Phase Variation in *Xenorhabdus nematophilus*

ANTONIA VOLGYI,^{1,2} ANDRAS FODOR,² ATTILA SZENTIRMAI,³ AND STEVEN FORST^{1*}

*Department of Biological Sciences, University of Wisconsin, Milwaukee, Wisconsin 53201,¹
and Department of Genetics, Eotvos Lorand University, Budapest,² and Department of
Microbiology, Kossuth Lajos University, Debrecen,³ Hungary*

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Xenorhabdus nematophilus is a symbiotic bacterium that inhabits the intestine of entomopathogenic nematodes. The bacterium-nematode symbiotic pair is pathogenic for larval-stage insects. The phase I cell type is the form of the bacterium normally associated with the nematode. A variant cell type, referred to as phase II, can form spontaneously under stationary-phase conditions. Phase II cells do not elaborate products normally associated with the phase I cell type. To better define phase variation in *X. nematophilus*, several strains (19061, AN6, F1, N2-4) of this bacterium were analyzed for new phenotypic traits. An analysis of pathogenicity in *Manduca sexta* larvae revealed that the phase II form of AN6 (AN6/II) was significantly less virulent than the phase I form (AN6/I). The variant form of N2-4 was also avirulent. On the other hand, F1/II and 19061/II were as virulent as the respective phase I cells. Strain 19061/II was found to be motile, and AN6/II regained motility when the bacteria were grown in low-osmolarity medium. In contrast, F1/II remained nonmotile. The phase II cells did not produce the outer membrane protein, OpnB, that is normally induced during the stationary phase. Both phase I and phase II cells were able to support nematode growth and development. These findings indicate that while certain phenotypic traits are common to all phase II cells, other characteristics, such as virulence and motility, are variable and can be influenced by environmental conditions.

Xenorhabdus nematophilus is a symbiotic and pathogenic bacterium belonging to the family *Enterobacteriaceae* (1, 2, 10, 12, 24, 25). It is harbored as a symbiont in a specialized intestinal vesicle of the infective juvenile stage of the nematode *Steinernema carpocapsae* (2). The bacteria are carried into susceptible insect larvae by the nematode and are subsequently released into the insect hemolymph, where they participate in the killing of the insect host (2, 20, 21, 25, 32). *X. nematophilus* proliferates within the hemolymph and eventually enters the stationary phase of its life cycle. During the stationary phase, *X. nematophilus* secretes several products, including broad-spectrum antibiotics, which hinder the multiplication of other microorganisms in the insect cadaver. *X. nematophilus* has not yet been shown to exist as a free-living organism in the soil environment. The symbiotic association with the nematode may enable the bacterium to survive outside the insect host. The bacteria, in turn, are essential for effective killing of the insect larvae and are required for the nematode to efficiently complete its life cycle, which involves developing into an infective juvenile stage. At this stage, the nematode-bacterium symbiotic pair leaves the insect cadaver in search of a new host.

The form of the bacterium that is normally isolated from the symbiotic nematode is referred to as phase I. A characteristic feature of phase I cells is the ability to bind specific dyes, such as bromothymol blue. During the stationary phase, the phase I cells produce proteases, phospholipases, antibiotics, and protoplasmic paracrystalline inclusions composed of crystal proteins (9, 10, 16, 17). The amino acid compositions and molecular weights of the crystal proteins of *Xenorhabdus* have been determined (16). The closely related symbiotic bacterium *Photorhabdus luminescens* also produces crystal proteins (8). However, the molecular properties of the crystal proteins of the two

