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Functional and regulatory analysis of the OmpF-like porin, OpnP, of the symbiotic bacterium *Xenorhabdus nematophilus*

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Summary

The function and novel regulation of OpnP of the symbiotic/pathogenic bacterium, *Xenorhabdus nematophilus* was studied. *In vitro* pore-function analysis of purified OpnP indicated that the single-channel-conductance values were similar to that measured for the porin protein, OmpF, of *Esherichia coli*. Nucleotide sequence analysis revealed that the mature OpnP protein contained 348 amino acid residues and shared 55% amino acid sequence identity with OmpF. Similar to *ompF*, *opnP* mapped between *asnS* and *aspC*. The 16 transmembrane β-sheet structures and the internal loop 3 were highly conserved, while the remaining external loop domains were more divergent. Primer extension analysis identified the start site of transcription of *opnP*. A σ^{70}-type promoter, a perfect 20 bp OmpR-binding site, and a binding site for the antisense molecule, *micF* RNA, were found in the upstream region of *opnP*. While the overall sequence identity of the *asn–opnP–aspC* region was high, the intergenic region between *asnS* and *opnP* had diverged markedly. The *asnS–opnP* region was 313 bp shorter than the intergenic region between *asnS* and *ompF* and lacked the OmpR-binding site that is required for *ompF* repression by high osmolarity in *E. coli*. Results from osmolarity-shift experiments indicated that OpnP was not repressed by high osmolarity. It was also found that OpnP was thermally regulated.

Introduction

The outer membrane of Gram-negative bacteria represents a diffusion barrier that regulates the passage of solutes into and out of the cell (Hancock, 1991; Nikaido, 1993). Small hydrophilic nutrients diffuse across the outer membrane of *Escherichia coli* by passing through water-filled pores formed by the outer membrane porin proteins, OmpF and OmpC (Nikaido, 1992). These general diffusion pores are formed by homotrimeric association of the respective porin proteins (Nikaido, 1993). Diffusion of nutrients through the OmpF pore is considerably faster than that of the OmpC pore. Besides forming a non-specific diffusion pore, OmpF also allows passage of several antibiotics, including β-lactams, across the outer membrane (Benz, 1994; Nikaido, 1994). The overall amino acid sequence identity and sequence conservation between OmpF and OmpC is 55% and 73%, respectively (Mizuno *et al.*, 1983). While the nucleotide sequences of *ompC* genes from several bacteria is known, the sequence of the *ompF* gene has only been determined, so far, in *E. coli* (Jeanteur *et al.*, 1994).

The three-dimensional structure of OmpF has been elucidated at 2.4 Å resolution (Cowan, 1994; Cowan *et al.*, 1992). The β-barrel structure formed by OmpF consists of 16 antiparallel transmembrane β strands surrounding a water-filled channel and eight extracellularly exposed loops. The longest loop, L3, bends into the channel forming the narrow constriction through which solutes must pass. Resolution of the crystal structures *of PhoE* (Cowan *et al.*, 1992), the protein that forms the anion-specific pore in *E. coli*, and the porin protein of *Rhodobacter capsulatus* (Weiss *et al.*, 1991), has shown that the 16 β-stranded structure of these porin proteins is highly conserved. Comparison of the deduced amino acid sequence of the porin proteins revealed that the β strands share a high degree of sequence identity, while the extracellular loop regions are more divergent.

