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Analysis of the PixA Inclusion Body Protein of *Xenorhabdus nematophila*

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The symbiotic pathogenic bacterium *Xenorhabdus nematophila* produces two distinct intracellular inclusion bodies. The *pixA* gene, which encodes the 185-residue methionine-rich PixA inclusion body protein, was analyzed in the present study. The *pixA* gene was optimally expressed under stationary-phase conditions but its expression did not require RpoS. Analysis of a *pixA* mutant strain showed that PixA was not required for virulence towards the insect host or for colonization of or survival within the nematode host, and was not essential for nematode reproduction. The *pixA* gene was not present in the genome of *Xenorhabdus bovienii*, which also produces proteinaceous inclusions, indicating that PixA is specifically produced in *X. nematophila*.

Xenorhabdus nematophila, a member of the family *Enterobacteriaceae*, forms a mutualistic association with the entomopathogenic nematode *Steinernema carpocapsae* (2, 12, 15). *X. nematophila* is carried in a specialized gut vesicle in the anterior portion of the intestine in the infective juvenile (IJ) form of the nematode (5). Upon invasion of the insect, the IJ enters the hemocoel and releases the bacterium into the hemolymph, where it secretes insect toxins that are involved in killing the insect host (12). In the insect cadaver, *X. nematophila* grows to high cell density and secretes antimicrobial and nematicidal products which protect the insect carcass from invasion by soil organisms. *Xenorhabdus nematophila* also produces exoenzymes that degrade insect tissues and macromolecules which contributes to the nutrient base that supports bacterial and nematode reproduction.

After several rounds of nematode reproduction, the infective juvenile form of the nematode develops, which possesses the gut vesicle that is colonized by the bacterium (5, 14). The colonized infective juvenile enters the soil environment, initiating a new life cycle upon invasion of an insect host. Each of the five established species of *Xenorhabdus* colonize a specific steinernematid nematode (6). These symbiotic nematode-bacterium pairs have been used as biological control agents against several agricultural pests (2).

Xenorhabdus spp. produce two distinct proteinaceous intracellular inclusions during stationary phase in culture and in insects (2, 10, 11). The sister taxon of *Xenorhabdus*, *Photorhabdus*, which engages in mutualistic associations with nematodes of the *Heterorhabditidae* family (1, 7, 13), also produces two morphologically distinct proteinaceous inclusion bodies (4, 8). The finding that intracellular crystalline inclusions are produced in both *Xenorhabdus* and *Photorhabdus* species suggests they are integral to the symbiotic pathogenic life cycles of these bacteria.

In *X. nematophila*, one inclusion body is composed of the acidic 26-kDa IP1 protein that possesses a high content of methionine residues (~8%), while the second inclusion is composed of the neutral 22-kDa IP2 protein which is not rich in methionine residues. Together, IP1 and IP2 represent >40% of the total cellular protein in stationary-phase cells (11). Immunodetection studies showed that IP1 was present in *X. nematophila* but not in other *Xenorhabdus* species (11).

The role that crystalline inclusion body proteins play in the life cycle of *Xenorhabdus* spp. is not presently known. In *Photorhabdus luminescens*, the type 1 crystalline inclusion is composed of the 11.3-kDa CipB protein, while the type 2 crystalline inclusion is composed of the methionine-rich 11.6-kDa CipA protein (4). Strains in which either *cipA* or *cipB* was inactivated displayed a pleiotropic phenotype and were incapable of supporting nematode growth in vitro but were still virulent towards the insect host (4). Whether the absence of CipA or CipB production per se or the loss of numerous phenotypic traits in the *cipA* and *cipB* strains accounted for the inability to support nematode growth remains unclear. Analysis of the genomic sequence of *Photorhabdus asymbiotica* (http://www.sanger.ac.uk/Projects/P_asymbiotica/) revealed it also possesses conserved *cipA* and *cipB* genes. Finally, spontaneously forming secondary variant cell types that lack numerous phenotypic traits, including inclusion body production, are formed by both *Xenorhabdus* spp. and *Photorhabdus* spp. (4, 6, 20, 21). The *X. nematophila* variant cells are able to support growth of *S. carpocapsae*, while *Heterorhabditis bacteriophora* nematodes are unable to grow on the secondary variant strains of *P. luminescens*.