bacteria are distinctly different, suggesting that the genes encoding these proteins were laterally acquired from disparate genetic origins (25). A variant form of *Xenorhabdus* spp. can arise spontaneously when the bacteria exist under nongrowing conditions. This so-called phase II cell type does not bind dye and produces markedly reduced levels of the stationary-phase products. While the phase II cells lack intracellular inclusion bodies (1, 2), production of the crystal proteins has not been directly analyzed in the variant forms of *X. nematophilus*. Phase variation in the genus *Xenorhabdus* apparently does not involve DNA rearrangement (5, 11, 15, 22, 24) and occurs in all five species of this genus (24, 37). It also occurs in *Photorhabdus luminescens* (10, 24). It has been proposed that phase II cells may be able to adapt to conditions in the soil and therefore represent a free-living form of *X. nematophilus* (35a). However, it is difficult to detect phase II cells in soil samples because they lack numerous phenotypic traits that are characteristic of the genus. Since phase variants appear when the bacteria are in a nongrowing state, the phase II cell type may also be a form that can multiply in larval-stage insects that provide suboptimal growth conditions (1). Phase I and phase II forms have been shown to be equally pathogenic for larvae of the greater wax moth, *Galleria mellonella* (2, 3). In contrast, the number of infective juvenile nematodes obtained from *G. mellonella* larvae infected with phase II cells was significantly lower than the number obtained from larvae infected with phase I cells (3).

Givaudan et al. showed that phase I cells of strain F1 were able to swarm on the surface of Luria-Bertani (LB) agar, while the phase II variant (strain F1/II) was nonmotile (29). In strain F1 the *fliCD* genes, which encode flagellar proteins, were expressed in phase I cells but not in phase II cells (29). It was also shown that cloned *fliCD* genes of phase I cells could not restore motility when transferred into phase II cells (30). Phase II cells of several *Xenorhabdus* species were also shown to be incapable of fimbrial production (7, 14). In addition, in the phase I form of strain AN6, but not in the phase II variant, the outer membrane protein, OpnB, was found to be induced

* Corresponding author. Mailing address: Department of Biological Sciences, University of Wisconsin, P.O. Box 413, Milwaukee, WI 53201. Phone: (414) 229-6373. Fax: (414) 229-3926. E-mail: sforst@csd.uwm.edu.

FIG. 1. Production of crystal proteins in *X. nematophilus*. Crystal proteins were isolated and separated by SDS-PAGE as described in Materials and Methods. Bacteria were grown to either the mid-logarithmic phase (lanes 1, 3, 5, 7, 9, 11, 13, and 15) or the stationary phase (lanes 2, 4, 6, 8, 10, 12, 14, and 16). The inclusion body proteins derived from 10^9 phase I cells are shown in lanes 1, 2, 5, 6, 9, 10, 13, and 14. The proteins derived from 10^9 phase II cells are shown in lanes 3, 4, 7, 8, 11, and 12. The crystal proteins obtained from N2-4/IV are shown in lanes 15 and 16.

under stationary-phase conditions (27, 33). However, it was not known whether all strains of *X. nematophilus* produced OpnB under stationary-phase conditions and whether their respective phase II cells were unable to produce this protein. In order to more thoroughly define phase variation in *X. nematophilus*, numerous phenotypic characteristics of different strains of this bacterium were examined in the present study.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The following *X. nematophilus* strains were used in this study: AN6/I (= ATCC 19061) (15) and AN6/II (from R. J. Akhurst); ATCC 19061/I and ATCC 19061/II (from R. E. Hurlbert [36]); F1/I and F1/II (from N. Boemare [10]); and N2-4/I and the variant form N2-4/IV (see below), which were provided by E. Szallas and A. Szentirmai. Bacteria were maintained on LB agar containing 60 μ g of ampicillin per ml, 0.0025% bromothymol blue, and 0.004% triphenyltetrazolium chloride (Sigma Chemical Co.). Phase I cells bind bromothymol blue to form blue colonies on this medium, while phase II cells remain red (10). Strain N2-4/IV was isolated as a red colony and originally produced less antibiotic than strain N2-4/I. In the present study, it was found that N2-4/IV was not a phase II variant but remained red on dye-binding plates. Strain AN6 is equivalent to strain ATCC 19061 (15); however, ATCC 19061/II was originally isolated in the laboratory of R. Hurlbert, while strain AN6/II was isolated by R. Akhurst. Hereafter, strain ATCC 19061 will be referred to simply as strain 19061. While the phase II form of strain F1 has been studied previously (10, 29), strains 19061/II and AN6/II have not been described previously. Finally, strains N2-4 and N2-4/v have not been described previously. Cultures of *X. nematophilus* were grown in LB medium containing 60 μ g of ampicillin per ml at 30°C in sidearm flasks (250 ml). Cells were also grown in Grace's insect cell culture medium (Gibco). The *Micrococcus luteus* strain used to assay antibiotic production was grown on LB agar plates. Unless otherwise stated, all growth media were obtained from Difco Laboratories. Sarkosyl was obtained from Sigma Chemical Co.