OmpF production is affected by numerous environmental conditions (Csonka, 1989; Forst and Roberts, 1994). The *ompF* gene is regulated both transcriptionally and post-transcriptionally. The two-component response regulator OmpR, and the sensor histidine kinase, EnvZ, regulate the expression of *ompF* at the transcriptional level. OmpR interacts with a high affinity binding region (−100/−70) to activate *ompF* expression (Forst and Roberts, 1994; Igo *et al.*, 1990; Mizuno and Mizushima, 1990). The kinase/phosphatase activities of EnvZ modulate the

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levels of OmpR-phosphate in the cell (Forst *et al.*, 1990; Russo and Silhavy, 1991). Under high-osmolarity conditions, the levels of OmpR-phosphate increase resulting in the binding of OmpR to a lower affinity site (−380/−350). Binding to this site is essential for the repression of *ompF* (Huang *et al.*, 1994; Rampersaud *et al.*, 1994). OmpF production is also regulated post-transcriptionally by the antisense RNA molecule, *micF* RNA, which forms an RNA–RNA hydrid with the untranslated region of *ompF* mRNA (Andersen *et al.*, 1989; Andersen and Delihas, 1990; Schmidt *et al.*, 1995). In cells exposed to increased temperatures (Coyer *et al.*, 1990), intermediate osmolarity (Ramani *et al.*, 1994), or oxidative stress (Chou *et al.*, 1993), the production of *micF* RNA is elevated and the level of *ompF* mRNA concommitantly decreases. The gene encoding *micF* RNA has been analysed in *Salmonella typhimurium*, *Klebsiella pneumoniae* (Esterling and Delihas, 1994) and *Serratia marcescens* (Hutsel and Worobec, 1994). A high degree of conservation of nucleotide sequence in the region of *micF* RNA that binds *ompF* RNA, exists between these species (Esterling and Delihas, 1994). However, the *ompF* gene has not yet been identified in these organisms.

Most studies on the regulation and function of porin proteins have focussed on bacteria that can exist in a free-living state. In this report, we describe the structural and regulatory features of OpnP from the symbiotic/pathogenic bacterium *Xenorhabdus nematophilus* (Akhurst and Boemare, 1990). We have recently shown that OpnP constitutes approximately 60% of the total outer membrane proteins of *X. nematophilus* (Leisman *et al.*, 1995). The first 27 residues of the OpnP were found to share 59% amino acid sequence identity with OmpF of *Escherichia coli*. *X. nematophilus*, a Gram-negative bacterium belonging to the family *Enterobacteriacea*, is carried in a species-specific symbiotic association in the gut of the infective juvenile stage of the entomopathogenic nematode, *Steinernema carpocapsae*. The infective juvenile nematode locates the larvae of several different insects, penetrates into the haemocoele of the host and releases the bacterial symbiont into the nutrient-rich haemolymph (Akhurst and Dunphy, 1993). The bacteria proliferate, secrete numerous extracellular products, and participate in the killing of the insect host. The developing nematode feeds on the bacteria and eventually leaves the insect cadaver carrying its symbiotic partner, *X. nematophilus*. The *ompR* and *envZ* genes of *X. nematophilus* have recently been isolated and nucleotide sequence analysis indicated that the OmpR proteins of *X. nematophilus* and *E. coli*, as well as the cytoplasmic phosphotransfer domain of the EnvZ proteins, were highly conserved (Tabatabai and Forst, 1995). In contrast, the periplasmic domain of the EnvZ proteins had completely diverged. In this study we showed that the amino acid sequences of OpnP and OmpF were strongly conserved, while the regulatory region involved in the repression of *ompF* had markedly diverged. The divergence of this regulatory pathway may play an important role in the ability of the symbiotic bacterium, *X. nematophilus*, to adapt to its unique ecological niche.

Results

Porin function of OpnP

We had previously purified the major outer membrane protein, OpnP, from *X. nematophilus* as a monomer (Leisman *et al.*, 1995). The apparent molecular mass of OpnP was 30 kDa. N-terminal sequence analysis of the purified protein revealed that 16 out of 27 amino acids were identical to OmpF. The amino acid composition of mature OpnP was shown to be similar to that of OmpF. To study the pore-forming ability of OpnP, we measured the stepwise conductance increases as the purified protein inserted into a planar lipid bilayer (Benz and Hancock, 1981). Stepwise conductance increases are considered to represent the insertion of individual proteins into the membrane. The magnitude of such conductance increases is related to the limiting diameter of the water-filled channel and its shape. Stepwise increases in conductance 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 centred on a single mean for each of the salt solutions used (Fig. 1). Experiments with different salt solutions showed that an increased salt concentration resulted in a proportional increase in conductance, which indicated that OpnP formed large, water-filled channels (Table 1). When the salt solution contained a large cation (a hydrated Li^+ ion) a slight decrease in mobility was seen, whereas the presence of a large anion (CH_3COO^-) did not decrease the

Table 1. Average single-channel-conductance measurements of OpnP and OmpF.

