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Role of the Histidine Kinase, EnvZ, in the Production of Outer Membrane Proteins in the Symbiotic-Pathogenic Bacterium *Xenorhabdus nematophilus*

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We show that inactivation of *envZ*, the gene encoding the histidine kinase sensor protein, EnvZ, of *Xenorhabdus nematophilus*, affected the production of several outer membrane proteins (Opns). *X. nematophilus* produced five major Opns during exponential growth. Insertional inactivation of *envZ* led to a decrease in the production of OpnP, the OmpF-like pore-forming protein which constitutes approximately 50% of the total outer membrane protein in *X. nematophilus*. OpnA production was also reduced, while the remaining Opns were produced normally. During the transition to stationary phase, three new outer membrane proteins, OpnB, OpnS, and OpnX, were induced in the wild-type strain. The *envZ*-minus strain, ANT1, did not produce OpnB and OpnX, while OpnS was induced at markedly reduced levels. These results suggest that EnvZ was required for the high-level production of OpnP during exponential growth and may be involved in the production of OpnB, OpnS, and OpnX during stationary-phase growth. We also show that ANT1 was more pathogenic than the wild-type strain when as few as five cells were injected into the hemolymph of the larval stage of the tobacco hornworm (*Manduca sexta*). The larvae died before significant numbers of bacteria were detectable in the hemolymph. These results are discussed in relation to the role of EnvZ in the life cycle of *X. nematophilus*.

Xenorhabdus nematophilus is a gram-negative bacterium belonging to the family *Enterobacteriaceae* (1, 2, 8, 12). It is harbored as a symbiont in a specialized intestinal vesicle of the entomopathogenic nematode *Steinernema carpocapsae* (4, 7, 21). The bacteria are carried into the susceptible insect larvae by the nematode and are subsequently released into the insect hemolymph, where they participate in the killing of the insect host (1). *X. nematophilus* proliferates within the hemolymph and eventually enters the stationary phase of its life cycle. During stationary-phase growth in culture, *X. nematophilus* secretes several products, including broad-spectrum antibiotics, which inhibit multiplication of other microorganisms in the insect cadaver (12, 17, 19). Since *X. nematophilus* apparently does not exist as a free-living organism in the soil environment, the symbiotic association with the nematode would enable the bacteria to survive outside the insect host (1). The bacteria, in turn, are essential for the effective killing of the larvae and are required for the nematode to efficiently complete its life cycle.

In the hemolymph, *X. nematophilus* encounters the cellular and humoral antibacterial defense systems of the host (9). During the early phase of the infection the larval hemocytes become activated (10). *X. nematophilus* adheres to the activated hemocytes, which in turn aggregate, forming nodules that function to entrap the infecting bacteria (9). *X. nematophilus* eventually escapes the defense systems of the host and ultimately grows to high concentrations in the hemolymph. Since the cell surface components of *X. nematophilus* are likely to play an important role in the survival of the bacteria within the insect hemolymph (15), we have begun to characterize the outer membrane proteins of this bacterium (16). The major outer membrane protein, OpnP, was shown to share 59% amino acid sequence identity with the pore-forming protein, OmpF, of *Escherichia coli* (13). The expression of *ompF* is controlled by the osmolarity sensor protein, EnvZ, and the cytoplasmic regulatory protein, OmpR (12, 14). EnvZ modulates the levels of the phosphorylated form of OmpR in response to changes in the osmolarity of the growth environment. The nucleotide sequences of the *ompR* and *envZ* genes of *X. nematophilus* have been determined (30). OmpR proteins of *X. nematophilus* ($OmpR^{X.n.}$) and *E. coli* ($OmpR^{E.c.}$) share 78% amino acid identity. The cytoplasmic domain of the EnvZ molecules shows 57% amino acid identity, while $EnvZ^{X.n.}$ lacks the large periplasmic domain found in $EnvZ^{E.c.}$.

While OmpF is repressed by high-osmolarity conditions, the OmpF-like porin OpnP was not repressed when *X. nematophilus* was grown under high-osmolarity conditions (13). The question arises concerning the role of EnvZ in the regulation of Opn production in *X. nematophilus*. To further study this question, an *envZ*-null strain was constructed. In this report we show that $EnvZ^{X.n.}$ was required for the high-level production of OpnP in exponentially growing cells and was also involved in the stationary-phase regulation of OpnB, OpnS, and OpnX.

MATERIALS AND METHODS

Strains and plasmids. The strains used in this study were *E. coli* S17-1 (28), *X. nematophilus* AN6 (ATCC 19061) (3), and *X. nematophilus* ANT1 (constructed in this study; see below). The plasmids used in this study were pNT51 (30), pSNT1 (31), pJQ200SK (p15A replicon; *sacB* Mob^+ Gm^r [23]), pSUP106 (oriV replicon of the IncQ plasmid RSF1010; Cm^r Tc^r [22]), and pJNT7 (constructed in this study; see below).

