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Characterization and Environmental Regulation of Outer Membrane Proteins in *Xenorhabdus nematophilus*

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We have examined the production of the outer membrane proteins of the primary and secondary forms of *Xenorhabdus nematophilus* during exponential- and stationary-phase growth at different temperatures. The most highly expressed outer membrane protein of *X. nematophilus* was OpnP. The amino acid composition of OpnP was very similar to those of the porin proteins OmpF and OmpC of *Escherichia coli*. N-terminal amino acid sequence analysis revealed that residues 1 to 27 of the mature OpnP shared 70 and 60% sequence identities with OmpC and OmpF, respectively. These results suggest that OpnP is a major porin protein in *X. nematophilus*. Three additional proteins, OpnA, OpnB, and OpnS, were induced during stationary-phase growth. OpnB was present at a high level in stationary-phase cells grown at 19 to 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. In addition, we show that the stationary-phase proteins OpnA and OpnB were not produced in secondary-form cells.

The genus *Xenorhabdus* (*Enterobacteriaceae*) consists of motile rods that are carried monoxenically in a specialized intestinal vesicle of a nonfeeding juvenile stage of an entomopathogenic nematode, *Steinernema carpocapsae*, in a species-specific symbiotic association (2). The foraging infective juvenile nematode locates the larvae 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 trehalose-rich hemolymph (17) and then presumably enter the stationary phase of its life cycle (2). During stationary-phase growth in culture, *Xenorhabdus* spp. secrete several products. These products include a protease and a lipase that are thought to play important roles in the killing and digestion of the insect larva (4). 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 (1, 22, 26). In addition, protein inclusion bodies form crystalline structures in the protoplasm of stationary-phase cells (2, 22). 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 (20).

Xenorhabdus spp. exhibit a phase variation that is also a growth phase-dependent phenomenon. The primary form produces protease, lipase, antibiotics, and crystalline protein and is able to bind specific dyes such as Congo Red (28). In the closely related *Photorhabdus luminescens* (5), the primary-form cells also produce an insect toxin (6) and a lipase (27) and are luminescent during stationary-phase growth (16). The primary cells are the predominant form of *Xenorhabdus* spp. present during exponential growth in culture. It is also the primary-form cell that is isolated from the nematode (2, 24). 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 (2). In addition, the secondary-form *Photorhabdus* cells are nonluminescent (16, 22). The primary form provides better conditions than the secondary form for nematode reproduction (2). Smigielski et al. have speculated that primary-form cells have optimally adapted to the conditions present in the nematode gut and insect hemolymph whereas the secondary-form cells are better adapted to conditions as a free-living organism (25).

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 (18, 23). In *Escherichia coli*, nutrients diffuse through water-filled channels formed by homotrimeric association of the porin proteins OmpF and OmpC (13, 14, 23). The *ompF* and *ompC* genes are regulated by several environmental stimuli including changes in the osmolarity and temperature of growth (3, 10, 14) and by oxidative stress (9). Exposure of *E. coli* to purified antibacterial hemolymph proteins (attacins) has been shown to repress OmpF and OmpC production (8). 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 (23). 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 is no information presently available describing the functions 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.

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.

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Akhurst) and *E. coli* MC4100 (F^- *lacU169 araD rpsL relA thi flbB*; from the Cold Spring Harbor Laboratory collection). Luria broth (LB) (0.5% NaCl) supplemented with 0.5 mM $MgSO_4$ was the medium used for all experiments except for maintaining *X. nematophilus*, 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 gyratory shakers at 30 and 37°C, respectively (or other temperatures when specified) in sidearm flasks. Growth curves were determined by measuring light scattering with a Klett-Summerson colorimeter (VWR, Chicago, Ill.) 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 × *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 10 min (1-min bursts at 120 W) in 400 μl of sodium phosphate buffer on ice. The disrupted cells were centrifuged for 3 min at low speed (15,800 × *g*) and then for 14 min at high speed (353,000 × *g* in a TL-100 ultracentrifuge; Beckman Instruments, Inc., Fullerton, Calif.). 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 the cytoplasmic membrane components and then centrifuged another 14 min at 353,000 × *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-sodium dodecyl sulfate (SDS)-polyacrylamide gels (8 M urea, 10% polyacrylamide) (15). To test heat modifiability, some samples were run without heating.

