

Characterization of Outer Membrane Proteins of *Xenorhabdus nematophilus*

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Abstract

The major outer membrane proteins of the primary and secondary phase variants of *Xenorhabdus nematophilus* produced during exponential and stationary phase growth were characterized. OpnP, the most highly expressed outer membrane protein of *X. nematophilus*, was purified as a monomer with a molecular weight of 30,000. The amino acid composition of OpnP was very similar to that of the porin proteins, OmpF and OmpC, of *Escherichia coli*. N-terminal amino acid sequence analysis revealed that residues 1-27 of the mature OpnP shared 60% sequence identity with OmpF. In vitro pore function analysis of purified OpnP indicated that the single channel conductance values were similar to that measured for OmpF. These results suggest that OpnP is the OmpF-like porin protein in *X. nematophilus*. Three additional proteins, OpnA, OpnB and OpnS were induced during stationary phase growth. We show that the stationary phase proteins, OpnA and OpnB, were not produced in secondary phase cells. OpnB was present at a high level in stationary phase cells grown at 19-30°C, and was repressed in cells grown at 34°C. OpnA was optimally produced at 30°C and was not present in cells grown at lower and higher temperatures. The production of OpnS was not dependent on growth temperature. In contrast, another outer membrane protein, OpnT, was strongly induced as the growth temperature was elevated from 19° to 34°C.

Keywords: OpnP, symbiosis, OmpF, porin, entomopathogenic nematode

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1. Introduction

The genus *Xenorhabdus* (Enterobacteriaceae) consists of motile rods that are carried monoxenically in a specialized intestinal vesicle of a nonfeeding juvenile stage of entomopathogenic nematode, *Steinernema carpocapsae*, in a species specific symbiotic association (Akhurst and Boemare, 1990). The foraging infective juvenile nematode locates the larval stage of many different insects, penetrates into the hemocoel of the host and releases the bacterial symbiont into the nutrient rich insect hemolymph. The bacteria proliferate within the hemolymph, and then presumably enter a stationary phase of their life cycle. During stationary phase growth in culture, *Xenorhabdus* spp. secrete several products. These include a protease and a lipase that are thought to play important roles in the killing and digestion of the insect larva (Boemare and Akhurst, 1988). Broad spectrum antibiotics, also optimally produced during stationary phase growth, inhibit the multiplication of other microorganisms within the insect cadaver, and help to establish conditions that are required for nematode reproduction (Akhurst, 1982; Neelson et al., 1990; Sundar and Chang, 1993). In addition, protein inclusion bodies form crystalline structures in the protoplasm of stationary phase cells (Akhurst and Boemare, 1990). The *Xenorhabdus* /nematode symbiotic pair can kill a broad range of insect pests. This system is currently being intensively studied for its usefulness as a biological control agent (Klein, 1990).

Xenorhabdus spp. exhibit a phase variation that is also a growth phase dependent phenomenon. The primary form produces protease, lipase, antibiotics, crystalline protein, and is able to bind specific dyes such as Congo Red (Xu et al., 1991). In the closely related bacterium, *Photorhabdus* (Boemare et al., 1993), the primary form cells also produce an insect toxin (Bowen et al., 1988) and are luminescent during stationary phase growth (Frackman and Neelson, 1990). The primary cells are the predominant form of *Xenorhabdus* present during exponential growth in culture. It is also the primary form cell that is isolated from the nematode (Poinar, 1990). The secondary form is isolated from cell cultures that are in stationary phase. The secondary form cells do not produce the growth phase-dependent products described above and possess altered dye binding properties (Akhurst and Boemare, 1990). In addition, the secondary form cells of *Photorhabdus* are nonluminescent (Frackman and Neelson, 1990). The primary form provides better conditions than the secondary form for nematode reproduction. Smigielski et al. (1994) have speculated that primary form cells have optimally adapted to the conditions present in the nematode gut and insect hemolymph whereas the secondary form variant is better adapted to conditions as a free-living organism.