Growth conditions. *E. coli* was routinely grown on Luria broth (LB) at 37°C as described previously (11). *X. nematophilus* strains were grown in Grace's insect cell culture medium (Gibco BRL, Gaithersburg, Md.) as previously described (13). The following antibiotics were added to LB or LB agar at the indicated concentrations: ampicillin, 50 μg/ml (Am_{50}); chloramphenicol, 25 μg/ml (Cm_{25}); and gentamicin, 30 μg/ml (Gm_{30}). AN6 and ANT1 were maintained on LB agar containing 0.004% 2,3,5-triphenyltetrazolium chloride and 0.0025% bromothymol blue (LBTA plates). Congo red containing LB agar was made by the addition of 0.01% (wt/vol) dye.

Construction of pJNT7. The interposon-inactivated *envZ*::Cm contained in pNT51 was subcloned by first digesting with *Mun*I (New England Biolabs), generating an 11.2-kb *envZ*-containing fragment. After the staggered ends were filled in with Klenow fragment, the DNA was digested with *Bam*HI, resulting in three fragments (a 5.5-kb *envZ* fragment, a 4.0-kb fragment and a 1.7-kb fragment) which were subsequently ligated into the vector, pJQ200SK, that was

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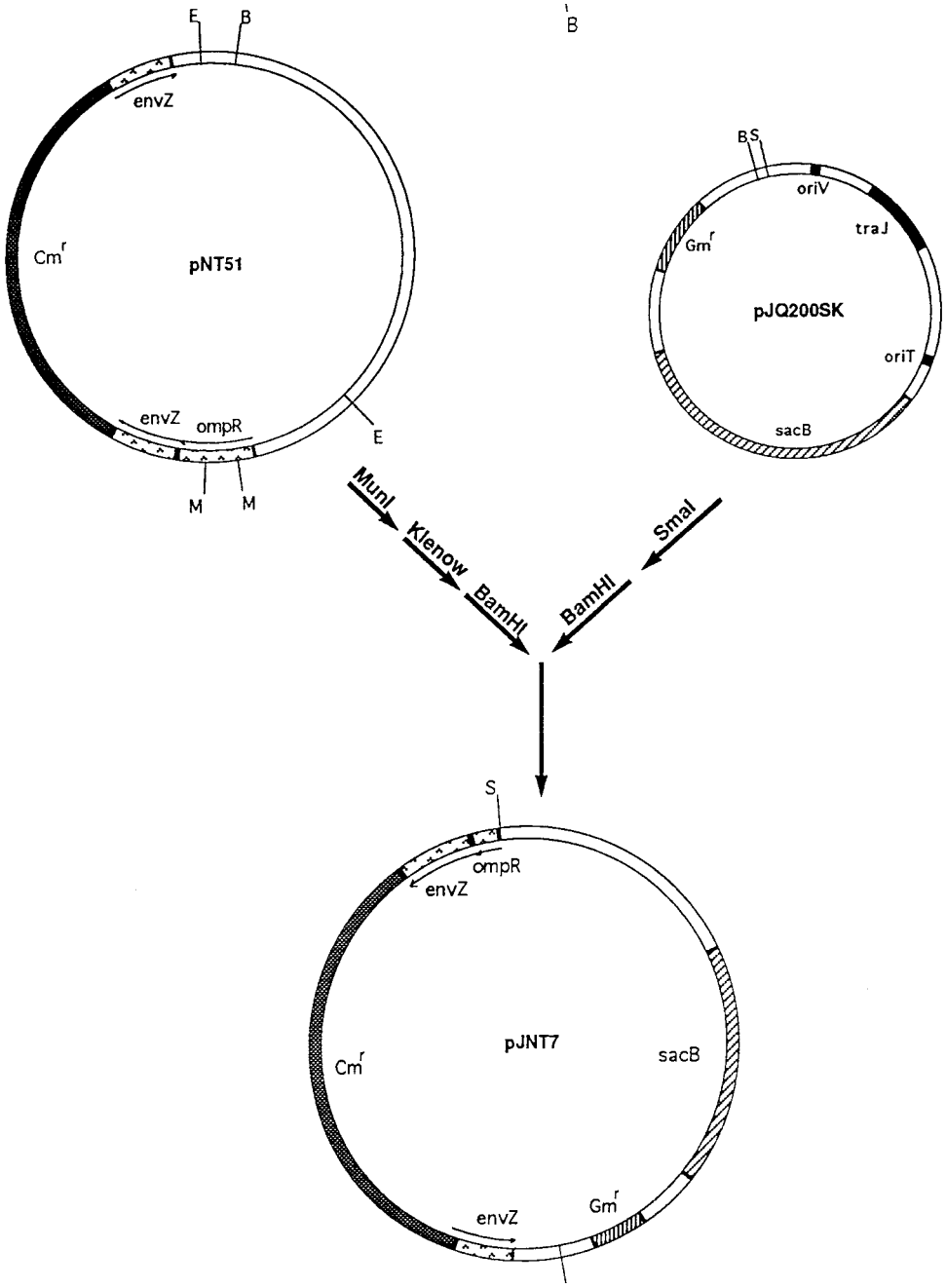

