematode colonization by *Xenorhabdus* species

Species	Presence of *nil* locus	Mean CFU/nematode[a]	Proportion of nematodes colonized[b]	Mean CFU/nematode[c]	Mean CFU/colonized nematode[d]
X. nematophila	−	<0.005	<0.001	<0.005	
X. nematophila	+	65.5 ± 9.7	0.97	37.7 ± 4.4	38.8
X. bovienii	−	<0.005	<0.001	<0.005	
X. bovienii	+	0.89 ± 0.2	0.026	0.6 ± 0.2	24.0
X. poinarii	−	<0.005	<0.001	<0.005	
X. poinarii	+	1.2 ± 0.3	0.121	1.1 ± 0.3	9.1

[a] Mean CFU/nematode for strains lacking the *nil* locus (*X. nematophila* Tn*7-nil* deletion strain HGB777 or *X. bovienii* or *X. poinarii* strains with a chromosomal control vector) or carrying the *nil* locus (wild-type *X. nematophila*, *X. bovienii* Tn*7-nil*, or *X. poinarii* Tn*7-nil*). The data represent the means and standard deviations of five replicates.

[b] Colonization frequency was determined by epifluorescence microscopic examination of 1,000 nematodes from duplicate populations reared in cocultures with the indicated species carrying the *nil* locus and expressing GFP.

[c] Mean CFU/nematode of the indicated species carrying the *nil* locus and expressing GFP. The data represent the means and standard deviations of five replicates.

[d] Calculated by dividing the mean CFU/nematode in column 4 by the proportion of nematodes colonized in column 3.

simply reflect an inherent limited colonization proficiency of *X. bovienii* and *X. poinarii* relative to that of *X. nematophila*. Alternatively, *X. nematophila* may possess additional unique genetic factors that contribute to its ability to reproduce within *S. carpocapsae*.

Individual *nil* locus genes play genetically similar roles in host colonization by *X. nematophila* and in cross-species colonization by noncognate *Xenorhabdus* spp. To assess the individual contributions of the four *nil* locus genes to colonization and host range, we engineered four separate null mutation constructs, each with a null mutation in a *nil* locus gene. We previously found that *X. nematophila nilB* and *nilC* transposon mutants are completely defective and a *nilA* transposon mutant is partially defective in colonizing *S. carpocapsae* nematodes. The *tn2* gene, which encodes a putative transposase, is not likely to encode a colonization factor, although its involvement in nematode colonization has not been directly examined (17). *X. nematophila*, *X. bovienii* Tn*7-nil*, and *X. poinarii* Tn*7-nil* carrying null mutations in either *nilB* or *nilC* were unable to colonize *S. carpocapsae* nematodes (Fig. 2), demonstrating that each of these two genes is essential for normal and cross-species colonization. A null mutation in *nilA*, however, had variable effects on colonization in different organisms. *X. nematophila* carrying a *nilA*-null mutation colonized to the same extent as a *nilA* transposon mutant (17), approximately 2.5% of that of the wild type. However, a *nilA*-null mutation did not significantly affect cross-species colonization by *X. bovienii* Tn*7-nil* and prevented cross-species colonization by *X. poinarii* Tn*7-nil* (Fig. 2), indicating that the importance of *nilA* in colonization and host range specificity is variable. As predicted, a null mutation in *tn2* had no significant effect on nematode colonization by any of the three species (Fig. 2).

The interactions between noncognate *Xenorhabdus* bacteria that carry the *nil* locus and *S. carpocapsae* nematodes are biologically relevant. In the experiments presented above, bacteria and nematodes were cocultivated on a synthetic growth medium rather than in their natural host, an insect cadaver. To test if the *nil* locus enables cross-species colonization within an insect cadaver, *X. bovienii* Tn*7-nil*, *X. poinarii* Tn*7-nil*, and control strains lacking the *nil* locus were coinjected with sterile