Purification of outer membrane proteins. Stationary-phase cells of *E. coli* (grown at 37°C) and *X. nematophilus* (grown at 30°C) were broken by passage through a French press and then centrifuged and treated with Sarkosyl as described above. The pellets from the second centrifugation at 353,000 × *g* were incubated either for 2 h or overnight at 37°C with TES (50 mM Tris-HCl [pH 7.2], 5 mM EDTA, 1% SDS) and centrifuged (14 min at 353,000 × *g*), and the resulting pellets were incubated overnight with TENS (50 mM Tris-HCl [pH 7.2], 5 mM EDTA, 400 mM NaCl, 1% SDS). OmpF from *E. coli* appeared in the TES supernatant fraction (19), while OpnP from *X. nematophilus* was found in the TENS supernatant fraction after centrifugation at 353,000 × *g*. Eluted OmpF and OpnP were chromatographed on a column (1.6 by 84 cm) of Sephacryl S-200 (Pharmacia, Inc., Piscataway, N.J.), using either TES (OmpF) or TENS (OpnP) as the elution buffer.

Amino acid analysis and N-terminal sequencing. Purified OpnP was electrotransferred from 15% polyacrylamide minigels to Immobilon-P (Millipore, Bedford, Mass.), using the Bio-Rad 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 (21). 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.

RESULTS

Characterization of outer membrane proteins of *X. nematophilus*. The outer membrane proteins of *X. nematophilus* (Opns) have not yet been characterized. To understand the roles that these proteins may play in the adaptive responses to the different environmental conditions that this bacterium encounters during its life cycle, we isolated and characterized the major outer membrane proteins of *X. nematophilus* AN6/1. Several proteins produced in exponentially growing cells were separated by electrophoresis in the presence of 8 M urea (Fig. 1A). OpnC, -D, -P, and -T were present at high levels in the outer membrane. The faster-migrating proteins may represent major lipoproteins and were not further characterized in this study. OpnP was the most highly expressed protein in the outer membranes of exponentially growing cells. The apparent molecular weights of OpnP and OpnT were 37,000 and 40,000, respectively. Two bands migrated with apparent molecular weights of approximately 46,000 and 48,000 and were assumed to consist of OpnC and OpnD. These results are representative of numerous separate experiments and outer membrane preparations.

Purification of OpnP. 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. The method used to purify OpnP was derived from that used to

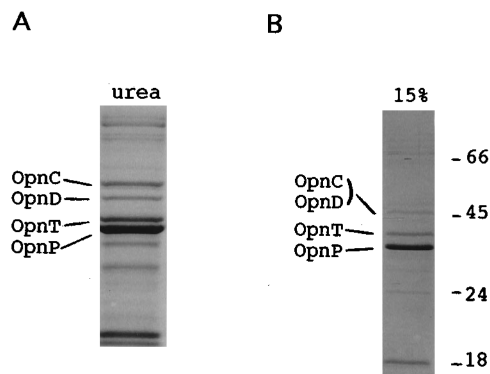

FIG. 1. Outer membrane proteins of mid-log *X. nematophilus*. Strain AN6/1 was grown in LB at 30°C, and outer membrane proteins were run on SDS-polyacrylamide gels and stained with Coomassie blue. (A) 8 M urea in 10% polyacrylamide; (B) 15% polyacrylamide.

purify outer membrane proteins of *E. coli*. After outer membrane pellets were obtained, 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 a molecular weight of 30,000. OpnP migrated as a 37,000-molecular-weight protein on SDS-polyacrylamide gels, even without being boiled, indicating that it was not a heat-modifiable protein. In contrast, it has been shown that OmpF and OmpC run as high-molecular-weight smears when the boiling step was omitted (19). A comparison of the properties of OpnP and OmpF with those of 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 with those of OmpF, OmpC, and OmpA of *E. coli* (Table 1) (29). The composition of OpnP was very similar to those of OmpF and OmpC and was distinctly different from

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

Amino acid[a]	Amino acid composition (no. of amino acid residues)			
	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[b]	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

[a] Asterisks indicate the amino acids that distinguish OpnP and OmpF or OmpC from OmpA.