Nematode strain, media, and preparation of axenic nematode eggs. The nematode strain used in this study was *Steinernema carpocapsae* All (Biosys). To grow nematodes in vitro, the *X. nematophilus* strains were first grown on oily agar plates (16 g of nutrient broth, 5 g of yeast extract, 5 g of commercially available vegetable oil, 15 ml of NaPO₃ buffer [pH 7.0], and 15 g of Bacto Agar [Difco] in a final volume of 1 liter). Axenic nematode eggs were placed on the bacteria and incubated at room temperature. To obtain axenic eggs, the following procedure was used. Adult nematodes were washed twice with distilled water and then placed in a solution containing 6.75 ml of distilled water, 2 ml of Clorox, and 1.25 ml of 5 N NaOH. The nematodes were incubated at room temperature until they disappeared (about 5 min), and the eggs, which were resistant to the base solution used, were pelleted by centrifugation at room temperature for 2 min at 900 $\times$ g with a Marathon model 21K/BR centrifuge (Fisher Scientific). The eggs were washed once with sterile distilled water and once with a physiological salt solution. To maintain the infective juvenile nematodes, the nematodes were grown on a bacterial lawn on an oily agar plate.

Swarming motility on soft agar. LB agar containing 0.5% Bacto Agar, 60 μ g of ampicillin per ml, 0.0025% bromothymol blue, and 0.004% triphenyltetrazolium chloride was used to observe swarming motility (29). The plates were dried at room temperature. The various strains of *X. nematophilus* were grown in Grace's medium for 24 h, and 1- μ l portions of the bacterial cultures were placed on the swarm plates. The plates were incubated at 30°C and continually observed for swarming motility.

Antibiotic production, protease activity, and hemolysis. Antibiotic activity was tested by previously described methods (4, 19). Briefly, 1 μ l of a 24-h bacterial culture was placed on an LB agar plate and then incubated for 48 h. The bacteria were then exposed to chloroform fumes for 30 min and air dried for 30 min. *Micrococcus luteus* (25 μ l of an overnight culture; $\sim 10^9$ cells/ml) was added to 6 ml of top agar, which was poured over the bacterial colony, and zones of growth inhibition were observed. Hemolysis was determined with standard sheep blood agar plates (9). Protease activity was measured by the gelatin plate assay as described previously (9). The lack of this activity in strains AN6/II and 19061/II was observed previously by R. Akhurst and R. Hurlbert, respectively.

Electron microscopy and phase-contrast microscopy. Negative staining for electron microscopy was accomplished as follows. Cells were grown in either LB medium or LB medium without NaCl to the mid-logarithmic stage, and 200 μ l of cells was pelleted, washed once in 400 μ l of 0.1 $\times$ Grace's medium, pelleted again, and resuspended in 400 μ l of 0.1 $\times$ Grace's medium. Then 3 μ l of the culture was placed on a 400-mesh grid, air dried for 2 min, and gently dabbed with Whatmann filter paper. The bacteria were stained with 1 drop of 0.2% uranyl acetate for 30 s and dried with Whatmann filter paper. Swimming motility was observed with an Olympus light microscope equipped with phase optics (magnification, $\times 40$).

SDS-polyacrylamide gel analysis of crystal proteins. A simple centrifugation protocol was developed in this study to isolate protein inclusion bodies. Bacterial cells were grown in LB medium and harvested either during mid-logarithmic growth or at the stationary phase. The cell pellets were washed once with fresh growth medium and resuspended in 200 μ l of 20 mM sodium phosphate buffer (pH 7.1). Cells were disrupted by sonification with a Branson Sonifier over a period of 10 min (2.5-min bursts at 120 W). The disrupted cells were centrifuged for 10 min at 15,800 $\times$ g, the pellet was solubilized in sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min, and the proteins were separated by electrophoresis with an SDS-15% polyacrylamide gel system.