In Gram-negative bacteria, the outer membrane functions as a permeability barrier permitting small water soluble nutrients to passively diffuse into the cell while excluding toxic substances (Nikaido, 1994). In *Escherichia coli*, nutrients diffuse through water-filled channels formed by homotrimeric association of the porin proteins, OmpF and OmpC (Forst and Inouye, 1988; Forst and Roberts, 1994). The *ompF* and *ompC* genes are regulated by several environmental stimuli including changes in the osmolarity and temperature of growth. A large amount of the monomeric protein, OmpA, also exists in the outer membrane. OmpA is considered to be a structural protein, although recent evidence has indicated that it allows very slow diffusion of solutes in a reconstituted system (Nikaido, 1994). In *Xenorhabdus* spp., the outer membrane proteins may provide additional functions that allow the bacterium to evade the insect host immune system, to transport molecules out of the cell, and to establish the symbiotic association with the nematode. There was no information previously available describing the function of the outer membrane proteins of *Xenorhabdus* spp. In this study we have characterized the major outer membrane proteins of *X. nematophilus*, and studied the environmental conditions that affect their production.

2. Materials and Methods

Strains and media

The following strains were used: *Xenorhabdus nematophilus* AN6/1 (primary form) and AN6/2 (secondary form) both from R.J. Akhurst and *Escherichia coli* MC4100 (F^- *lac U169 araD rpsL relA thi flbB*) from the Cold Spring Harbor collection. LB (0.5% NaCl) supplemented with 0.5 mM MgSO₄ was the medium used for all experiments except for the maintenance of *Xenorhabdus* which was done on L-Agar with 0.0025% bromthymol blue (Sigma, St. Louis, MO) and 0.004% triphenyltetrazolium chloride (Sigma).

Growth conditions

Cultures of *X. nematophilus* and *E. coli* were grown on gyrorotary shakers at 30 and 37°C, respectively (or other temperatures when specified) in side arm flasks. Growth curves were determined by measuring light scattering using a Klett-Summerson colorimeter (VWR, Chicago, IL) equipped with a red filter.

Preparation of outer membrane proteins using Sarkosyl

Cells were harvested in different phases of growth by centrifugation at 10,000

x g. The cell pellets were washed once with 20 mM sodium phosphate (pH 7.1; *E. coli*) or LB (*X. nematophilus*); the latter prevented lysis of the *X. nematophilus* cells. Sonication was performed over a period of ten min (one min bursts at 120 watts) in 400 μ l of sodium phosphate buffer on ice. The disrupted cells were centrifuged 3 min at low speed (15,800 x g) and then 14 min at high speed (353,000 x g in a TL-100 ultracentrifuge, Beckman Instruments, Inc., Fullerton, CA). These crude membrane pellets were suspended over a period of 30 to 60 min in 0.5% Sarkosyl (N-lauroyl-sarcosine, Sigma) in 20 mM phosphate in order to solubilize cytoplasmic membrane components and then centrifuged another 14 min at 353,000 x g. The resulting outer membrane pellets were solubilized in Laemmli sample buffer, heated for 5 min at 100°C and electrophoresed on 15% polyacrylamide gels or on urea-SDS-polyacrylamide gels (8 M Urea, 10% polyacrylamide). To test heat modifiability, some samples were run without heating.

Purification of outer membrane proteins

X. nematophilus (grown at 30°) were passaged through a French Press, centrifuged and treated with Sarkosyl. The pellets from the second 353,000 x g centrifugation were incubated 2 hr at 37° with TES (50 mM Tris-HCl pH 7.2/5 mM EDTA/1% SDS), centrifuged and the resulting pellets were incubated overnight with TENS (50 mM Tris-HCl pH 7.2/5 mM EDTA/400 mM NaCl/1% SDS). OpnP was found in the TENS 353,000 x g supernatant fraction. Eluted OpnP was chromatographed on a 1.6 x 84 cm column of Sephacryl S-200 (Pharmacia, Inc., Piscataway, NJ) using TENS (OpnP) as the elution buffer.

Amino acid analysis and N-terminal sequencing

Purified OpnP was electrotransferred from 15% polyacrylamide mini-gels to Immobilon-P (Millipore, Bedford, MA) using the Biorad Trans Blot Cell for 1 h at 78 V (constant) at 4°C in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid, Sigma), pH 11.0. The bands were visualized by staining with 0.1% amido black. The amino acid analysis and N-terminal amino acid sequencing were performed at the Protein/Nucleic Acid Shared Facility at the Medical College of Wisconsin.

Pore function assay

A planar lipid bilayer system (1.5% w/v of oxidized cholesterol in n-decane) as described previously (Benz and Hancock, 1981) was used to study OpnP pore properties. Single channel conductance measurements were obtained after

forming a lipid bilayer across a 0.2 mm² hole. Approximately 20 ng of OpnP, solubilized in 0.1% Triton X-100 was added to one compartment and 50 mV was applied across the lipid bilayer. Zero current membrane potential experiments were performed in a chamber with a 2.0 mm² hole separating the compartments. 50 ng of protein was added to one compartment and a voltage of 10 mV was applied. Approximately 200 channels were allowed to insert into the bilayer and then the voltage was removed. An aliquot of 100 µl of 3.0 M KCl was added to one side of the membrane while 100 µl of 0.1 M KCl was added to the other. Eight subsequent additions were made, and differences in potential due to preferential diffusion of one ion species, creating a potential that opposed the concentration gradient, were measured after each addition.