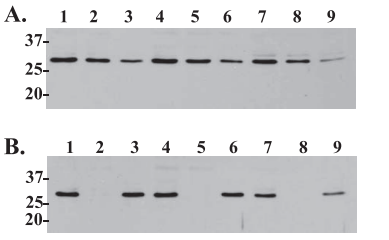

FIG. 3. Expression and localization of NilC in three *Xenorhabdus* spp. containing the *nil* gene. (A) Twofold serial dilutions of stationary-phase *X. nematophila* (lanes 1 to 3), *X. bovienii* Tn*7-nil* (lanes 4 to 6), and *X. poinarii* Tn*7-nil* (lanes 7 to 9) were immunoblotted for NilC. The *X. nematophila* and *X. bovienii* Tn*7-nil* samples were diluted with one additional twofold dilution prior to loading. Triplicate samples showed results identical to those shown. (B) Stationary-phase *X. nematophila* (lanes 1 to 3), *X. bovienii* Tn*7-nil* (lanes 4 to 6), and *X. poinarii* Tn*7-nil* (lanes 7 to 9) were washed and lysed by French pressure lysis. The cell-free supernatants (lanes 1, 4, and 7) were separated into soluble (lanes 2, 5, and 8) and insoluble (lanes 3, 6, and 9) fractions and immunoblotted for NilC. As can be seen, NilC is found in the insoluble fractions in all *Xenorhabdus* spp. tested and is therefore likely to be properly membrane associated.

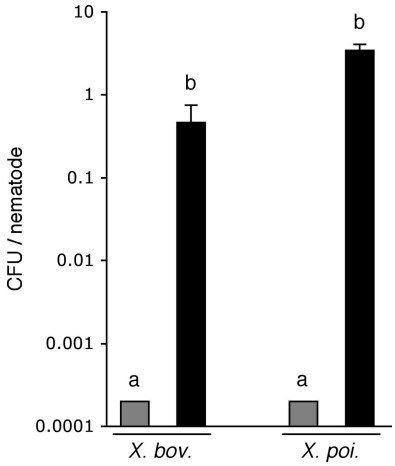

FIG. 4. *X. bovienii* (*X. bov.*) and *X. poinarii* (*X. poi.*) colonize *S. carpocapsae* nematodes in vivo in the presence (black bars) of the *nil* locus, but not in its absence (gray bars). For trials in which no colonizing bacteria were detected, the colonization level is shown as the limit of detection, 0.0002 CFU/nematode. Results are shown as means ± standard deviations ($n = 5$ replicates per trial). The letters (a and b) above the bars indicate values that are significantly different ($P < 0.001$) from each other by analysis of variance and Tukey's test with a 95% confidence interval.