Analysis of outer membrane proteins. Cells were harvested at either the mid-logarithmic phase or the stationary phase. Outer membrane proteins were extracted with Sarkosyl as described previously (26, 33). Briefly, total membrane pellets, prepared as described above, were incubated for 30 to 60 min in 0.5% Sarkosyl-20 mM phosphate buffer. The outer membrane proteins were collected by centrifugation for 14 min at 353,000 $\times$ g with a model TL100 ultracentrifuge (Beckman Instruments). The resulting membrane pellets were solubilized in 40 μ l of SDS sample buffer (28) and boiled for 5 min, and the proteins were separated by electrophoresis with a urea-SDS-polyacrylamide gel electrophoresis (PAGE) system.

In vivo pathogenicity assay. *Manduca sexta* larvae were reared at 26°C on an artificial diet (27) by using a 16 h of light-8 h of darkness cycle. The various *Xenorhabdus* strains were grown to the mid-exponential phase in Grace's medium and diluted in Grace's medium, and various amounts of bacteria were injected into four to six individual larvae per experiment, as described previously (27). Control larvae were injected with Grace's medium. Bacterial concentrations were determined by dilution plating on LB agar containing bromothymol blue (LBTA) plates. The growth-inhibitory effect was monitored by weighing the larvae at regular intervals. The LT₅₀ (time at which 50% of the injected larvae had died) was calculated for each bacterial strain tested. Experiments were repeated at least three times. The results of a representative experiment are shown below.

Nematode production. Bacteria were grown in Grace's medium for 24 h, and a 100- μ l aliquot was placed in the center of a petri dish (diameter, 6 cm) containing 15 ml of oily agar. The bacteria were grown for 24 h, enough time to allow a large colony to form. Then axenic eggs (ca. 2,000 eggs) were placed on the bacterial colony. After 14 days, the 6-cm-diameter petri dish was placed into a 10-cm-diameter petri dish, and 10 ml of tap water was added to the larger dish. The dauer juveniles climbed out of the smaller dish into the water of the larger dish, where they could be counted over time with a Bausch & Lomb dissecting microscope.

RESULTS

Crystal protein production. As shown in Fig. 1, crystal proteins were not produced during log-phase growth (Fig. 1, lanes 1, 5, and 9) of the phase I cells but were produced during stationary-phase growth (lanes 2, 6, and 10). Both the 22- and 26-kDa proteins could be identified by SDS-PAGE gel analysis. The quantities of the crystal proteins obtained from different strains appeared to differ; F1 produced larger amounts of the crystal proteins (lane 10), while strains AN6 and 19061 produced lower amounts of these proteins (lanes 2 and 6, respectively). In contrast, none of the phase II cells produced crystal proteins during stationary-phase growth (lanes 4, 8, and

TABLE 1. Phenotypic characteristics of phase I, phase II, and variant forms of *X. nematophilus* strains^a

Strain	Phase	Dye binding	Crystal protein	Antibiotics	Motility			OpnB	Virulence	Nematode production
					Soft agar	LB medium	LB medium without NaCl			
AN6	I	B	+	+	+	+	+	+	+	+
	II	R	—	—	—	—	+	—	—	+
F1	I	B	+	+	+	+	+	+	+	+
	II	R	—	—/+	—	—	—	—	+	+
19061	I	B	+	+	+	+	+	+	+	+
	II	R	—	—	—	+	+	—	+	+
N2-4	I	B	+	+	+	+	+	+	+	+
	Iv	R	+	+	+	+	+	+	—	+

^a B, blue; R, red; +, characteristic apparent; —, characteristic not apparent; —/+, characteristic weakly present. The assays used to determine individual characteristics are described in Materials and Methods.

12). We also found that both the phase I form (lane 14) and the variant form (lane 16) of strain N2-4 produced crystal proteins. Since the N2-4 variant form possessed properties that are normally absent in phase II cells (Table 1), it was designated N2-4/Iv (variant form) rather than a phase II form. When the various strains were grown in Grace's insect medium, intracellular inclusion bodies were difficult to see in the phase I cells when phase-contrast microscopy was used, but the crystal proteins were detected by the SDS-PAGE gel analysis (data not shown).