In the present study the gene encoding the IP1 protein of *X. nematophila*, here referred to as the *p*rotein *i*nclusion of *X*enorhabdus (PixA), was sequenced and a mutant strain in which *pixA* was inactivated was analyzed. The *pixA* strain did not display a pleiotropic phenotype, was virulent towards *Manduca sexta* larvae, and was able to colonize and survive within the nematode.

Nucleotide sequence accession number. The nucleotide sequences of the *pixA* and *cob* genes described in this study were deposited in GenBank and were assigned accession number AY56156.

Sequence analysis of the *pixA* gene. It was shown previously that insoluble inclusion body-containing fractions of the F1 strain of *X. nematophila* contained high levels of PixA which

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MRNIDIMLAVKAEEIMNDYGKLSKDINKPV
LITPSILNKNYLRLLPEDDSVVCVDEELTLS
IKANIGDMIRLNVRSLNIETTYSAALVKIEP
MNVMRDGVVSMVSLPVVESVVRSVATVD
MKNTVDVDINTMKDYHWMIKVNDLPSVD
RISTMYYLSTIAIYRDKMLMGYIALESGLIL
DHTVMM

FIG. 1. Amino acid sequence of PixA. PixA contains 185 amino acid residues. The 15 methionine residues are shown in bold.

could be separated from contaminating proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (20). In the present study PixA derived from the F1 strain was electrotransferred to an Immobilon-P membrane (Millipore) and subsequently subjected to N-terminal amino acid sequence analysis, which identified the first 30 residues of PixA. To obtain the full-length *pixA* gene sequence, the 5′ end of *pixA* was amplified using degenerate primers (18) designed to the N-terminal sequence of PixA, followed by an arbitrary PCR approach (9).

A PCR fragment containing the *pixA* gene was used to isolate a *pixA*-bearing clone from a plasmid library derived from the ATCC 19061 type strain of *X. nematophila* (see below). The nucleotide sequences of *pixA* of the ATCC 19061 and F1 strains were found to be identical. The *pixA* gene encoded a protein of 185 amino acids (Fig. 1), 15 of which were methionine residues (8.1%). Eleven of the methionine residues were located in the C-terminal half of PixA. The average methionine content in genomes related to *X. nematophila* is 2.58% (www.tigr.org). Blastp analysis against the nonredundant protein databases revealed that PixA did not share significant sequence similarity to any known protein.

The calculated pI of PixA was 4.76 and the A+T content of *pixA* was 65.8%, quite distinct from the 55% A+T content of the rest of the genome (Forst et al., unpublished). Nucleotide sequence analysis of the flanking downstream region revealed that the *cob* operon (*cobUSCT*), which encodes enzymes for cobalamine (vitamin B_{12}) synthesis, was convergently transcribed relative to *pixA*. Subsequent to the completion of the above analysis, a genome sequencing project was initiated for the ATCC 19061 strain (http://xenorhabdus.org/). Analysis of the *X. nematophila* genome confirmed the nucleotide sequence of *pixA* and the existence of a single copy of *pixA* in the genome. Finally, the genomic sequence of another species of *Xenorhabdus*, *X. bovienii*, was recently completed (http://xenorhabdus.org/). *X. bovienii* produces intracellular protein inclusions, but a *pixA* homologue was not identified in the *X. bovienii* genome.

Expression of the *pixA* gene. The expression of *pixA* at different phases of growth was analyzed by reverse transcription (RT)-PCR (Fig. 2). *pixA* mRNA was not detected during the early (Fig. 2, lane 1) or mid-exponential (Fig. 2, lane 2) phase and was first apparent in cells grown to the late exponential phase (Fig. 2, lane 3). The expression of *pixA* reached high levels in 18-h stationary-phase cells (Fig. 2, lane 4). To determine whether the stationary-phase sigma factor RpoS was required for the expression of *pixA*, PixA production was examined in the *rpoS* strain HGB151 (19). The temporal expression and level of production of PixA in the parent and *rpoS* strains were indistinguishable, indicating that stationary-phase expression of *pixA* does not require RpoS (data not shown).