3. Results

Characterization of outer membrane proteins of X. nematophilus

To understand the role that the outer membrane proteins play in the adaptive response to the different environmental conditions that this bacterium encounters during its lifecycle, we isolated and characterized the major outer membrane proteins of *X. nematophilus* strain, AN6/1. Several proteins produced in exponentially growing cells were separated by electrophoresis in the presence of 8M urea (Fig. 1A, lane 3). Opn C, D, P and T were present at high levels in the outer membrane. OpnP was the most highly expressed protein in the outer membrane of exponentially growing cells. Since OpnP was a predominant outer membrane protein that would significantly contribute to the cell surface properties of *X. nematophilus*, we purified this protein in order to further characterize its physical properties. OpnP was solubilized in the high salt buffer (0.4 M NaCl) and subsequently applied onto a size exclusion column. OpnP eluted as a single peak with an approximate molecular weight of 30,000. On SDS-PAGE gels OpnP migrated as 37,000 molecular weight protein, even without being boiled. In contrast, it has been shown that OmpF and OmpC run as high molecular weight smears when the boiling step was omitted. A comparison of the properties of OpnP and OmpF/OmpC of *E. coli* revealed that these proteins have similar molecular weights but OmpF and OmpC purify as trimeric proteins, are heat modifiable, and are not soluble in 0.4 M NaCl. In contrast, OpnP purified as a monomer, was not heat modifiable and was soluble in 0.4 M NaCl. The amino acid composition of OpnP was determined and compared to OmpF, OmpC and OmpA of *E. coli* (Table 1). The composition of OpnP was very similar to OmpF and OmpC, and was distinctly different from that of OmpA. In particular OpnP, OmpF and OmpC contained a single

histidine residue, a relatively low level of proline residues, and a high level of phenylalanine.

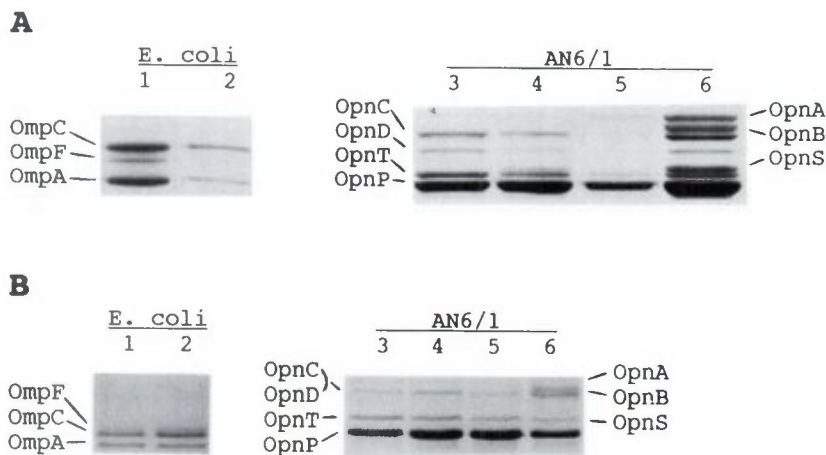


Figure 1. Growth phase regulation of outer membrane proteins. Panel A: 8 M urea in 10% polyacrylamide, Panel B: 15% polyacrylamide. *E. coli* (MC4100) grown in LB at 37°C, was harvested at: lane 1, Mid log phase; lane 2, Stationary phase. *X. nematophilus* strain AN6/1, grown in LB at 30°C, was harvested at: lane 3, Early log phase; lane 4, Mid log phase; lane 5, Late log phase; and lane 6, Stationary phase.