		Average Single-channel Conductance		
Salt	n[a]	OpnP	OmpF (trimer)	OmpF[b] (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

a. n represents the number of OpnP channels observed for each salt solution.
b. OmpF (monomer) represents the predicted value calculated as one third of the conductance of the trimer. The OmpF porin measurements were obtained from Benz *et al.* (1985).

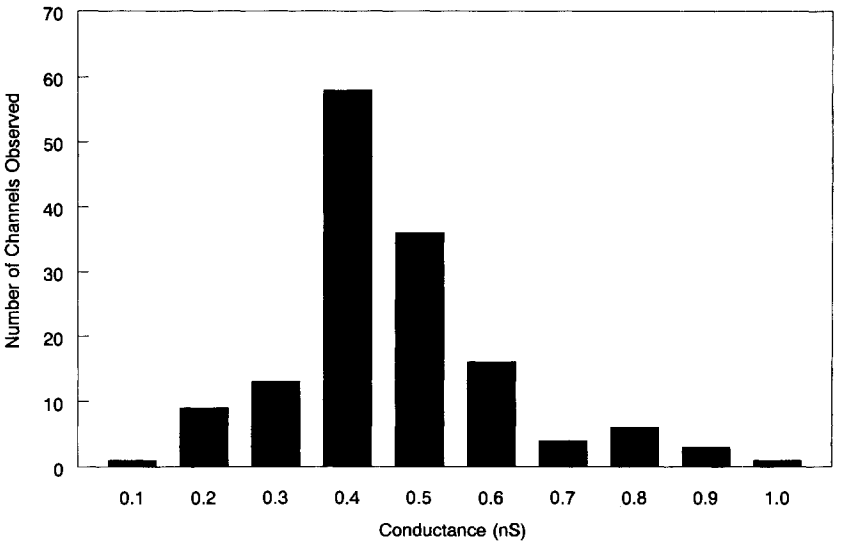


Fig. 1. Single-channel-conductance measurements. Histogram showing distribution of channel sizes observed with OpnP in 1.0 M KCl.

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 (Benz *et al.*, 1985), which showed a permeability ratio of cations to anions of 1.8:1. The average single-channel-conductance values for the OpnP monomer were similar to the predicted values for an OmpF monomer (Benz *et al.*, 1985).