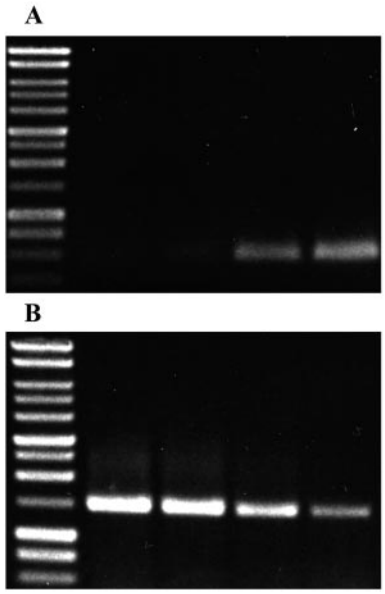

FIG. 2. Analysis of steady-state levels of *pixA* mRNA by RT-PCR. (A). Total RNA was isolated from the parental *X. nematophila* strain at different times during growth. Lane 1, early exponential phase; lane 2, mid-exponential phase; lane 3, late exponential phase; and lane 4, stationary phase. PCR primers directed to internal sequences of *pixA* were used in the RT-PCR. (B). Internal control reaction using total RNA as above and primers directed to the 16S rRNA.

Insertional inactivation of *pixA*. To study the role of PixA in *X. nematophila*, *pixA* was insertionally inactivated. A pST-Blue clone carrying a 400-bp PCR fragment encoding an internal region encompassing amino acids 42 to 175 of PixA was restriction digested with XbaI and PstI and the purified *pixA*-containing fragment was ligated into the same sites in the suicide vector pKNOCK-Cm^r (3). The recombinant plasmid was electroporated into *Escherichia coli* S17-1 λpir and subsequently transferred by conjugation into strain ATCC 19061. Disruption of *pixA* in candidate mutant strains that were resistant to chloramphenicol and had the *pixA*-containing pKNOCK plasmid integrated into the chromosome was confirmed by Southern blot analysis. One of the confirmed strains, designated NMI1, was chosen for further analysis.

The formation of inclusion bodies in the parental and NMI1 strains was compared by transmission electron microscopy (Fig. 3). Inclusions were observed in most median longitudinal sections of the bacteria. The majority of ATCC 19061 cells examined contained two inclusion bodies (Fig. 3). The inclusions appeared to be in direct contact with each other and were not homogenous in appearance, having distinctive staining properties. These results indicated that two distinct crystalline inclusions can be produced in an individual *X. nematophila* cell. In contrast, only one crystalline inclusion was present in the NMI1 cells (Fig. 3).

A *pixA*-containing low-copy-number plasmid (16, 17) was used to complement the mutant strain. SDS-polyacrylamide gel analysis showed that the parental strain produced both PixA and IP2 (Fig. 4, lane 1) while the NMI1 strain lacked