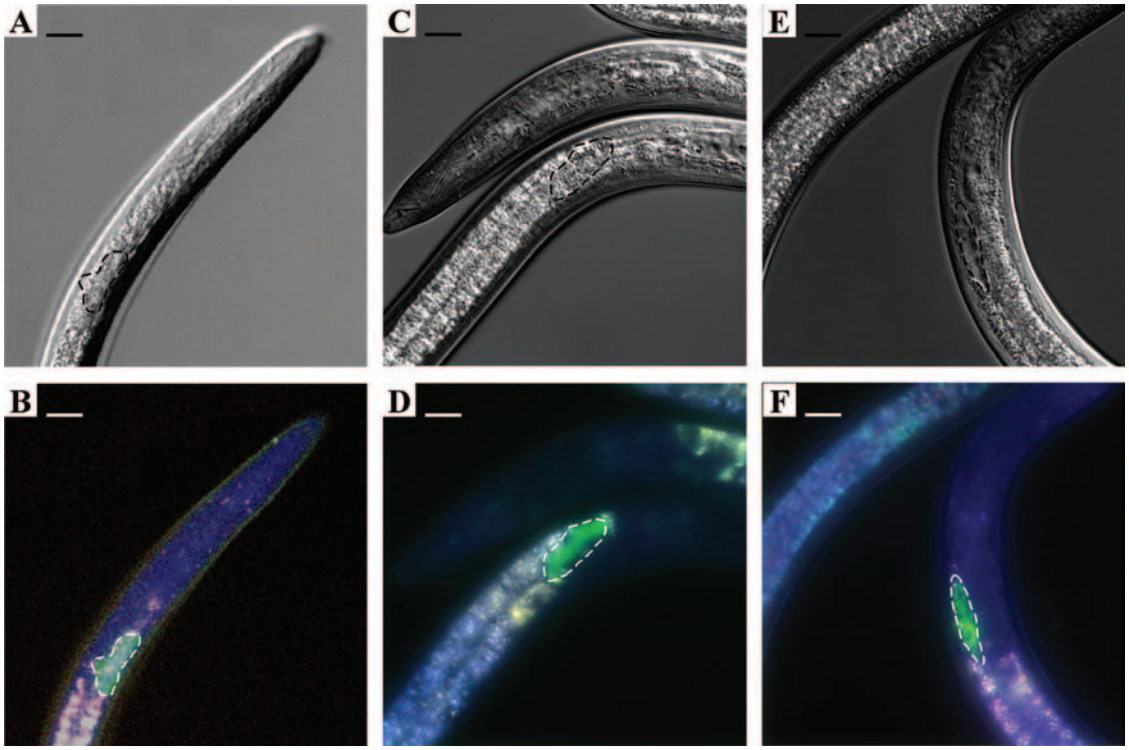

FIG. 5. *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* colonize the vesicle of *S. carpocapsae* nematodes. Visible light (A, C, and E) and fluorescence (B, D, and F) micrographs of *S. carpocapsae* nematodes colonized by GFP-expressing *X. nematophila* (A and B), *X. bovienii* Tn*7-nil* (C and D), and *X. poinarii* Tn*7-nil* (E and F). All images were taken at a magnification of ×600, and a 10-μm scale bar is shown. Dashed lines indicate the borders of the vesicles. The images are oriented with each nematode's digestive tract below the vesicle (apparent as white autofluorescence).

S. carpocapsae nematodes into *Galleria mellonella* insect larvae. The emergent nematodes were colonized by *X. bovienii* and *X. poinarii* only in trials where the *nil* locus was present (Fig. 4), confirming that the ability to colonize *S. carpocapsae* is species specific in a natural setting and that the *nil* locus confers this specificity.

Xenorhabdus bacteria colonize a specific region, termed the vesicle, of their *Steinernema* nematode hosts (Fig. 5) (3). To determine if *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* colonize the *S. carpocapsae* vesicle, these strains were engineered to express GFP and examined by using epifluorescence microscopy. *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* bacteria, but not control strains lacking the *nil* locus, were observed exclusively within the *S. carpocapsae* vesicle (Fig. 5). This result indicates that cross-species-colonizing bacteria exhibit the same tissue tropism as *X. nematophila*, the native *S. carpocapsae* symbiont.

Colonization levels of cross-species-colonizing bacteria and their distribution within host populations. Typically, *X. nematophila* colonizes >90% of the *S. carpocapsae* nematodes in a population (31, 37); however, this is not the case for all *Xenorhabdus-Steinernema* pairs. For example, *X. poinarii* colonizes only 50% of the nematodes in populations of its native host, *S. glaseri* (1, 3, 9). We examined the proportion of colonized nematodes, using epifluorescence microscopy, and found that 2.6% and 12.1% of nematodes were colonized by *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil*, respectively (Table 4). Therefore, it is possible that species-specific characteristics of *Xenorhabdus* bacteria determine their colonization initiation efficiency regardless of host. Alternatively, these results could indicate a requirement for additional colonization factors. Next, by calculating the average number of bacteria per colonized nematode, we determined that *X. bovienii* Tn*7-nil* and *X. poinarii* Tn*7-nil* colonize *S. carpocapsae* nematodes to levels that are approximately 62% and 24%, respectively, of those achieved by *X. nematophila* (Table 4).

The *nil* locus encodes proteins involved in initiating colonization and either bacterial outgrowth or colonization maintenance. The *X. nematophila* colonization process is thought to have both initiation and outgrowth stages. Newly formed nematodes have few bacteria that grow to fill the colonization site, and the final population of bacteria in a mature, fully colonized nematode (Fig. 5) represents 1 to 2 individual clones (23). These findings have led to the working model of colonization in which very few individual cells initiate colonization (although the process of initiation is not understood) and then divide to fill the colonization site. Our data demonstrate that the *nil* locus enables colonization of *S. carpocapsae* nematodes by *Xenorhabdus* bacteria but do not reveal if NilA, NilB, and NilC function in the initiation and/or outgrowth stages of nematode colonization, either or both of which could contribute to the specificity of the natural interaction.