FIG. 1. Construction of pJNT7. The 5.5-kb fragment containing the insertionally inactivated *envZ* gene of pNT51 (30) was cloned into the *sacB*-containing plasmid pJQ200SK, creating pJNT7. The *traJ*, *oriV*, and *oriT* loci derived from pJQ200SK are present on pJNT7 (not shown) and allow for conjugal transfer into and replication within *X. nematophilus*. B, *Bam*HI; E, *Eco*RI; M, *Mun*I; S, *Sma*I; Cm^r and Gm^r, chloramphenicol resistance and gentamicin resistance, respectively.

previously digested with *Sma*I and *Bam*HI. The ligation reaction was used to transform *E. coli* S17, and the *envZ*-containing recombinant plasmid was isolated by plating transformed cells on Cm_{25}-Gm_{30}-containing LB agar plates. The plasmid designated pJNT7 was confirmed by restriction analysis. Unless otherwise noted, reagents were obtained from Promega, Madison, Wis.

Construction of the *envZ*-null strain, ANT1. pJNT7 contained in *E. coli* S17 was conjugally transferred into AN6 as follows. Fresh cultures of S17/pJNT7 and AN6 were grown to early log phase in Cm25Gm30 and LB, respectively. Cells were washed once in LB and resuspended in 0.5 ml of LB. Equal volumes (0.2 ml) of each strain were added together, gently mixed, placed on LB agar, and incubated overnight at 30°C. The cells were harvested, diluted in LB, and spread on $Cm_{25}Am_{50}$ agar containing 5% sucrose. Since AN6 is naturally resistant to ampicillin, this antibiotic was used to counterselect against *E. coli* S17. Cells in which the *envZ*::Cm of pJNT7 had recombined into the chromosome and had lost the plasmid would be resistant to chloramphenicol and survive in the presence of sucrose. The loss of the plasmid, pJNT7, in a putative *envZ*-null strain (ANT1) was assessed by patching onto LB agar containing $Gm_{30}Am_{50}$–5% sucrose. The strain was sensitive to gentamicin, indicating that it did not contain the plasmid. Interestingly, we found that putative *envZ*-null strains grown overnight without the 5% sucrose selection sometimes retained pJNT7, so the sucrose was included in all media used after the original conjugation. Southern blot analysis of chromosomal DNA of both AN6 and ANT1 digested with *Eco*RI confirmed the recombination of *envZ*::Cm into the chromosome of ANT1. In AN6 an *envZ* probe hybridized to the 4.5-kb *envZ*-containing DNA fragment

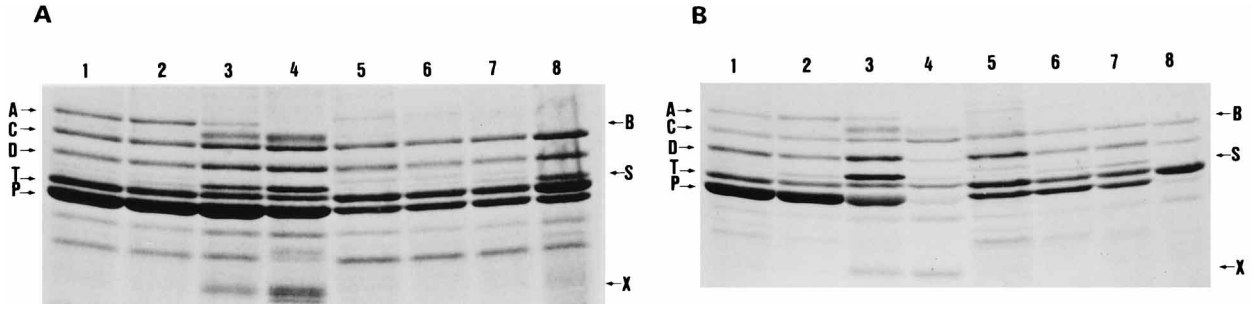

FIG. 2. Separation of outer membrane proteins of *X. nematophilus* by urea-SDS-polyacrylamide gel electrophoresis. AN6 (lanes 1 to 4) and ANT1 (lanes 5 to 8) were grown in Grace's medium at 30°C. At indicated times during growth, cells were pulse-labeled with radioactive methionine-cysteine, and the Opns were prepared and stained as described in Materials and Methods. (A) Coomassie blue-stained Opns derived from cells grown to mid-log phase (lanes 1 and 5), late log phase (lanes 2 and 6), or transition into stationary phase (lanes 3 and 7) and 24-hour stationary-phase cells (lanes 4 and 8). (B) Autoradiograph of Coomassie blue-stained gel in panel A. Opns produced during log phase growth are indicated on the left. Opns induced in cells under stationary-phase conditions are indicated on the right.