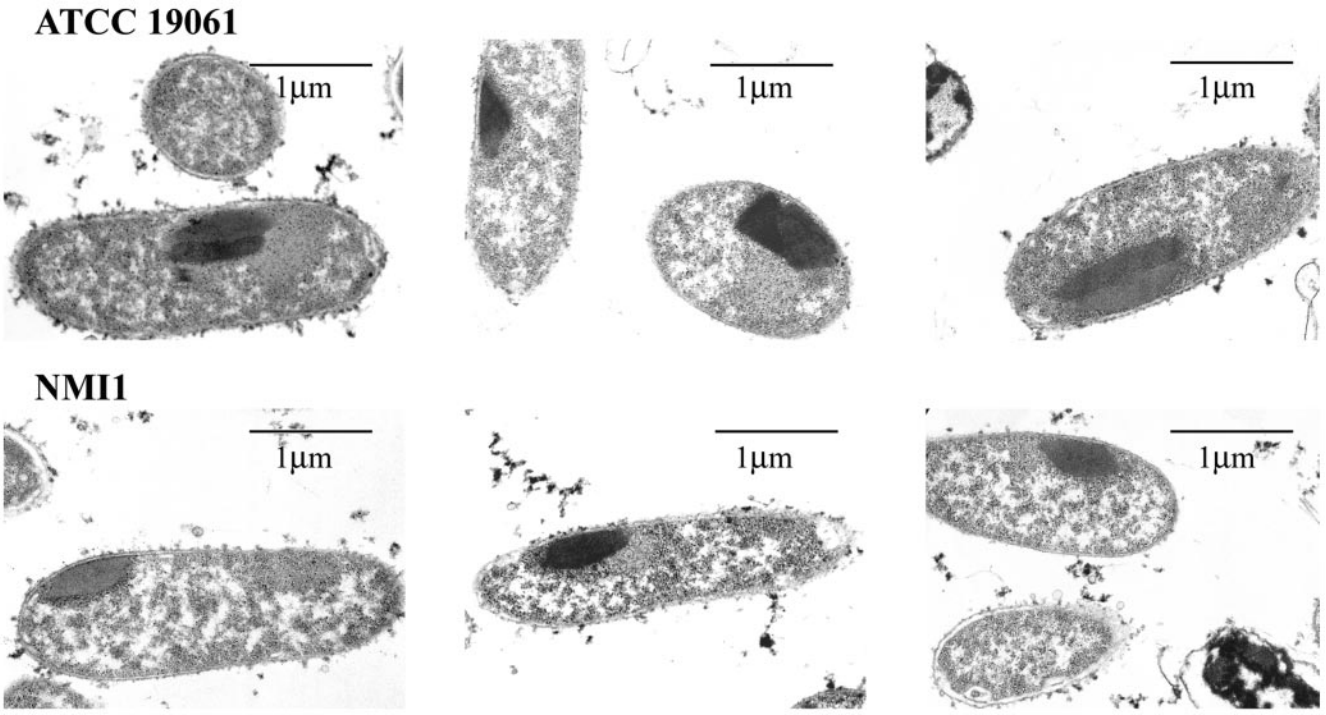

FIG. 3. Transmission electron microscopic analysis of inclusion bodies in the parental and NMI1 strains. Thin sections of stationary-phase cells were prepared as described in the text. Representative sections from ATCC19061 (top) and NMI1 (bottom) are shown.

PixA (Fig. 4, lane 2). PixA was found to be highly expressed in the complemented strain grown to mid-exponential phase (Fig. 4, lane 3). Early exponential growth of the complemented strain was similar to that of the parental strain. However, as the cells entered the mid-exponential phase, the growth rate was markedly reduced. This negative effect on growth of the complemented strain precluded its use in this study.

Phenotypic characterization of the *pixA* strain. The phenotypic traits examined in the NMI1 strain, such as growth rate, motility, hemolysis, lipolysis, proteolysis, and dye binding, were indistinguishable from those of the parent strain. These results were distinctly different from the *cipA* and *cipB* mutant strains of *Photorhabdus luminescens*, in which many of these traits were affected (4). In addition, inactivation of *pixA* did not affect virulence to fourth-instar *Manduca sexta* insects. The time at which 50% of the insect population died after injection of ~200 cells of either the parental or NMI1 strain was 27 and 28 h, respectively.

In vitro analysis of nematode colonization. To determine whether PixA was involved in either nematode colonization or the ability of *X. nematophila* to survive within the intestinal vesicle, the average number of bacteria per individual live nematode colonized with either the parental or NMI1 strain was monitored over a 140-day period (Fig. 5). We added 1,000 axenic surface-sterilized IJs (20) to a bacterial lawn of either the parental or NMI1 strain. After approximately 8 to 10 days colonized IJs were collected in White traps containing tap water (22). The number of IJs produced on bacterial lawns containing either the parental or NMI1 strain was comparable,

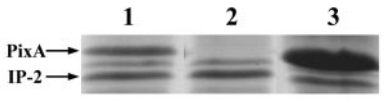

FIG. 4. Analysis of inclusion body protein production in the parental, NMI1, and complemented NMI1 strains. Inclusion bodies isolated from either ATCC 19061 (lane 1), the NMI1 strain (lane 2), or the complemented NMI1 strain (lane 3) were analyzed by SDS-polyacrylamide gel electrophoresis.

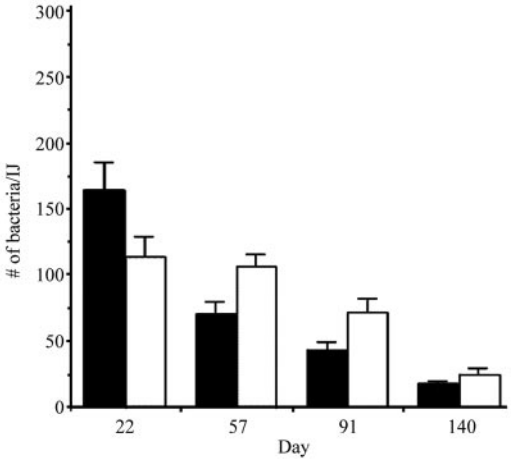

FIG. 5. Individual colonization of IJs grown on bacterial lawns. The average number of CFU per live IJ was determined for 20 live individual nematodes at each time point indicated. The results from IJs grown on ATCC 19061 and NMI1 are shown as black and white bars, respectively.