Amino terminal sequence analysis of OpnP

The amino acid composition of OpnP suggested that it may share sequence similarity with OmpF and OmpC. To assess this possibility, the amino acid sequence of the N-terminal region of OpnP was determined. The first 27 residues of OpnP are compared with the N-terminal sequence of OmpF and OmpC (Fig. 2) revealing that OpnP shared 59% and 70% sequence identity with OmpF and OmpC, respectively. We have recently isolated the gene coding for OpnP (Forst et al., 1995). Initial DNA sequence analysis has allowed us to deduce the amino acid sequence of the signal peptide of OpnP (Fig. 2). Comparison of the deduced amino acid sequence of the signal peptides of OpnP, OmpF and OmpC showed a remarkable degree of sequence identity. OpnP shared 86% and 59% sequence identity with OmpF and OmpC, respectively. This analysis also showed that the signal peptide cleavage site was identical in all three proteins. Based on the strong sequence similarity between the signal peptide and N-terminal regions of OpnP and the *E. coli* porin proteins, we conclude that OpnP is a major porin protein in the outer membrane of *X. nematophilus*.

Table 1. Comparison of amino acid compositions of OpnP, OmpC, OmpF and OmpA

	OpnP	OmpC	OmpF	OmpA
Lys	17	15	18	17
His*	1	1	1	5
Arg	11	13	11	13
Asx	51	62	57	41
Thr	25	24	21	21
Ser	15	16	16	16
Glx	32	32	27	29
Pro*	5	3	4	19
Gly	38	47	48	37
Ala	30	24	29	29
Cys	ND	0	0	2
Val	23	21	23	25
Met	4	3	3	5
Ile	12	10	12	14
Leu	32	22	20	22
Tyr	24	29	29	17
Phe*	16	19	19	8
Trp	ND	4	2	5

ND = not determined. Residues indicated by "*" highlight amino acids that distinguish OpnP and OmpF/C from OmpA.

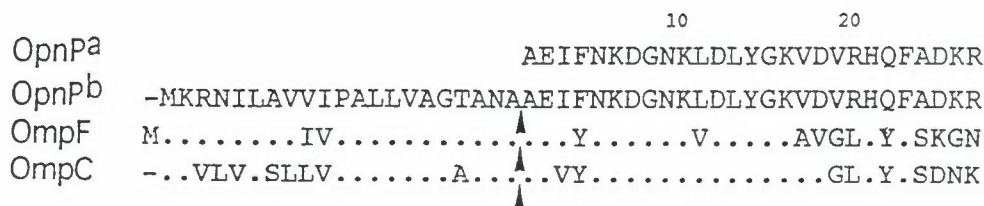


Figure 2. N-terminal amino acid sequence of porin proteins. In the sequence of OmpF and C a dot indicates identity and a dash indicates a deletion. OpnP^a: N-terminal sequence of OpnP protein. OpnP^b: deduced amino acid sequence from nucleotide sequence of *opnP*. Arrows show the signal peptide cleavage site.

Porin Function of OpnP

Porin proteins can be characterized *in vitro* by measuring stepwise conductance increases as the purified protein inserts into a planar lipid bilayer (Benz and Hancock, 1981). To study its pore forming ability purified OpnP was analyzed *in vitro*. Stepwise increases in conductance across a lipid bilayer were observed when Triton X-100 solubilized OpnP monomers were added to the model membrane system, but not when detergent alone was added (data not shown). This indicated that OpnP functioned as a porin by reconstituting channels in the bilayer. Single channel conductance measurements showed a distribution of channel sizes centered on a single mean for each of the salt solutions used (Table 2). Experiments with different salt solutions showed that an increase salt concentration resulted in a proportional increase in conductance, which indicated that OpnP channels were large and water filled. When the salt solution contained a large cation (hydrated Li^+ ion) a slight decrease in mobility was seen, whereas the presence of a large anion (CH_3COO^-) did not decrease the conductance relative to that observed with Cl^- . These data were consistent with OpnP being a weakly cation selective channel. This was demonstrated directly by measuring the zero current membrane potential, as calculated according to the Goldman-Hodgkin-Katz equation, which showed a permeability ratio of cations to anions of 1.8:1. It is important to note that the average single channel conductance values for the OpnP monomer were very similar to the predicted values for an OmpF monomer.

Table 2. Stepwise increases in conductances of OpnP channels. Conductances indicate the incorporation of channel forming proteins into the lipid bilayer. The channels were formed after the addition of 20 ng of OpnP (solubilized in 0.1% Triton X-100) to a 1.0 M KCl solution that was bathing the bilayer.