(30) while the Cm^r probe did not hybridize to this fragment. In contrast, both the *envZ* and the Cm^r probes hybridized to the same 8.8-kb fragment derived from the chromosomal DNA of ANT1.

Physiological and biochemical characterization of ANT1. Phase-contrast microscopy was used to examine bacterial size, motility, and the presence of crystalline inclusion bodies in stationary-phase cells. Protease activity was determined with both gelatin broth (25) and milk agar (3). Production of antibiotics was determined by use of *E. coli* SB221 (11), *Bacillus subtilis*, or *Micrococcus luteus* as sensitive target organisms (2). Lipase activity was evaluated on Tween 20 and Tween 80 agars (8). chromeazurol S agar plates were used to detect siderophore activity. DNase activity and erythrocyte hemolysis were determined by standard methods. Previously described methods were used to show that both AN6 and ANT1 were negative for the following properties: catalase, ability to reduce nitrate to nitrite, sucrose and lactose fermentation, methyl red, Voges-Proskauer, citrate utilization, growth on mannitol salts, ability to hydrolyze esculin, indole reaction, presence of urease, and formation of H_2S (31).

Preparation and pulse-labeling of outer membrane proteins. Forty-milliliter cultures of AN6 and ANT1 were grown in Grace's medium. During the mid-exponential growth phase 40 μCi of [^{35}S] protein labeling mix (1,000 Ci/mmol; NEN-DuPont) was added to a 15-ml aliquot of either the AN6 or the ANT1 culture. Radiolabel incorporation was continued for 2 min and was followed by the addition of 5 μM L-methionine and 20 μg of sodium azide per ml (final concentrations). The radiolabeling was also performed with cells grown to late exponential phase (7.5 ml) and cells entering the transition to stationary phase (6.0 ml). To radiolabel the 24-h stationary-phase-grown cells, 80 μCi was added for 10 min. Outer membrane proteins were prepared by sonifying cells in phosphate buffer, solubilizing the inner membrane with Sarkosyl, and harvesting the insoluble outer membrane by high-speed centrifugation (11). The outer membrane proteins were analyzed by urea-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described previously (11). The gels were stained with Coomassie brilliant blue, and the dried gel was processed for autoradiography. The autoradiographs were scanned on a GS 300 transmittance/reflectance scanning densitometer from Hoefer Scientific Instruments. Scan data was analyzed with the GS370 1-D data system, version 2.0 (Hoefer Scientific Instruments).

In vivo pathogenicity assay. Day 1 fifth-instar *Manduca sexta* larvae (ca. 2 to 3 g), reared on an artificial diet (6) on an 18:6 light/dark cycle at 26°C were fed for 24 h a natural diet of tobacco leaves. Cultures of AN6 and ANT1 grown to mid-exponential phase were diluted in Grace's medium, and various amounts of bacteria were injected into eight individual larvae per experimental condition. For injection the larva was chilled on ice for 5 min and surface sterilized with ethanol. One hundred microliters of bacterial culture was injected dorsal to the posteriormost hind limb with a 27-gauge sterile needle. Grace's medium was injected into the control larvae. Bacterial concentrations were determined both by direct counting with a Petroff-Hausser counting chamber under phase-contrast microscopy and by dilution plating on LBTA plates. The growth-inhibitory effect was monitored by weighing the larvae at regular intervals. Data collected from eight individuals per experimental condition were used to obtain mean and standard error values with a multivariate repeated-measures analysis program. To monitor the progression of infection, 5 μl of hemolymph was repetitively withdrawn from each larva and examined by both phase-contrast microscopy and plating on LBTA agar.

RESULTS

Construction of the *envZ*-null strain, ANT1. Gene replacement had not been previously reported in *X. nematophilus*. In order to create the *envZ*-null strain, we used a negative-selection plasmid that could be transferred by conjugation, since we found that the transfer of plasmids into the AN6 strain of *X. nematophilus* by either transformation or electroporation was problematic. The *envZ* gene carried on a pBR322-derived plasmid was previously inactivated by inserting a chloramphenicol resistance cassette (Cm^r) into the coding sequence of *envZ*, creating pNT51 (30) (see Fig. 1). To create the *envZ*-null strain of *X. nematophilus*, the inactivated *envZ*::Cm allele of pNT51 was first cloned into the *sacB*-containing plasmid pJQ200SK, resulting in pJNT7 (Fig. 1). The *sacB* gene encodes levansucrase, which is toxic when expressed in *X. nematophilus*, as well as in many gram-negative bacteria, grown in the presence of sucrose (33). As expected, *X. nematophilus* containing pJQ200SK could not grow in sucrose-containing medium (data not shown). We conjugally transferred pJNT7 into the wild-type strain, AN6, and subsequently selected for the homologous recombination of the *envZ*::Cm allele into the chromosome by growing cells on medium containing chloramphenicol and sucrose. An *envZ* mutant strain, ANT1, was isolated by this approach. ANT1 was shown to be chloramphenicol resistant and gentamicin sensitive and did not contain pJNT7. Southern blot analysis of chromosomal DNAs of the wild-type strain and mutant strain confirmed that the *envZ*::Cm allele had replaced the wild-type *envZ* gene (see Materials and Methods). Finally, to ensure that the *ompR* gene was not altered in the *envZ* mutant strain, DNA fragments, encompassing the 5′ end of *ompR* to the 5′ region of *envZ*, from both AN6 and ANT1 were amplified by PCR. A DNA fragment of the anticipated size (664 bp) was obtained from both strains, indicating that *ompR* of ANT1 remained intact.