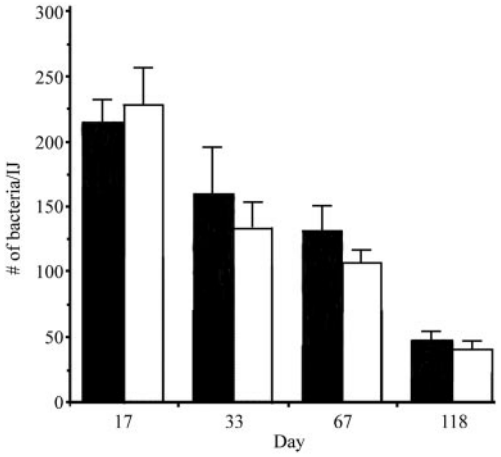

FIG. 6. Individual colonization of IJs derived from natural infections. The average number of CFU per live IJ was determined for 20 individuals at each time point indicated. The results from IJs grown on ATCC 19061 and NMI1 are shown as black and white bars, respectively.

indicating that PixA was not required for nematode reproduction in vitro.

To assess the level of colonization in individual nematodes a 1-ml aliquot of IJs was surface sterilized and resuspended in 0.5 ml of sterile LB broth. Single live nematodes were pipetted into 100 μl of LB broth in a sterile 1.5-ml microcentrifuge tube and homogenized for 70 seconds with a sterile motor-driven polypropylene pestle (Kontes). The homogenate (50 μl) was plated onto LB agar and incubated overnight. The level of colonization was determined for 20 individual IJs at each time point. At 22 days, an average of 160 and 114 CFU were recovered from nematodes grown on the parental or NMI1 strain, respectively. The CFU/IJ decreased progressively over time in both strains. With the exception of the 22-day time point, the average CFU/IJ was slightly higher for the NMI1 strain. By 140 days, an average of 9 and 12 CFU were recovered from IJs carrying the parental or NMI1 strain, respectively. These findings indicate that PixA production was not essential for either colonization of or survival in the infective juvenile.

In vivo analysis of nematode colonization. To assess whether the results obtained in the in vitro experiments accurately reflect the events that occur in vivo, the ability of the parental and NMI1 strains to colonize nematodes during natural infection of insect larvae was examined (Fig. 6). A 200-μl suspension containing 150 IJs colonized with either the parental or NMI1 strain was aliquoted into a container lined with moist filter paper. Three fourth-instar *Manduca sexta* larvae were then added to the container. After the insects died (24 to 36 h), they were moved to a White trap. IJs began to emerge from the insect cadaver 10 days after transfer to the White trap and were collected over a 118-day time period. IJs were surface sterilized and individual live nematodes were homogenized as described above.

At the 17-day time point, IJs derived from insects infected with the parental or NMI1 strain contained an average of 211 and 227 CFU/IJ, respectively. Interestingly, the level of colonization of IJs derived from infected insects was consistently greater than when the IJs were grown on bacterial lawns. By 118 days, an average of 48 and 37 CFU were recovered from IJs carrying the parental1 or NMI1 strain, respectively, indicating that PixA production was not required for either colonization of or survival within the nematode in vivo.

The above in vivo assay was used to determine whether PixA was required for nematode reproduction in the insect. *M. sexta* larvae were infected with IJs containing either the parental or NMI1 strain and the number of new IJs that emerged from the insect cadaver was monitored over a 20-day period. The results from five independent experiments showed that while the timing of IJ emergence varied between experiments the number of IJs produced in insects infected with the NMI1 strain was comparable to that produced in insects infected with the parental strain. Thus, nematodes are able to grow and reproduce in insects in the presence of *X. nematophila* organisms which lack PixA.

Finally, a competitive colonization experiment was also conducted in which a 1:1 mixture of IJs carrying either the parental or NMI1 strain was used to infect *M. sexta* larvae. Under these conditions, >95% of the IJs recovered were monoclonally colonized with NMI1. Almost identical results were obtained in an in vitro competitive colonization experiment in which IJs were raised on bacterial lawns containing a 1:1 mixture of the parental and NMI1 strains (data not shown). These findings suggest that *X. nematophila* cells that do not produce the PixA crystal protein have a selective advantage for colonization of and/or maintenance within the gut vesicle of the nematode.

Concluding remarks. We show that the *pixA* gene is unique to *X. nematophila* and was highly expressed during stationary-phase independent of RpoS. Unlike the inactivation of *cipA* in *Photorhabdus luminescens*, inactivation of *pixA* did not produce a pleiotropic phenotype and the *pixA* strain was able to support nematode reproduction and colonization. These finding raise several intriguing questions: What is the mechanism of stationary-phase regulation of *pixA*? What is the nature of the differences between the inactivation of inclusion body genes in *Xenorhabdus* and *Photorhabdus* spp.? What is the function of PixA in the life cycle of *X. nematophila*?