[b] ND, not determined.

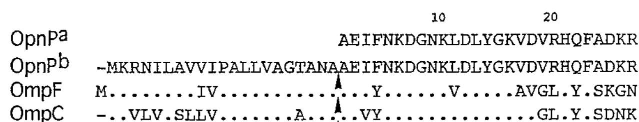

FIG. 2. N-terminal amino acid sequences of porin proteins. In the sequences of OmpF and OmpC (29), a dot indicates identity and a dash indicates a deletion. The N-terminal sequence of OpnP protein (OpnP[a]) and the deduced amino acid sequence from the nucleotide sequence of *opnP* (OpnP[b]) are shown. Arrowheads show the signal peptide cleavage site.

that of OmpA. In particular, OpnP, OmpF, and OmpC contained a single histidine residue, relatively few proline residues, and many phenylalanine residues.

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 sequences of OmpF and OmpC (Fig. 2) (29), revealing that OpnP shared 59 and 70% sequence identities with OmpF and OmpC, respectively. We have recently isolated the gene coding for OpnP (27a). 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 sequences of the signal peptides of OpnP, OmpF, and OmpC showed a remarkable degree of sequence identity (Fig. 2). OpnP shared 86 and 59% sequence identities with OmpF and OmpC, respectively. This analysis also showed that the signal peptide cleavage site was identical in all three proteins. On the basis of 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.*

Growth phase regulation. *X. nematophilus* is exposed to very different environments in the nematode gut and in the insect hemolymph. 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-polyacrylamide gel system (Fig. 3A). OpnC, -D, -T, and -P were produced in cells grown at 30°C and harvested during early-log-phase growth (Fig. 3A, 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 blot analysis using an *opnP* probe suggested that the steady-state level of *opnP* mRNA was markedly reduced during stationary-phase growth (12a). In contrast, *E. coli* did not exhibit a growth phase-dependent modulation of outer membrane protein production (Fig. 3A, lanes 1 and 2). The apparent molecular weights of OpnA, OpnB, and OpnS were 52,000, 49,000 and 45,000, respectively (Fig. 3B).

Temperature regulation. One of the environmental conditions that the bacterium and nematode must adapt to is changes in both the daily and seasonal ambient temperature. Outer membrane proteins in *E. coli* have been shown to be thermally regulated (3). In order to look for temperature-

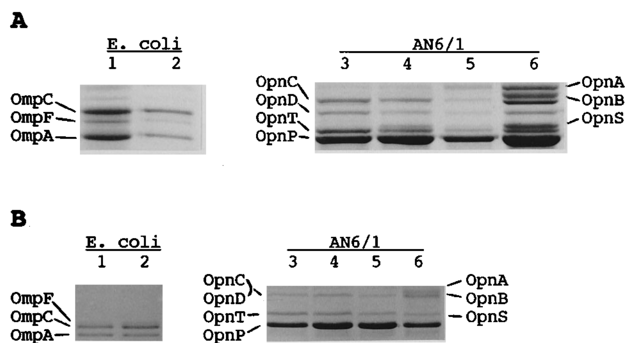

FIG. 3. Growth phase regulation of outer membrane proteins. (A) 8 M urea in 10% polyacrylamide; (B) 15% polyacrylamide. *E. coli* (MC4100), grown in LB at 37°C, was harvested at mid log phase (lane 1) and stationary phase (lane 2). *X. nematophilus* AN6/1, grown in LB at 30°C, was harvested at early log phase (lane 3), mid log phase (lane 4), late log phase (lane 5), and stationary phase (lane 6).

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). The optimal growth temperature of *X. nematophilus* in LB was 30°C. The production of several outer membrane proteins was dramatically modulated by changes in growth temperature. OpnB was produced at 19 to 30°C but was markedly repressed in cells growing at 34°C. In contrast, OpnT was strongly induced by elevated growth temperatures. This protein was not expressed in cells grown at 19 and 23°C, was present in cells grown at 27°C, and was produced at higher levels in cells grown at 30 and 34°C. OpnA exhibited a stringent temperature-dependent expression, being produced only in cells grown at 30°C.