Motility on soft agar and in liquid culture. The phase I cells were motile on LB agar (Fig. 2A and C), while the phase II cells were not motile (Fig. 2B). The N2-4/Iv cells were found to be motile (Fig. 2C). Surprisingly, AN6/II exhibited motility on LB agar plates when the final concentration of NaCl in the medium was reduced to less than 0.2%. This suggested that the motility of phase II cells might be a variable trait that is influenced by environmental conditions. Phase II cells were examined for motility in LB liquid medium by phase-contrast microscopy. Unexpectedly, we repeatedly observed that 19061/II was able to swim in LB liquid medium while AN6/II and F1/II remained nonmotile (Table 1). Furthermore, when the AN6/II cells were grown in LB liquid medium lacking added NaCl, motility was clearly observed (Table 1).

To assess the production of flagella in AN6/II grown in low-salt medium, an electron microscopic analysis was conducted. AN6/I cells grown in normal LB medium produced peritrichous flagella (Fig. 3A), but AN6/II cells grown in LB

medium did not (Fig. 3B). However, when AN6/II cells were grown in LB medium lacking NaCl, cellular appendages that resembled thin flagella were clearly visible (Fig. 3C).

Outer membrane protein production. Figure 4 shows that in all four *Xenorhabdus* strains OpnB was produced when cells were maintained under stationary-phase conditions (Fig. 4, lanes 1, 4, 8, and 12). In contrast, OpnB was not produced during the stationary phase in the phase II cells of strains AN6, 19061, and F1 (lanes 2, 6, and 10, respectively). N2-4/Iv did produce OpnB under stationary-phase conditions (lanes 12 and 14).

An analysis of other outer membrane proteins in the various strains of *X. nematophilus* revealed considerable differences in the production of specific proteins. For example, OpnS was produced only in AN6/I and 19061/I grown to the stationary phase (Fig. 4, lanes 1 and 4, respectively), while in F1/I and N2-4/I OpnS was produced in both log-phase cells (lanes 7 and 11) and stationary-phase cells (lanes 8 and 12). In addition, a new protein that migrated slightly slower than OpnS was detected in strains F1 and N2-4. This protein was designated OpnN.

Pathogenicity. Table 2 shows that 20 cells of AN6/I killed fifth-instar *Manduca sexta* larvae, with an LT₅₀ of 29 h. All six individuals injected died within 44 h. In contrast, injection of 100 cells of AN6/II did not kill the larval host, and injection of 400 cells killed only 50% of the larvae, with an LT₅₀ of 85 h. These results represent the first reported demonstration of significant differences in the virulence properties of phase I

FIG. 2. Motility on 0.5% agar. Agar plates were dried, and 1 μ l of each bacterial culture was placed on the agar surface. (A) Phase I cells. Colony 1, AN6; colony 2, F1; colony 3, 19061. (B) Phase II cells. Colony 1, AN6; colony 2, F1; colony 3, 19061. (C) Strain N2-4. Colony 1, AN6/II; colony 2, N2-4/Iv; colony 3, N2-4/I.

FIG. 3. Electron micrographs of AN6/I (A), AN6/II (B), and AN6/II grown in the absence of added NaCl (C). (A) Bar = 2 μ m. (B and C) Bar = 1 μ m.

and phase II cells of *X. nematophilus*. N2-4/Iv was shown to be avirulent for *Manduca sexta* larvae; injection of 320 cells did not kill the larval host. Thus, while N2-4/Iv was similar to phase I cells with respect to many phenotypic characteristics, it appeared to be missing an essential characteristic(s) required for pathogenicity for *Manduca sexta*. On the other hand, strains F1/II and 19061/II were similar in virulence to their respective phase I cells.