To determine if the *nil* locus gene products function in colonization initiation and/or outgrowth within nematodes hosts, we examined the distribution of *S. carpocapsae* nematodes colonized by an *X. nematophila nilA* mutant expressing GFP. *nilA* mutants

are attenuated but not completely deficient in colonization and therefore allow these aspects of colonization to be determined. Of 500 nematodes examined (24), 65% were empty and 35% were visibly colonized, in contrast to the distribution for wild-type *X. nematophila* expressing GFP, where 3.8% of nematodes were observed to be empty and 96% were visibly colonized. The finding that a *nilA* mutant colonizes a lower percentage of nematodes than the wild type indicates that *nilA* functions in colonization initiation or in the very early stages of colonization, with uncolonized nematodes representing those in which initiation failed. Compared to the gross colonization levels for *nil*-null mutants (Fig. 2 and above), this result also suggests that the *nilA* mutant is defective in outgrowth, since the proportion of nematodes colonized by the *nilA* mutant is over 10-fold greater (35% colonized) than that which would be expected from its overall colonization levels (~2.5% of wild-type levels). In other words, if the *nilA* mutant were defective only in initiation, one would expect that only 2.4% of all nematodes would be colonized. Instead, each of the 35% *nilA*-colonized nematodes must contain fewer bacteria (due to defective outgrowth) than wild-type-colonized nematodes to explain the relatively low average of *nilA* CFU/nematode.

The *nil* locus was likely acquired through a horizontal gene transfer event. The absence of *nil* genes in other *Xenorhabdus* species and the presence of sequences similar to *nilB* in other bacterial genera (17) prompted us to investigate whether some or all of the *nil* locus may have been horizontally acquired. In support of this idea, both the entire *nil* locus (35.7% G+C) and the *nilAB* portion of the *nil* locus (30.7% G+C) are of lower G-plus-C content than average, compared to that of the entire *X. nematophila* genome (43.3% G+C) (Fig. 1). To gauge the likelihood that the entire *nil* locus or the *nilAB* portion of the *nil* locus was horizontally acquired, we calculated the G-plus-C content of all positions within the *X. nematophila* genome, using a sliding window that was identical in size to that of the entire *nil* locus or the *nilAB* portion of the *nil* locus. Indeed, 95.2% of all similarly sized fragments had a higher G-plus-C content than that of the *nil* locus, and 98.7% of all similarly sized fragments had a higher G-plus-C content than that of the *nilAB* portion of the *nil* locus.

To assess if the *nil* genes are part of a larger genetic island, we used the MaGe (Magnifying Genomes microbial genome annotation system; https://www.genoscope.cns.fr/agc/mage/) genomic island search tool to identify regions of the *X. nematophila* genome greater than 5 kb that contain open reading frames (ORFs) lacking reciprocal best hits in comparison genomes: *Xenorhabdus bovienii* (Jollieti), *Photorhabdus luminescens* TT01, *Yersinia pestis* CO92, *Yersinia pseudotuberculosis* IP32953, *Salmonella enterica* LT2, and *Escherichia coli* K-12. This analysis identified a 20-kb region that includes the *nil* genes, three transposase-like elements, and five other ORFs. None of these five ORFs have homologs in either *Pasteurella multocida* (str Pm70) or *Haemophilus influenzae* (86-028NP), two bacteria with NilB homologs. The region is flanked by conserved genes predicted to encode succinylornithine transaminase (left side; GenBank accession no. AAL79613) and phosphotransacetylase (right side) but not by insertion sequences or tRNA elements (12). Based on the atypical GC content, the presence of mobile elements, and the presence of genes involved in symbiosis, the *nil* region can be considered part of the flexible gene pool and a genomic island (12).

DISCUSSION

We have expanded the host ranges of animal-associated bacteria by supplying a single genetic locus. Although the *nil* locus is not present in any other *Xenorhabdus* spp. examined, sequences similar to *nilB* occur in several pathogens with distinct host range specificities, including *Moraxella catarrhalis*, *Actinobacillus pleuropneumoniae*, *Haemophilus* spp., and *Neisseria* spp. (17). Based on our results, which demonstrate that *nilB* is a species specificity determinant in a mutualistic relationship between an animal and a bacterium, it will be of interest to determine if the genes of these pathogens that are similar to *nilB* also contribute to the host range in a homologous manner. If so, it would lend further support to the idea that common molecules mediate interactions between hosts and microbes, regardless of whether the outcome is pathogenesis or mutualism (19).