Salt	n	Average single channel conductance		
		OpnP	OmpF (trimer)	OmpF (monomer)
0.3 M KCl	103	0.09	0.54	0.18
1.0 M KCl	147	0.44	1.9	0.63
3.0 M KCl	102	1.07	4.3	1.43
1.0 M LiCl	101	0.22	0.62	0.21
1.0 M CH_3COOK	98	0.25	1.1	0.37

Growth phase regulation

X. nematophilus is exposed to very different environments in the gut of nematodes and in the hemolymph of insects. Furthermore, the bacteria are actively growing in the insect hemolymph and may be in a nongrowing state in the nematode gut. To adapt to these different environments the bacterium may modulate the production of its outer membrane proteins. To examine this possibility, we analyzed the production of outer membrane proteins at different stages of the *Xenorhabdus* growth cycle by electrophoresis on the urea/SDS-PAGE gel system (Fig. 1A). Opn C, D, T and P were produced in cells grown at 30°C and harvested during early log phase growth (Fig. 1A, lane 3). During mid log (lane 4) and late log (lane 5) phase growth, OpnA and OpnS began to be produced at higher levels. In stationary phase cells OpnA and OpnS were strongly induced. A new protein, OpnB, also appeared in the outer membranes of stationary phase cells. OpnC, D, T and P were apparently not growth phase regulated although Northern analysis using an *opnP* probe suggested that the steady state level of *opnP* mRNA was markedly reduced during stationary phase growth (L. Esterling and S. Forst, unpublished data). In contrast, *E. coli* did not exhibit a growth-phase dependent modulation of outer membrane protein production (Fig. 1A, lanes 1 and 2). The apparent molecular weights of OpnA, OpnB and OpnS were 52,000, 49,000 and 45,000, respectively (Fig. 1B).

Opn production in secondary form variant

Since most of the secondary metabolites of *X. nematophilus* exhibit a form-dependent pattern of expression, we examined the regulation of the Opns in the secondary form variant. One of the most striking results was that OpnA and OpnB were expressed at a much lower level in the secondary form cells (Fig. 3, lane 2) than in the primary form cells (lane 3). We also noted that OpnC was growth phase inducible in the secondary form cells. On the other hand, growth phase induction of OpnS did occur in the secondary form cells.

Temperature regulation

One of the environmental conditions that both the bacterium and nematode must adapt to is changes in ambient temperature. OmpF in *E. coli* is thermally regulated. At elevated growth temperatures (above 37°C) the production of OmpF production decreases (Andersen et al., 1989). In order to look for temperature controlled expression of the outer membrane proteins, strain AN6/1 was grown to stationary phase at 19, 23, 27, 30 and 34°C (Fig. 4). OpnB was produced at 19–30°C but was markedly repressed in cells growing at 34°C. In

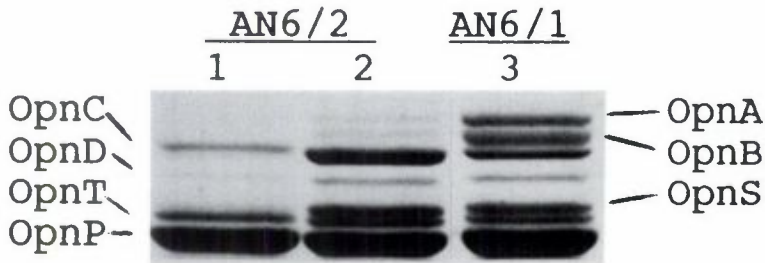


Figure 3. Secondary vs primary form variant of *X. nematophilus*. Strain AN6/2 (secondary form): lane 1, Mid log; lane 2 Stationary phase. Strain AN6/1 (primary form): lane 3, Stationary phase.

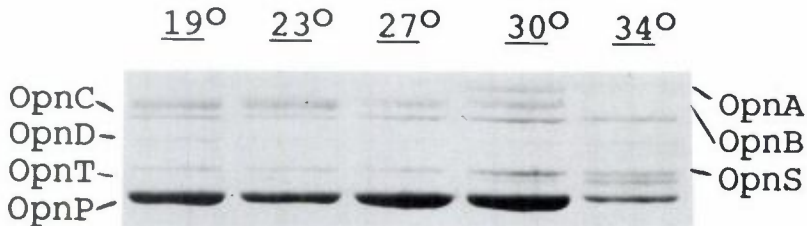


Figure 4. Temperature regulation of outer membrane proteins. Cells were grown in LB to stationary phase at indicated temperatures ($^{\circ}\text{C}$). Note the induction of OpnA and OpnT at 30°C , and the repression of OpnA and enhanced production of OpnT at 34°C .

contrast, OpnT was strongly induced by elevated growth temperature. This protein was not expressed in cells grown at 19°C and 23°C , was present in cells grown at 27°C , and was produced at higher levels in cells grown at 30°C and 34°C . OpnA exhibited a stringent temperature-dependent expression being produced only in cells grown at 30°C .