Nucleotide sequence analysis of OpnP

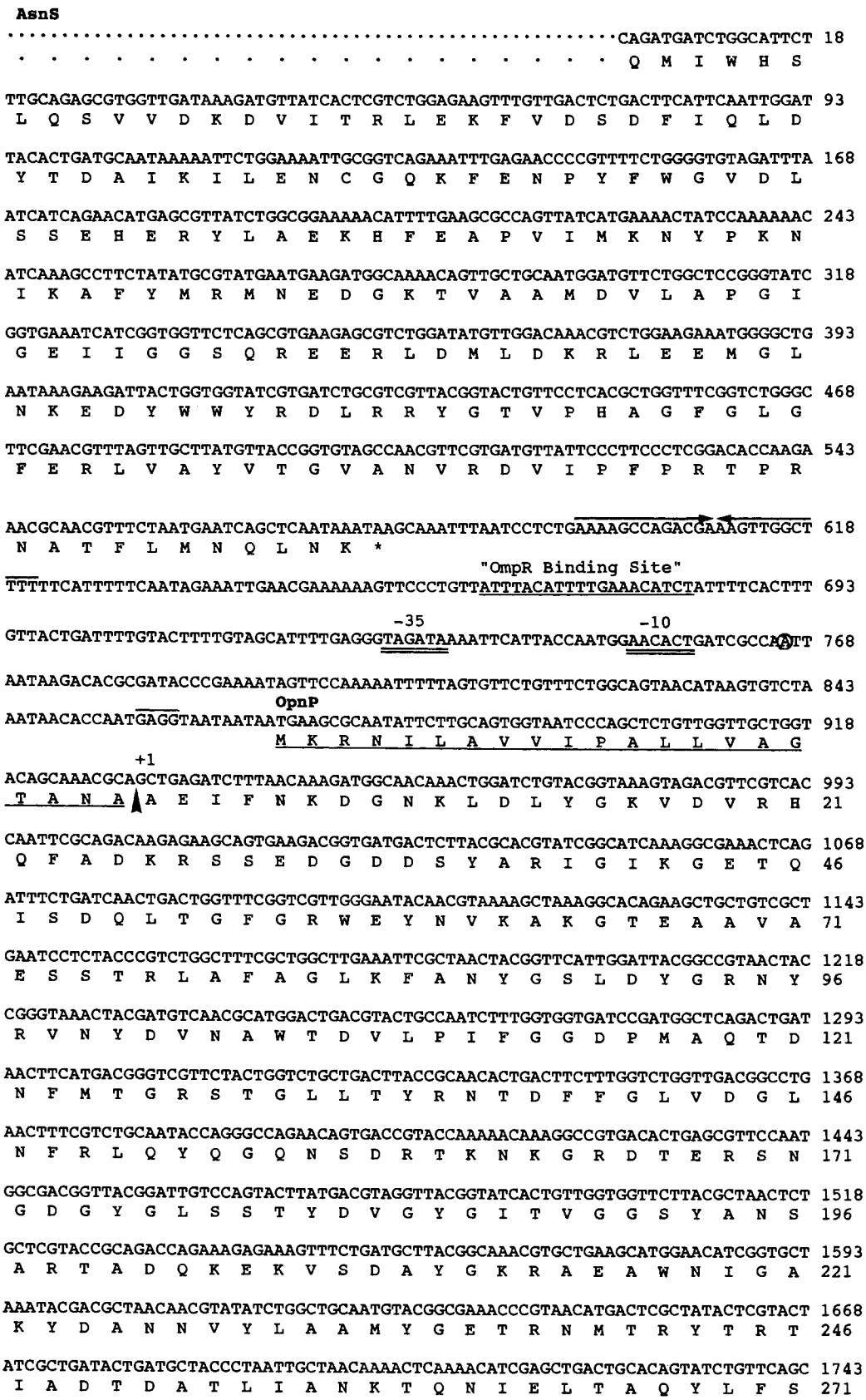
The above pore-function studies suggested that the OpnP protein formed OmpF-like pores in the outer membrane of *X. nematophilus*. To further analyse OpnP, we isolated the *opnP* gene by screening our λgt10 *X. nematophilus* library with a DNA fragment containing *E. coli ompF* as a probe (see the *Experimental procedures*). A subclone containing *opnP* and the flanking open reading frames (ORFs), on a 3.2 kb *Eco*R1–*Hin*dIII fragment, was isolated in this manner.

The nucleotide sequence of *opnP* and the partial sequences of the upstream and downstream ORFs were determined (Fig. 2). The deduced amino acid sequence indicated that OpnP contained 369 residues. The N-terminal amino acid sequence deduced from the nucleotide sequence analysis of *opnP* was identical to that obtained from the analysis of the purified OpnP protein (Leisman *et al.*, 1995). Cleavage of the signal peptide occurred between the Ala residue at −1 and the Ala at +1 (Fig. 2). Comparison of the signal peptide sequence of OpnP and OmpF indicated that 19 out of 21 amino acid residues were identical (Fig. 3). The mature OpnP protein contained 348 amino acid residues. The C-terminal residue encoded by *opnP* was found to be phenylalanine, which is found in all porin genes sequenced so far (Jeanteur *et al.*, 1994).