Outer membrane protein production in AN6 and ANT1. Figure 2A shows the production of outer membrane proteins of AN6 (lanes 1 to 4) and ANT1 (lanes 5 to 8) grown in Grace's medium. The cells were harvested during early log phase (lanes 1 and 5), mid-log phase (lanes 2 and 6), and the transition into stationary phase (lanes 3 and 7) and from 24-h stationary-phase growth (lanes 4 and 8). As shown previously, OpnP was the most predominant protein produced in the outer membrane and the steady-state levels of OpnP did not change as a function of growth phase (13). Likewise, OpnC, -D, and -T were produced in the outer membrane during all stages of bacterial growth. The level of OpnD appeared to increase as the cells entered stationary phase, while OpnA was produced in exponentially growing cells but was not present in stationary-phase cells (lane 4). In contrast, two new outer membrane proteins, OpnB and OpnS, were produced when the bacteria entered stationary phase (lane 3) and were also present in the

24-hour stationary-phase cells (lane 4). A smaller-molecular-weight protein(s), designated OpnX, was also induced as the cells entered stationary phase. These results show that, unlike in *E. coli*, outer membrane protein production was markedly altered as *X. nematophilus* entered stationary phase.

Inactivation of *envZ* in ANT1 affected the level of production of numerous outer membrane proteins (Fig. 2A, lanes 5 to 8). The level of OpnP production in ANT1 was significantly reduced, relative to the wild-type levels, during all stages of growth. This result suggested that EnvZ was required to phosphorylate OmpR, which in turn regulated the expression of *opnP*. In addition, OpnA was not produced in ANT1, while OpnC, -D, and -T were synthesized in the absence of *envZ* function. EnvZ was also found to be involved in the production of stationary-phase-induced outer membrane proteins. Figure 2A shows that OpnB, OpnS, and OpnX were not produced in ANT1 during the transition into stationary phase (compare lanes 3 and 7).

We next examined the de novo synthesis of outer membrane proteins by pulse-labeling cells during different stages of growth. Densitometric scanning of the autoradiograph shown in Fig. 2B indicated that OpnP constituted approximately 50% of the total proteins present in the outer membrane of *X. nematophilus*. In AN6, OpnB and OpnS were induced as the cells entered stationary phase (lane 3) and were synthesized at markedly reduced levels in 24-h stationary-phase cells (lane 4). OpnX was also induced during the transition to stationary phase but was continually synthesized in the 24-h stationary-phase cells. In addition, the de novo synthesis of OpnD increased noticeably as the cells entered stationary phase (lane 3). The pattern of de novo synthesis changed considerably in the *envZ*-minus strain (lanes 5 to 8). Densitometric scanning analysis revealed that in the ANT1 strain OpnP synthesis was reduced by approximately 60% (see lanes 1 and 5) while the de novo synthesis of OpnC, -D, and -T did not change significantly. In contrast, OpnB and OpnX were not synthesized in the *envZ*-null strain during the transition to stationary phase and OpnS was produced at markedly reduced levels (lane 7). Taken together, these results indicated that EnvZ was required for optimal synthesis of OpnP and was involved in the stationary-phase production of OpnB, OpnS, and OpnX.

Complementation of ANT1 with *envZ*-containing plasmids. If EnvZ is required to phosphorylate OmpR, which in turn regulates *opn* genes, then normal Opn production should be restored by complementing the ANT1 strain with an *envZ*-containing plasmid. To test this prediction, we cloned $envZ^{X.n.}$ into two different conjugable plasmids (pSUP106 and pJQ200; see Materials and Methods). The $envZ^{X.n.}$ gene was initially cloned into pSUP106, and the resulting plasmid, pSNT1, was shown to fully complement an *envZ*-null strain of *E. coli*, indicating that pSNT1 produced a functional EnvZ protein (31). pSNT1 was next transferred into ANT1, and the cells were grown in Grace's medium at 30°C. As shown in Fig. 3, ANT1 containing the control plasmid (pSUP106) produced low levels of OpnP (lane 3), while cells containing pSNT1 were able to produce normal levels of OpnP (lane 5). This finding confirmed that *envZ* was essential for the high-level production of OpnP.