In vitro growth of axenic eggs of *Steinernema carpocapsae*. The production of infective juvenile nematodes raised on either phase I or phase II cells was studied with an in vitro system. Sterile nematode eggs were placed on different bacterial lawns, and the cumulative production of infective juvenile nematodes was monitored over a 60-day period. The results shown in Fig. 5 indicate that the nematodes grew and developed equally well on phase I and phase II cells. AN6/I and AN6/II were reisolated from the respective nematodes and were assessed for crystal protein and OpnB production. The bacteria isolated from nematodes grown on phase I cells produced both crystal proteins and OpnB, while the bacteria isolated from nematodes grown on phase II cells produced neither of these types of proteins (data not shown). This result indicated that the phase II variant was retained by the nematode and did not revert to phase I. The nematodes were unable to grow on heat-killed AN6/I (data not shown), indicating that viable bacteria were essential for growth. *Steinernema carpocapsae* could grow and develop efficiently on a lawn of *Escherichia coli* (23; data not shown) but could not grow on *Photobacterium luminescens*, suggesting that the latter bacterium may produce a specific nematocidal toxin.

Antibiotic production, hemolysis, and protease activity. Phase I cells of several strains of *X. nematophilus*, such as F1, were shown previously to produce antibiotics and proteases and to cause hemolysis of sheep erythrocytes, while phase II cells were deficient in these activities (1–3, 9, 12). This was found to be the case for strains AN6 and 19061 (Table 1 and data not shown). In contrast, N2-4/Iv cells were able to produce antibiotics (Table 1) and protease and caused hemolysis on blood agar plates (data not shown).

DISCUSSION

An important question regarding the possible biological significance of phase variation is whether phase II variants are pathogenic for insect larvae. In the present study, we show that AN6/II and N2-4/v were markedly less virulent towards *Manduca sexta* than the respective phase I cells. It is not known whether the reduced virulence of the phase II cells toward *Manduca sexta* results from the loss of a single property or from changes in several properties of the bacterium. It is conceivable that *X. nematophilus* secretes a potent insect toxin and that avirulent phase II cells are defective in the production and/or secretion of the putative toxin. Recently, a gene from *X. nematophilus* that encodes a putative protein toxin has been identified (25). Furthermore, a high-molecular-weight protein toxin (molecular weight, >900,000), consisting of several protein subunits ranging in molecular weight from 23,000 to 200,000, has been identified in *Photobacterium luminescens* (13). Alternatively, reduced virulence could be due to an inability of phase II cells to adapt to the host environment. For example, it was recently shown that *X. nematophilus* produces hydroxybutanoyl homoserine lactone and that avirulent strains are unable to produce this quorum-sensing molecule (19). AN6/II and N2-4/Iv may be defective in the production of hydroxybutanoyl homoserine lactone. In any event, further studies on phase II cells should increase our understanding of the factors contributing to the insect pathogenicity of *X. nematophilus*. The extreme potency of *X. nematophilus* as an insect pathogen is illustrated by the fact that injection of 10 cells of AN6/I was sufficient to kill fifth-instar larvae of *Manduca sexta* (27), while the larvae were able to survive injection of large numbers (>10⁵ cells) of other enteric bacteria (18). The *X. nematophilus*-nematode system is presently being explored to determine its effectiveness as a bioinsecticidal agent (25).

While strains 19061 and AN6 are equivalent, the virulence properties of the respective phase II forms differed markedly in the *Manduca sexta* virulence assay system. It is not clear whether the observed differences were present when the phase II cells were originally isolated or whether the reduced viru-

FIG. 4. Outer membrane proteins separated on a urea-SDS-polyacrylamide gel. The proteins produced during the mid-logarithmic phase are shown in lanes 3, 5, 7, 9, 11, and 13. The proteins produced during the stationary phase are shown in lanes 1, 2, 4, 6, 8, 12, and 14. The phase I cell products are shown in lanes 1, 3, 4, 7, 8, 11, and 12. The outer membrane proteins derived from phase II cells are shown in lanes 2, 5, 6, 8, 9, and the outer membrane proteins derived from N2-4/Iv are shown in lanes 13 and 14. The various outer membrane proteins are represented by A, B, S, T, N, and P.