A consensus ribosome-binding site (GAGG) was identified nine nucleotides upstream of the translation initiation codon (AUG) of OpnP. The deduced amino acid sequence of the C-terminal 192 residues of the upstream ORF was found to share 80% identity with AsnS (asparaginyl tRNA synthetase) of *E. coli*. A putative transcriptional termination sequence was identified immmediately downstream of the TAA stop codon of AsnS of *X. nematophilus* (Huang *et al.*, 1994). The deduced amino acid sequence of the 23 N-terminal residues of the downstream ORF was found to share 87% identity with AspC (aspartate aminotransferase) of *E. coli*. These results indicated that the *opnP* gene mapped between *asnS* and *aspC* (Fig. 2), which are the same genes that flank *ompF* in *E. coli*.

Sequence comparsion of OpnP and OmpF

Comparison of the deduced amino acid sequences of OpnP and OmpF revealed that they shared 55% identity (Fig. 3). Secondary-structure predictions of OpnP indicated that this protein also contained a high degree of β-strand structure (data not shown). Resolution of the X-ray crystal structure showed that OmpF was composed of 16 antiparallel transmembrane β strands (Cowan *et al.*, 1992). Based on the high degree of amino acid sequence identity between OpnP and OmpF, the OpnP protein sequence could be superimposed on the known secondary structure of OmpF. This analysis revealed that the two proteins shared 63% amino acid identity within the transmembrane β-sheet sequences (see the underlined sequences in Fig. 3) and that only transmembrane region 9 (M9) contained amino acid insertions. This pattern of structural