4. Discussion

In this study, we have shown that during exponential growth the outer membrane of *X. nematophilus* contains several proteins that are expressed at high levels. The N-terminal amino acid sequence of the most highly expressed protein, OpnP, was found to be similar to that of the porin proteins, OmpF and OmpC, of *E. coli*. Based on its high level of expression and the strong sequence

similarity to OmpF and OmpC, OmpN appears to be the predominant porin protein in the outer membrane of *X. nematophilus*. Using planar lipid bilayer analysis OmpN was found to form relatively non-specific, water-filled channels. The OmpN channel displayed a slight selectivity for cations as demonstrated by the single channel conductance measurements using Li^+ as the mobile cation. The cation to anion permeability ratio of 1.8:1, as measured by zero current membrane potential analysis, further demonstrated that the OmpN channel was slightly cation selective. OmpN was isolated as a stable monomer while OmpF is functional as a trimer. The subunit:subunit interactions of the putative OmpN trimer may be weaker than that of the OmpF trimer. In this case extraction from the outer membrane may have resulted in the dissociation of the OmpN trimer. Each monomer of the OmpF trimer contains a nonspecific channel that functions in an independent fashion (Cowan et al., 1992). The OmpF trimer has a single channel conductance of 1.9 nS in 1 M KCl so that each monomer would contribute a conductance of approximately 0.63 nS. This value was reasonably close to the 0.43 nS value observed with OmpN monomers. That porin proteins may exist in trimeric form in the outer membrane, but can function in vitro as monomers, is supported by recent studies on several porin proteins of *Pseudomonas aeruginosa* (Hancock et al., 1991).

During stationary phase growth, an additional protein, OmpB, was induced in the primary form but not in the secondary form cells. This protein was not produced in exponentially growing cells and was repressed by growth at elevated temperatures (34°C). The pattern of expression of OmpB in cells growing in culture media may mimic that in cells growing in their natural environment, the insect hemolymph. We speculate that OmpB is not present in the outer membranes of cells growing exponentially in the hemolymph, and is induced during the stationary phase of the bacterial life cycle. This pattern of expression is consistent with that of a cell surface protein that may be involved in the association of the bacteria with the nematode. At some point during stationary phase growth, the ingested bacteria may be retained in the intestine and are then able to colonize the nematode gut. It is interesting to note that OmpB is not induced in the secondary form cells. If OmpB is essential for the association of the bacteria with the nematode, the secondary form cells would poorly colonize the nematode gut. Other factors such as pili and glycocalyx were also produced more predominantly in the primary as compared with the secondary form cells (Brehelin et al., 1993). The incomplete development of nematodes grown on the secondary form of *X. nematophilus* may be due, in part, to the absence of OmpB and other cell surface molecules that play a role in adhesion and colonization.

Two other outer membrane proteins, OmpA and OmpS, are produced at low levels in early and midlog phase growth and appear as major constituents of the

outer membrane of stationary phase cells. OpnA was highly produced in stationary phase primary cells grown at 30°C. The more restricted production of OpnA suggests that it would not be expressed in the insect hemolymph unless the temperature reaches 30°C. Since the ambient temperature is usually considerably lower than 30°C when bacteria are growing within the insect host, OpnA would not be a strong candidate for a cell surface protein that is important to the nematode/bacteria symbiosis. In contrast, OpnS was strongly induced in stationary phase cells grown at a broad range of temperatures. Another outer membrane protein, OpnT, was thermally regulated. The strong thermal regulation of OpnT suggests that this protein may play a role in the ability of the bacteria to adapt to changes in the growth temperature.

The properties of the outer membrane have been shown to contribute to the antihemocytic nature of *X. nematophilus* (Dunphy, 1994). Removal of lipopolysaccharide (LPS) from the bacterial cell surface, and treatment of the bacteria with carbohydrases and proteases, resulted in increased adherence of the bacteria to hemocytes and an increased rate of removal from the hemolymph of nonimmune larvae. Mutant strains that contained a reduced level of outer membrane protein, and an elevated level of the 3-deoxy-D-mannooctulosonic acid (KDO) moiety of LPS were found to be less virulent than wild type cells (Dunphy and Webster, 1991). Modulation of outer membrane protein production could significantly alter the cell surface properties of *X. nematophilus* and play a major role in the interaction of the bacteria with the nematode, and in the survival of the bacteria in the insect hemolymph.

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