TABLE 2. Pathogenicity of *X. nematophilus* strains: injection of bacteria into the hemocoels of fifth-instar *Manduca sexta* larvae

Strain	Phase	No. of bacteria injected	Avg wt of injected <i>Manduca sexta</i> larvae (g)	LT ₅₀ (h)	Mortality ^a	Time of death of last larvae (h)
AN6	I	20	1.85	29	6/6	44
	II	10	1.59		1/5	
	II	100	1.73		1/5	
	II	400	1.77	85	3/6	
F1	I	250	2.00	30	4/4	30
	II	280	2.09	29	4/4	30
19061	I	260	2.15	34	4/4	46
	II	270	1.95	29	4/4	32
N2-4	I	250	1.87	29	5/6	34
	Iv	320	1.35		1/6	

^a Number of larvae that died/number of larvae injected.

lence of AN6/II arose subsequently, possibly during storage and repeated passaging of the cells. These findings point to the importance of establishing standard assays and criteria for characterizing the phase II phenotype of *X. nematophilus* (9) and for monitoring whether the phenotype changes over time.

It was shown that 19061/II was motile in liquid medium, while AN6/II and F1/II were nonmotile. While strain 19061/II was able to swim in liquid media, it was unable to swarm on agar. A similar motility phenotype has been observed recently in *Proteus mirabilis*, in which inactivation of the *flgN* gene resulted in the loss of swarming but not swimming motility (31). It was proposed that FlgN increases the efficiency of flagellar filament assembly in *Proteus mirabilis*. In the genus *Proteus*, it is the hyperflagellation of the swarmer cell type that accounts for the ability of the organisms to swarm (31). Perhaps 19061/II is able to produce sufficient flagella for swimming, but not for swarming. The finding that motility was stimulated in AN6/II grown under low-osmolarity conditions indicated that the regulatory and structural genes required for flagellin synthesis were not lost or irreversibly inactivated in this strain. One possible explanation for the stimulation of flagellar synthesis in AN6/II is enhanced production of the master flagellar regulatory complex, FlhDC, under low-osmo-

larity conditions (27, 34, 35). Finally, it has been proposed that strain F1/II is missing one or more positively acting regulatory factors that are required for flagellar synthesis (30). In *Shigella dysenteriae* and *Shigella flexneri*, lack of motility was apparently due to insertion of an IS1 element into the *flhDC* operon of these bacteria (6). It will be interesting to determine whether a similar phenomenon has occurred in strain F1/II. Taken together, these results indicate that in phase II cells motility is a variable trait that can be influenced by environmental conditions. However, all phase II cells lacked the ability to swarm on an agar surface (29; this study).

In the present study we show that axenic eggs of *Steinernema carpocapsae* not only grew to the adult stage on phase II cells of *X. nematophilus*, but were able to develop into infective juvenile nematodes. Nematodes were unable to grow on heat-killed bacteria. These in vitro results indicate that viable phase II cells provide a nutrient base that permits efficient nematode development. However, the situation in vivo is quite different. It has been reported previously that low numbers of infective *Steinernema carpocapsae* juveniles were produced in *G. mellonella* larvae infected with phase II cells of *X. nematophilus* (4). This difference may reflect an in vivo requirement for the stationary-phase products, such as protease and crystal proteins, that are produced by phase I cells but not by phase II cells.

We show that OpnB and crystal proteins were not produced in phase II cells. These phenotypic traits, together with the lack of dye binding and swarming, the inability to produce antibiotic and protease activity, and the absence of hemolysis (9), can be used to define phase variant forms of *X. nematophilus*. In addition, the results of this study show that phase variation can affect the virulence properties of *X. nematophilus*, that phase II cells can be motile, and that *Steinernema carpocapsae* nematodes develop in vitro to the same extent on phase I and phase II cells. While phase variation occurs in all *Xenorhabdus* species, the molecular mechanism and biological significance of this phenomenon remain unknown. These questions should provide a fertile area of research for future studies on *Xenorhabdus* spp.

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FIG. 5. Cumulative production of infective juvenile nematodes grown on *X. nematophilus* strains. Data points represent average values from triplicate samples. The experiment was repeated twice with very similar results.

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