The complementation of ANT1 with pSNT1 proved to be more complex with respect to the other Opns. Most noticeably, OpnB and OpnS were not produced in the complemented cells grown under stationary-phase conditions (lane 6). One explanation for this result was that the pSUP106 vector was specifically inhibiting production of OpnB and OpnS. To assess this possibility, we transferred pSUP106 into the wild-type strain. As shown in Fig. 4, OpnB and OpnS were not produced in the

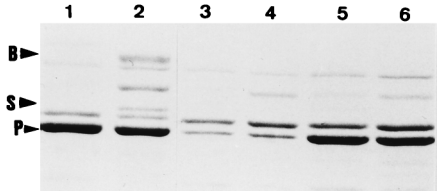

FIG. 3. Complementation of ANT1 with the *envZ*-containing plasmid pSNT1. Opns were prepared from cells grown in Grace's medium and harvested either during mid-log phase growth (lanes 1, 3, and 5) or from 24-h stationary-phase cells (lanes 2, 4, and 6). Opns derived from AN6 (lanes 1 and 2), ANT1/pSUP106 (lanes 3 and 4), and ANT1/pSNT1 (lanes 5 and 6) are shown. OpnP and the stationary-phase-induced OpnB and OpnS are indicated.

AN6/pSUP106 cells grown under stationary-phase conditions (lane 4). We repeated this experiment with the pJQ200 vector and obtained nearly identical results (10a). These findings suggested that a selective inhibitory effect of the vector itself may have masked the ability of *envZ*-containing plasmids to complement the ANT1 strain with respect to OpnB and OpnS production. Since pSUP106 and pJQ200 are derived from different parental plasmids and contain different origins of replication and antibiotic resistances (see Materials and Methods), it is unlikely that the inhibitory effect was due to a common factor, such as the presence of a specific origin of replication. The nature of the inhibitory effect remains unclear.

Physiological and biochemical characterization of ANT1. To determine whether the absence of *envZ* function and the reduced level of *Opns* affected bacterial growth in vitro, the doubling rates for AN6 and ANT1 grown in Grace's media were compared. ANT1 and AN6 doubled at the same rate (1.5 h), suggesting that the reduction in the number of OpnP pores in the outer membrane of ANT1 did not produce a growth-rate-limiting decrease in nutrient uptake. Inactivation of *envZ* did not affect motility, the dye binding properties, or colony morphology of *X. nematophilus*. The production of several proteins and secondary metabolites, such as protease, crystalline inclusion bodies, and antibiotics, that are induced during late log phase growth, was also unaffected by the inactivation of *envZ*. In addition, erythrocyte hemolysis and siderophore production were indistinguishable between the AN6 and ANT1 strains. Finally, we show that, while AN6 grew in medium containing 200 μg of ampicillin per ml, ANT1 grew in the presence of 700 μg/ml. Ampicillin diffuses across the outer membrane by passing through OmpF-like pores (20). Thus, as far as we could determine, the inactivation of *envZ* selectively affected the production of outer membrane proteins in the ANT1 strain and did not result in a pleiotropic phenotype for the characteristics we examined.

Characterization of the pathogenic properties of ANT1. Since the marked reduction in Opn production in ANT1 could

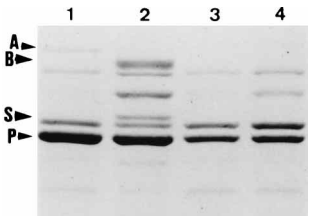

FIG. 4. Inhibitory effect of pSUP106 on the production of Opns in AN6. Opns were prepared from AN6 (lanes 1 and 2) and AN6/pSUP106 (lanes 3 and 4). The cells were harvested either during mid-log phase growth (lanes 1 and 3) or under 24-h stationary-phase conditions (lanes 2 and 4). Opns are indicated.