Our data demonstrate that a relatively small genetic locus can significantly alter the ability of specific mutualistic partners to recognize each other, even to the exclusion of other strains or species. Similarly, species specificity in the relationship between *Rhizobiaceae* bacteria and Phaseolae legumes can be manipulated through minor genetic alterations (15). However, the specificity of the *Rhizobia* is dictated through minor variations in genes that are uniformly present among members of the genus. In contrast, in the *Xenorhabdus* genus, *nil* genes appear to be restricted to *X. nematophila*, indicating either that this locus was horizontally acquired or that other species of *Xenorhabdus* have lost *nil* gene homologs. Given the comparatively low G-plus-C content of the *nil* locus and *nilAB* portion of the *nil* locus relative to that of the *X. nematophila* genome, we believe that the former hypothesis is more likely correct. Although the *tn2* gene is not required for nematode colonization (Fig. 2), its presence within the *nil* locus suggests that a transposition event may have mediated the horizontal gene transfer event that introduced the *nilAB* genes, or perhaps the entire *nil* locus, into the *X. nematophila* genome from an unrelated organism.

A similar horizontal transfer of a single gene may have contributed to the evolution of *Yersinia pestis*. Unlike its close relative *Yersinia pseudotuberculosis*, *Yersinia pestis* is able to colonize the flea midgut, enabling it to be transmitted to humans through the bite of a flea. Two genetic elements are known to be involved in *Y. pestis*-flea interactions: *hms*, necessary for production of an extracellular matrix, and *ymt*, which encodes a murine toxin with phospholipase D activity that protects the bacteria in the midgut of the flea from blood meal-derived antimicrobial activity. Although *Y. pseudotuberculosis* has functional *hms* genes, it appears that *Y. pestis* acquired *ymt* through a horizontal gene transfer. Thus, acquisition of a small genetic element may have had a profound impact on the environmental niche that can be occupied by this bacterium (18). Therefore, it is prudent to consider the impact that a similar transfer of host range specificity determinants could have on global health (6, 27).

In the mutualism between *X. nematophila* and *S. carpocapsae*, a successful association appears to require contributions from both the host and the microbe. We have shown here that *X. nematophila* requires specific factors to colonize its nematode host, while the nematode may control levels of colonizing

bacteria (23). Similarly, the initiation and maintenance of *Euprymna scolopes-Vibrio fischeri* mutualism is characterized by bacterial factors necessary for initiation (40) and host mechanisms to control bacterial population size (26). This suggests that animal-microbe mutualisms in general may be characterized by host-imposed restrictions that select for specific bacteria and host-imposed controls that prevent the unchecked growth of the colonizing mutualist.

