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 nema-
todes 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 protein inclusion of *Xenorhabdus* (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
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 (cobUSC7), which encodes enzymes for cobalalmine (vitamin B₁₂) 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).

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 transferring into strain ATCC 19061. Disruption of pixA in candidate mutant strains that were resistant to chloramphenicol and had the pixA-containing 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
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,
indicates 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 within the nematode 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. The results between the inactivation of inclusion body genes in X. nematophila and Photorhabdus spp. bacteria symbiotically associated with the insect pathogenic nematodes Neoaplectana and Heterorhabditis. J. Gen. Microbiol. 124:503–509.

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 findings 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