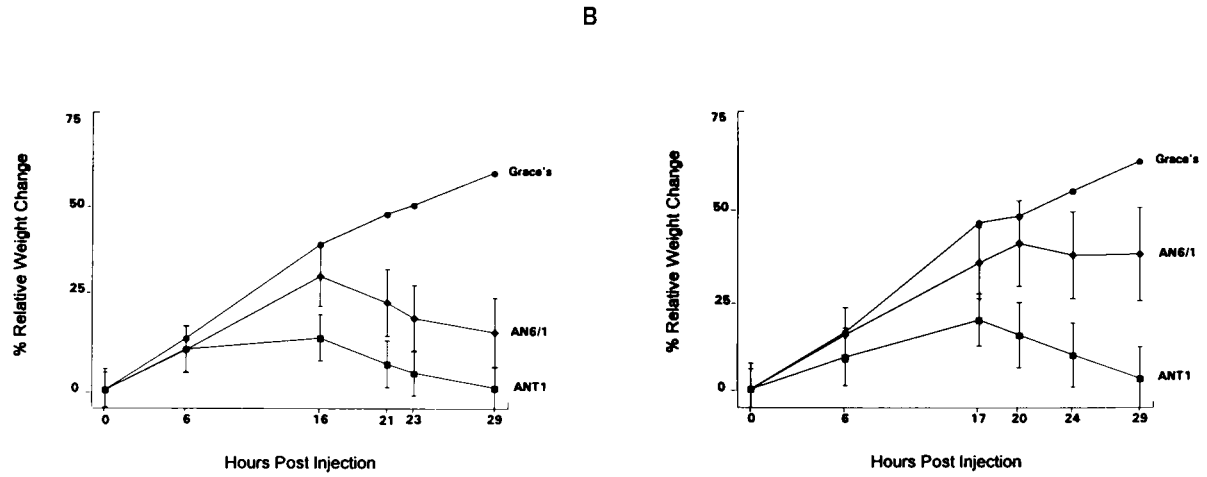

FIG. 5. Growth-inhibitory effects of AN6 and ANT1 injected into the hemocoel of *M. sexta* larvae. (A) Fifth-instar larvae were injected with approximately 70 bacterial cells. Each experimental condition represents injection of eight larvae. Mean percent changes in larval weight relative to the preinjection values are plotted with standard errors. Grace's, control individuals injected with Grace's medium. The experiment was repeated three times, and results of a representative experiment are shown. (B) Fifth-instar larvae injected with approximately five bacterial cells.

affect the ability of the bacterium to survive in the natural biological environment of the insect hemolymph, we compared the growth and virulence levels of AN6 and ANT1 introduced into the fifth-instar larvae of the tobacco hornworm, *Manduca sexta*. Approximately 70 bacterial cells were injected into the hemocoel of *M. sexta*. Eight individual larvae were injected per experimental condition. Relative weight change and evidence of bacterial infection were monitored over a 30-h period (Fig. 5A). By 16 h postinjection, larvae infected with ANT1 showed a significant reduction in the mean growth rate relative to the Grace's medium-injected control individuals. In contrast, the AN6-injected larvae grew at a rate that was similar to that for the control insects. At 29 h postinjection, four ANT1-injected larvae had died while all of the AN6-injected individuals were still alive. By 40 h postinjection, all eight of the ANT1-infected larvae were dead while three of the AN6-injected insects were still alive (31).

To further analyze the apparent enhanced virulence of the ANT1 strain, the pathogenicity experiment was repeated with approximately five bacterial cells injected per individual larva (Fig. 5B). The inhibition of larval growth when five ANT1 cells were injected was essentially identical to that observed when 70 bacteria were injected. By 40 h, seven of the eight ANT1-injected larvae injected had died. This result emphasized the extreme virulence of ANT1 in *M. sexta*. On the other hand, injecting fewer wild-type cells produced a noticeable delay in the growth-inhibitory effect. The AN6-injected cells continued to grow for 20 h, and only four of the AN6-injected larvae had died by 40 h.

During the course of the experiment in which 70 bacterial cells were injected into the hemocoel, hemolymph samples from a representative larva were examined by phase-contrast microscopy. By 8 h postinjection, free bacteria were not detectable in the hemolymph (31). A few bacteria were evident at 18 h postinjection in the ANT1-infected, but not in the AN6-injected, larval insect (Fig. 6). At 23 h, bacteria were not observed in the hemolymph in either the AN6- or the ANT1-injected larvae. By 30 h postinjection, the ANT1-injected larvae had died and a few bacteria were observed in the hemolymph. Bacteria were not present in the hemolymph of the AN6-injected larva at this time. By 40 h, high concentrations of bacteria ($>10^8$/ml) were clearly visible in the ANT1-injected larvae, while only a few bacteria were present in the AN6-injected-larva hemolymph (31). Taken together, these results showed that the *envZ*-null strain was more pathogenic than the wild-type strain. In addition, we show that the larvae were killed before significant numbers of bacteria were detectable in the hemolymph.

DISCUSSION

We demonstrate that EnvZ was required for optimal production of OpnP in *X. nematophilus*. These findings suggest that, in *X. nematophilus*, EnvZ functions to phosphorylate OmpR, which in turn stimulates the binding of this regulatory protein to the activation sequence located between −103 and −84 of *opnP* (13). In *E. coli*, growth under high-osmolarity conditions results in elevated OmpR-phosphate levels, which in turn enhance the binding of OmpR to a repression sequence located between −380 and −360 of *ompF* (4, 24). This repression sequence was found to be lacking in the upstream region of *opnP* (13), a finding which may explain why OpnP production is not repressed by high-osmolarity conditions. While *envZ* appears to be essential for high-level production of OpnP, this porin protein was still produced in the ANT1 strain. This production could be due to cross talk phosphorylation of OmpR by acetyl phosphate (18, 32). Although *opnP* does not appear to be osmoregulated, it is conceivable that the EnvZ/OmpR system in *X. nematophilus* regulates other genes that are responsive to changes in the osmolarity of the growth environment. In this regard, we note that, in *E. coli*, OmpR-phosphate has been proposed to function as a repressor of *flhDC*, a regulatory locus required for flagellum synthesis (27) and that flagellum synthesis is reduced under high-osmolarity conditions (26).