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REFERENCES

1. **Akhurst, R. J.** 1983. *Neoaplectana* species: specificity of association with bacteria of the genus *Xenorhabdus*. Exp. Parasitol. **55:**258–263.
2. **Bao, Y., D. P. Lies, H. Fu, and G. P. Roberts.** 1991. An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. Gene **109:**167–168.
3. **Bird, A. F., and R. J. Akhurst.** 1983. The nature of the intestinal vesicle in nematodes of the family Steinernematidae. Int. J. Parasitol. **13:**599–606.
4. **Boemare, N. E., R. J. Akhurst, and R. G. Mourant.** 1993. DNA relatedness between *Xenorhabdus* spp. (*Enterobacteriaceae*), symbiotic bacteria of entomopathogenic nematodes, and a proposal to transfer *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. Int. J. Syst. Bacteriol. **43:**249–255.
5. **Bourzack, K. M., and K. Guillemin.** 2005. *Helicobacter pylori*-host cell interactions mediated by type IV secretion. Cell. Microbiol. **7:**911–919.
6. **Brown, N. F., M. E. Wickham, B. K. Coombes, and B. B. Finlay.** 2006. Crossing the line: selection and evolution of virulence traits. PLoS Pathog. **2:**346–353.
7. **Cowles, C. E., and H. Goodrich-Blair.** 2004. Characterization of a lipoprotein, NilC, required by *Xenorhabdus nematophila* for mutualism with its nematode host. Mol. Microbiol. **54:**464–477.
8. **Cowles, C. E., and H. Goodrich-Blair.** 2006. *nilR* is necessary for co-ordinate repression of *Xenorhabdus nematophila* mutualism genes. Mol. Microbiol. **62:**760–771.
9. **Dunphy, G. B., T. A. Rutherford, and J. M. Webster.** 1985. Growth and virulence of *Steinernema glaseri* influenced by different subspecies of *Xenorhabdus nematophilus*. J. Nematol. **17:**476–482.
10. **Fischer-Le Saux, M., E. Arteaga-Hernández, Z. Mrácek, and N. Boemare.** 1999. The bacterial symbiont *Xenorhabdus poinarii* (Enterbacteriaceae) is harbored by two phylogenetic related host nematodes: the entomopathogenic species *Steinernema cubanum* and *Steinernema glaseri* (Nematoda: Steinernematidae). FEMS Microbiol. Ecol. **29:**149–157.
11. **Fischer-Le Saux, M., H. Mauleon, P. Constant, B. Brunel, and N. Boemare.** 1998. PCR-ribotyping of *Xenorhabdus* and *Photorhabdus* isolates from the Caribbean region in relation to the taxonomy and geographic distribution of their nematode hosts. Appl. Environ. Microbiol. **64:**4246–4254.
12. **Gal-Mor, O., and B. B. Finlay.** 2006. Pathogenicity islands: a molecular toolbox for bacterial virulence. Cell. Microbiol. **8:**1707–1719.
13. **Goodrich-Blair, H.** 2007. They've got a ticket to ride: *Xenorhabdus nematophila-Steinernema carpocapsae* symbiosis. Curr. Opin. Microbiol. **10:**225–230.
14. **Goodrich-Blair, H., and D. J. Clarke.** 2007. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Mol. Microbiol. **64:**260–268.
15. **Gualtieri, G., and T. Bisseling.** 2000. The evolution of nodulation. Plant Mol. Biol. **42:**181–194.
16. **Hartland, E. L., V. Huter, L. M. Higgins, N. S. Goncalves, G. Dougan, A. D. Phillips, O. C. MacDonald, and G. Frankel.** 2000. Expression of intimin γ from enterohemorrhagic *Escherichia coli* in *Citrobacter rodentium*. Infect. Immun. **68:**4637–4646.
17. **Heungens, K., C. E. Cowles, and H. Goodrich-Blair.** 2002. Identification of *Xenorhabdus nematophila* genes required for mutualistic colonization of *Steinernema carpocapsae* nematodes. Mol. Microbiol. **45:**1337–1353.
18. **Hinnebusch, B. J., A. E. Rudolph, P. Cherepanov, J. E. Dixon, T. G. Schwan, and Å. Forsberg.** 2002. Role of Yersinia murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. Science **296:**733–735.
19. **Koropatnick, T. A., J. T. Engle, M. A. Apicella, E. V. Stabb, W. E. Goldman, and M. J. McFall-Ngai.** 2004. Microbial factor-mediated development in a host-bacterial mutualism. Science **306:**1186–1188.
20. **Lengyel, K., E. Lang, A. Fodor, E. Szallas, P. Schumann, and E. Stackebrandt.** 2005. Description of four novel species of Xenorhabdus, family Enterobacteriaceae: *Xenorhabdus budapestensis* sp. nov., *Xenorhabdus ehlersii* sp. nov., *Xenorhabdus innexi* sp. nov., and *Xenorhabdus szentirmaii* sp. nov. Syst. Appl. Microbiol. **28:**115–122.