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REFERENCES

1. **Akhurst, R. J.** 1980. Morphological and functional dimorphism in Xenorhabdus spp. bacteria symbiotically associated with the insect pathogenic nematodes Neoaplectana and Heterorhabditis. J. Gen. Microbiol. **121:**303–309.
2. **Akhurst, R. J., and N. E. Boemare.** 1990. Biology and taxonomy of *Xenorhabdus*, p. 75–90. *In* R. Gaugler and H. K. Kaya (ed.), Entomopathogenic nematodes in biological control. CRC Press, Boca Raton, Fla.
3. **Alexceyev, M. F.** 1999. The pKnock series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. BioTechniques **26:**824–828.
4. **Bintrim, S. B., and J. C. Ensign.** 1998. Insertional inactivation of genes encoding the crystalline inclusion proteins of *Photorhabdus luminescens* results in mutants with pleiotropic phenotypes. J. Bacteriol. **180:**1261–1269.
5. **Bird, A. F., and R. J. Akhurst.** 1983., The nature of the intestinal vesicle in nematodes of the family *Steinernematidae.* Int. J. Parasitol. **16:**511–518.
6. **Boemare, N. E., and R. J. Akhurst.** 1988. Biochemical and physiological characterization of colony form variants in *Xenorhabdus spp.* (Enterobacteriaceae). J. Gen. Microbiol. **134:**751–761.

7. **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.
8. **Bowen, D. J., and J. C. Ensign.** 2001. Isolation and characterization of intracellular protein inclusions produced by the entomopathogenic bacterium *Photorhabdus luminescens.* Appl. Environ. Microbiol. **67:**4834–4841.
9. **Caetano-Anolles, G.** 1993. Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl. **3:**85–94.
10. **Couche, G. A., P. R. Lehrach, R. G. Forage, G. C. Cooney, D. R. Smith, and R. P. Gregson.** 1987. Occurrence of intracellular inclusions and plasmids in *Xenorhabdus spp.* J. Gen. Microbiol. **133:**967–973.
11. **Couche, G. A., and R. P. Gregson.** 1987. Protein inclusions produced by the entomopathogenic bacterium *Xenorhabdus nematophilus* subsp. *nematophilus*. J. Bacteriol. **169:**5279–5288.
12. **Forst, S., B. Dowds, N. Boemare, and E. Stackebrandt.** 1997. *Xenorhabdus* spp. and *Photorhabdus* spp.: bugs that kill bugs. Annu. Rev. Microbiol. **51:**47–72.
13. **Forst, S., and D. Clarke.** 2002. Bacteria-nematode symbiosis, p. 57–78. *In* R. Gaugler (ed.), Entomopathogenic nematology. CABI Publishing, Wallingford, United Kingdom.
14. **Martens, E. C., K. Heugens, 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.
15. **Poinar, G. O.** 1966. Significance of *Achromobacter nematophilus* Poinar and Thomas (Achromobacteraceae: Eubacteriales) in the development of the nematode, DD-136. (Neoaplectana sp. Steinernematidae). Parasitology **56:** 385–390.
16. **Quandt, J., and M. F. Hynes.** 1983. Versatile suicide vectors which allow direct selection for gene replacement in Gram-negative bacteria. Gene **127:** 15–21.
17. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1:**784–791.
18. **Skanter, A, M., and L. K. Carta.** 2000. Amplification of Hsp90 homologs from plant-parasitic nematodes using degenerate primers and ramped annealing PCR. BioTechniques **29:**1182–1186.
19. **Vivas, E., and H. Goodrich-Blair.** 2001. *Xenorhabdus nematophila* as a model for host-bacteria interactions: *rpoS* is necessary for mutualism with nematodes. J. Bacteriol. **183:**4687–4693.
20. **Volgyi, A., A. Fodor, A. Szentirmai, and S. Forst.** 1998. Phase variation in *Xenorhabdus nematophilus.* Appl. Environ. Microbiol. **64:**1188–1193.
21. **Volgyi, A., A. Fodor, and S. Forst.** 2000. Inactivation of a novel gene produces a phenotypic variant cell and affects the symbiotic behavior of *Xenorhabdus nematophila.* Appl. Environ. Microbiol. **66:**1622–1628.
22. **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, Ark.