Opn production in *X. nematophilus* was strongly influenced by growth phase. OpnB, OpnS, and OpnX were induced, and OpnD production was increased, under stationary-phase conditions. *X. nematophilus* produces numerous molecules, such as antibiotics, protease(s), and intracellular crystalline proteins, under stationary-phase conditions (1, 12, 29). Modulation of the protein composition of the outer membrane is therefore another aspect of the adaptive response to stationary-phase growth. The elimination of OpnB, OpnS, and OpnX induction

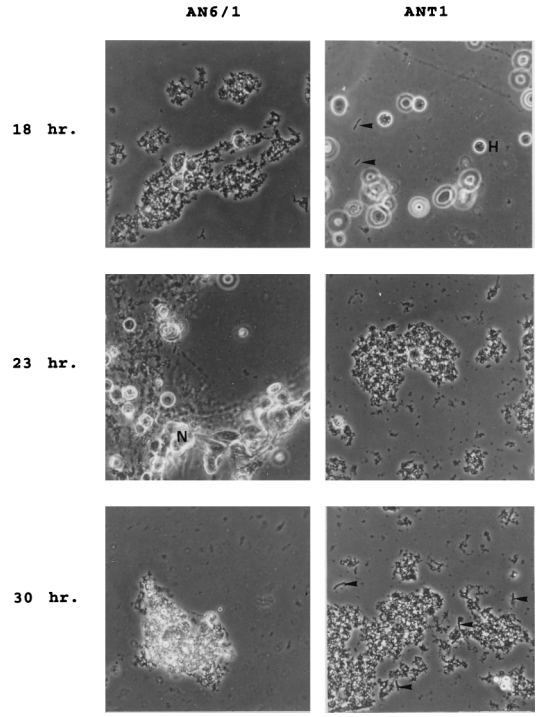

FIG. 6. Representative samples of hemolymph derived from *M. sexta* larvae infected with either AN6 or ANT1 (as indicated). A 5-μl sample was drawn at the indicated times postinjection. Arrowheads, bacterial cells. H, hemocyte; N, nodule.

in the *envZ*-null strain raised the possibility that EnvZ was involved in stationary-phase-induced synthesis of these proteins. We found that introduction of the conjugable plasmids pSUP106 and pJQ200 into the wild-type strain specifically inhibited OpnB and OpnS synthesis but did not affect the production of other Opns. Therefore, to address the question of whether EnvZ is a "stationary phase sensor" in *X. nematophilus*, it may be necessary to integrate a wild-type *envZ* allele into the chromosome of ANT1. Whether EnvZ is directly involved in the expression of OpnB, OpnS, and OpnX via phosphorylation of OmpR or indirectly affects stationary-phase production of Opns remains to be determined. EnvZ in *X. nematophilus* may represent a unique example of a single histidine kinase sensor that is involved in signal transduction events both in exponentially growing cells and in cells entering stationary phase. Induction of Opns during stationary-phase growth may provide the cell with specialized porin function and/or unique transport proteins which are required for growth in the hemolymph of the insect cadaver. For example, secretion of proteases by *X. nematophilus* during stationary-phase conditions in the hemolymph could generate a source of peptides. The uptake of these peptides would require specific transport functions in the outer membrane which may be provided by OpnB, OpnS, and OpnX. Another intriguing function for stationary-phase-induced outer membrane proteins would be to provide cell surface components which are required for establishing the symbiotic association between the bacterium and the nematode.

We had shown previously that the production of OpnB was repressed by elevated temperature (16), high-osmolarity conditions (10a), and sublethal concentrations of chloramphenicol (31). The highly regulated nature of OpnB suggests that its production is sensitive to numerous environmental stresses. Additionally, OpnB was not produced in phase II variants of AN6, which arise spontaneously in cells maintained under stationary-phase conditions (5, 16). It should be noted that nematode growth and reproduction were found to be less efficient if nematodes were cultured with phase II cells (1). In this regard, it will be of interest to determine whether OpnB plays a role in the symbiotic interaction between the nematode and bacterium. Since it is possible to prepare axenic eggs from *Steinernema carpocapsae* (4), the abilities of the parental and mutant strains of *X. nematophilus* to establish the symbiotic association between the nematode and the bacteria can be compared.

The finding that growth inhibition and insect mortality occurred before significant numbers of bacteria were present in the hemolymph of *M. sexta* suggests that *X. nematophilus* produces insect toxins which act early in the infectious process. A putative insect toxin has recently been identified in *X. nematophilus* A24 (reference 8 in the review of Forst and Nealson [12]). It is therefore conceivable that alterations in the bacterial outer membrane in the ANT1 strain resulted in an elevated amount of toxin released into the insect hemolymph. Alternatively, the enhanced virulence of the ANT1 strain may be related to an increase in released lipopolysaccharide, which has been proposed to be a virulence factor in *X. nematophilus* (10). Finally, since the bacterial cell surface plays a role in the interaction with activated hemocytes and in the formation of nodules, the mutant strain could more effectively evade the host defense systems.

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