21. **Litt, D. J., H. M. Palmer, and S. P. Borriello.** 2000. *Neisseria meningitidis* expressing transferrin binding proteins of *Actinobacillus pleuropneumoniae* can utilize porcine transferrin for growth. Infect. Immun. **68:**550–557.
22. **Marrs, C. F., G. P. Krasan, K. W. McCrea, D. L. Clemans, and J. R. Gilsdorf.** 2001. *Haemophilus influenzae*—human specific bacteria. Front. Biosci. **6:**E41–E60.
23. **Martens, E. C., K. Heungens, and H. Goodrich-Blair.** 2003. Early colonization events in the mutualistic association between *Steinernema carpocapsae* nematodes and *Xenorhabdus nematophila* bacteria. J. Bacteriol. **185:**3147–3154.
24. **Martens, E. C., F. M. Russell, and H. Goodrich-Blair.** 2005. Analysis of *Xenorhabdus nematophila* metabolic mutants yields insight into stages of *Steinernema carpocapsae* nematode intestinal colonization. Mol. Microbiol. **51:**28–45.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Nyholm, S. V., and M. McFall-Ngai.** 2004. The winnowing: establishing the squid-*Vibrio* symbiosis. Nat. Rev. Microbiol. **2:**632–642.
27. **Onstad, D. W., and M. L. McManus.** 1996. Risks of host range expansion by parasites of insects. BioScience **46:**430–435.
28. **Plant, L., and A. B. Jonsson.** 2003. Contacting the host: insights and implications of pathogenic *Neisseria* cell interactions. Scand. J. Infect. Dis. **35:**608–613.
29. **Raffatellu, M., Y.-H. Sun, R. P. Wilson, Q. T. Tran, D. Chessa, H. L. Andrews-Polymenis, S. D. Lawhon, J. F. Figueiredo, R. M. Tsolis, L. G. Adams, and A. J. Bäumler.** 2005. Host restriction of *Salmonella enterica* serotype Typhi is not caused by functional alteration of SipA, SopB, or SopD. Infect. Immun. **73:**7817–7826.
30. **Riely, B. K., J.-M. Ané, R. V. Penmetsa, and D. R. Cook.** 2004. Genetic and genomic analysis in model legumes bring Nod-factor signaling to center stage. Curr. Opin. Plant Biol. **7:**408–413.
31. **Sicard, M., J.-B. Ferdy, S. Pages, N. LeBrun, B. Godelle, N. Boemare, and C. Moulia.** 2004. When mutualists are pathogens: an experimental study of the symbioses between *Steinernema* (entomopathogenic nematodes) and *Xenorhabdus* (bacteria). J. Evol. Biol. **17:**985–993.
32. **Sicard, M., N. Le Brun, S. Pages, B. Godelle, N. Boemare, and C. Moulia.** 2003. Effect of native *Xenorhabdus* on the fitness of their *Steinernema* hosts: contrasting types of interactions. Parasitol. Res. **91:**520–524.
33. **Spiridonov, S. E., K. Krasomil-Osterfeld, and M. Moens.** 2004. *Steinernema jollieti* sp. n. (Rhabditida: Steinernematidae), a new entomopathogenic nematode from the American Midwest. Russ. J. Nematol. **12:**85–95.
34. **Stabb, E. V., and E. G. Ruby.** 2002. RP4-based plasmids for conjugation between *Escherichia coli* and members of the Vibrionaceae. Methods Enzymol. **358:**413–426.
35. **Tailliez, P., S. Pages, N. Ginibre, and N. Boemare.** 2006. New insight into diversity in the genus *Xenorhabdus*, including the description of ten new species. Int. J. Syst. Evol. Microbiol. **56:**2805–2818.
36. **Tobe, T., and C. Sasakawa.** 2002. Species-specific cell adhesion of enteropathogenic *Escherichia coli* is mediated by type IV bundle-forming pili. Cell. Microbiol. **4:**29–42.
37. **Vivas, E. I., and H. Goodrich-Blair.** 2001. *Xenorhabdus nematophilus* as a model for host-bacterium interactions: *rpoS* is necessary for mutualism with nematodes. J. Bacteriol. **183:**4687–4693.
38. **Woodring, J. L., and H. K. Kaya.** 1988. Steinernematid and Heterorhabditid nematodes: a handbook of biology and techniques. Southern Cooperative Series bulletin 331. Arkansas Agricultural Experiment Station, Fayetteville, AR.
39. **Xu, J., and R. E. Hurlbert.** 1990. Toxicity of irradiated media for *Xenorhabdus* spp. Appl. Environ. Microbiol. **56:**815–818.
40. **Yip, E. S., K. Geszvain, C. R. DeLoney-Marino, and K. L. Visick.** 2006. The symbiosis regulator *rscS* controls the *syp* gene locus, biofilm formation and symbiotic aggregation by *Vibrio fischeri*. Mol. Microbiol. **62:**1586–1600.