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 growth phase 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. 5, 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.

DISCUSSION

X. nematophilus inhabits two distinct ecological niches, the gut of nonfeeding entomopathogenic nematodes and the hemolymph of the larvae of many different insects. The outer membrane of *X. nematophilus* presumably provides important functions that allow the bacterium to adapt to and survive under these different environmental conditions. Since the outer membrane proteins had not been characterized in *X. nematophilus*, we have studied their production in this bacte-

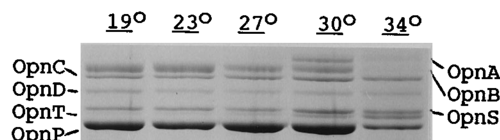

FIG. 4. Temperature regulation of outer membrane proteins. Cells were grown in LB to stationary phase at the temperatures (in degrees Celsius) over the lanes. Note the induction of OpnA and OpnT at 30°C and the repression of OpnA and enhanced production of OpnT at 34°C.

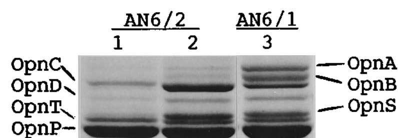

FIG. 5. Secondary-form versus primary-form variant of *X. nematophilus*. Strain AN6/2 (secondary form) at mid log phase (lane 1) and stationary phase (lane 2) and strain AN6/1 (primary form) at stationary phase (lane 3) are shown.

rium. We show that cells growing exponentially at 30°C produce several outer membrane proteins. The most highly expressed protein, OpnP, was purified, and its N-terminal sequence was determined. Protein sequence analysis reveals that the N-terminal sequence of OpnP is strongly similar to the porin proteins OmpF and OmpC of *E. coli*. On the basis of its high level of expression and the strong sequence similarity to OmpF and OmpC, OpnP is predicted to be the predominant porin protein in the outer membrane of *X. nematophilus*. The outer membrane proteins designated OpnC, OpnD, and OpnT were also produced during exponential growth conditions. We found that OpnC, OpnD, and OpnT were not recognized by antisera raised against purified OpnP (unpublished data). This result suggests that these proteins are not homologous to OpnP. Characterizing the pore properties of OpnP and elucidating the functions of the other Opns expressed during exponential growth will help define the role that cell surface properties of *X. nematophilus* plays in the unique life cycle of this bacterium.

During stationary-phase growth, an additional protein, OpnB, was induced in the primary-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 OpnB in cells growing in culture media may mimic the stationary-growth-phase environment that occurs in the insect hemolymph. We speculate that OpnB 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. Two other outer membrane proteins, OpnA and OpnS, were produced at low levels in early- and mid-log-phase growth and appear as major constituents of the outer membranes of stationary-phase cells. OpnS, like OpnB, was strongly induced in stationary-phase cells grown at a broad range of temperatures. OpnA was highly produced in stationary-phase primary cells grown at 30°C but was not produced at lower growth temperatures. 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 lower than 30°C, OpnA would not be a strong candidate for a cell surface protein that is important in establishing and/or maintaining the nemotode-bacterium symbiosis.

We also found that OpnB is not induced in the secondary-form cells grown under stationary-phase conditions. If OpnB is essential for the association with or survival within the nematode gut, secondary-form cells may not efficiently enter the symbiotic interactions with the nematode. The inefficient production of nematodes grown on the secondary form of *X. nematophilus* (2) may be due, in part, to the absence of OpnB and other cell surface molecules that play a role in adhesion and colonization (7).

The properties of the outer membrane have been shown to contribute to the antihemocytic nature of *X. nematophilus* (11). Treatment of the bacterium with carbohydrases and proteases result 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 moiety of lipopolysaccharide were found to be less virulent than wild-type cells (12). Avirulent forms of *X. nematophilus* have also recently been isolated by a Tn*5* mutagenesis approach (28). It will be of interest to determine whether the outer membrane proteins we have characterized play a role in virulence and in the ability of the bacterium to evade the host nonself response.

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