AsnS
CAGATGATCTGGCATTCT 18
Q M I W H S
TTGCAGAGCGTGGTTGATAAAGATGTTATCACTCGTCTGGAGAAGTTTGTTGACTCTGACTTCATTCAATTGGAT 93
L Q S V V D K D V I T R L E K F V D S D F I Q L D
TACACTGATGCAATAAAAATTCTGGAAAATTGCGGTCAGAAATTTGAGAACCCCGTTTTCTGGGGTGTAGATTTA 168
Y T D A I K I L E N C G Q K F E N P Y F W G V D L
ATCATCAGAACATGAGCGTTATCTGGCGGAAAAACATTTTGAAGCGCCAGTTATCATGAAAACTATCCAAAAAAC 243
S S E H E R Y L A E K H F E A P V I M K N Y P K N
ATCAAAGCCTTCTATATGCGTATGAATGAAGATGGCAAAACAGTTGCTGCAATGGATGTTCTGGCTCCGGGTATC 318
I K A F Y M R M N E D G K T V A A M D V L A P G I
GGTGAAATCATCGGTGGTTCTCAGCGTGAAGAGCGTCTGGATATGTTGGACAAACGTCTGGAAGAAATGGGGCTG 393
G E I I G G S Q R E E R L D M L D K R L E E M G L
AATAAAGAAGATTACTGGTGGTATCGTGATCTGCGTCGTTACGGTACTGTTCCTCACGCTGGTTTCGGTCTGGGC 468
N K E D Y W W Y R D L R R Y G T V P H A G F G L G
TTCGAACGTTTAGTTGCTTATGTTACCGGTGTAGCCAACGTTCGTGATGTTATTCCCTTCCCTCGGACACCAAGA 543
F E R L V A Y V T G V A N V R D V I P F P R T P R
AACGCAACGTTTCTAATGAATCAGCTCAATAAATAAGCAAATTTAATCCTCTGAAAAGCCAGACGAAAGTTGGCT 618
N A T F L M N Q L N K *
"OmpR Binding Site"
TTTTTCATTTTTCAATAGAAATTGAACGAAAAAAGTTCCCTGTTATTTACATTTTGAAACATCTATTTTCACTTT 693
-35
-10
GTTACTGATTTTGTACTTTTGTAGCATTTTGAGGGTAGATAAAATTCATTACCAATGGAACACTGATCGCCAATT 768
AATAAGACACGCGATACCCGAAAATAGTTCCAAAAATTTTTAGTGTTCTGTTTCTGGCAGTAACATAAGTGTCTA 843
OpnP
AATAACACCAATGAGGTAATAATAATGAAGCGCAATATTCTTGCAGTGGTAATCCCAGCTCTGTTGGTTGCTGGT 918
M K R N I L A V V I P A L L V A G
+1
ACAGCAAACGCAGCTGAGATCTTTAACAAAGATGGCAACAAACTGGATCTGTACGGTAAAGTAGACGTTCGTCAC 993
T A N A A E I F N K D G N K L D L Y G K V D V R H 21
CAATTCGCAGACAAGAGAAGCAGTGAAGACGGTGATGACTCTTACGCACGTATCGGCATCAAAGGCGAAACTCAG 1068
Q F A D K R S S E D G D D S Y A R I G I K G E T Q 46
ATTTCTGATCAACTGACTGGTTTCGGTCGTTGGGAATACAACGTAAAAGCTAAAGGCACAGAAGCTGCTGTCGCT 1143
I S D Q L T G F G R W E Y N V K A K G T E A A V A 71
GAATCCTCTACCCGTCTGGCTTTCGCTGGCTTGAAATTCGCTAACTACGGTTCATTGGATTACGGCCGTAACTAC 1218
E S S T R L A F A G L K F A N Y G S L D Y G R N Y 96
CGGGTAAACTACGATGTCAACGCATGGACTGACGTACTGCCAATCTTTGGTGGTGATCCGATGGCTCAGACTGAT 1293
R V N Y D V N A W T D V L P I F G G D P M A Q T D 121
AACTTCATGACGGGTCGTTCTACTGGTCTGCTGACTTACCGCAACACTGACTTCTTTGGTCTGGTTGACGGCCTG 1368
N F M T G R S T G L L T Y R N T D F F G L V D G L 146
AACTTTCGTCTGCAATACCAGGGCCAGAACAGTGACCGTACCAAAAACAAAGGCCGTGACACTGAGCGTTCCAAT 1443
N F R L Q Y Q G Q N S D R T K N K G R D T E R S N 171
GGCGACGGTTACGGATTGTCCAGTACTTATGACGTAGGTTACGGTATCACTGTTGGTGGTTCTTACGCTAACTCT 1518
G D G Y G L S S T Y D V G Y G I T V G G S Y A N S 196
GCTCGTACCGCAGACCAGAAAGAGAAAGTTTCTGATGCTTACGGCAAACGTGCTGAAGCATGGAACATCGGTGCT 1593
A R T A D Q K E K V S D A Y G K R A E A W N I G A 221
AAATACGACGCTAACAACGTATATCTGGCTGCAATGTACGGCGAAACCCGTAACATGACTCGCTATACTCGTACT 1668
K Y D A N N V Y L A A M Y G E T R N M T R Y T R T 246
ATCGCTGATACTGATGCTACCCTAATTGCTAACAAAACTCAAAACATCGAGCTGACTGCACAGTATCTGTTCAGC 1743
I A D T D A T L I A N K T Q N I E L T A Q Y L F S 271

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GATTTAGGCCTGAAGCCATCTCTGGCATACGTTCAGTCTAAAGGTAAAGACTTGACTGAAGGTAAAGGCTTCAAT 1818
 D  L  G  L  K  P  S  L  A  Y  V  Q  S  K  G  K  D  L  T  E  G  K  G  F  N  296

GGAGATTTGGTTAAATACGTTTCTGTAGGTACTTACTACTACTTTAACAAAAACCTGTCTACTTACGTTGATTAC 1893
 G  D  L  V  K  Y  V  S  V  G  T  Y  Y  Y  F  N  K  N  L  S  T  Y  V  D  Y  321

AAAATCAATCTGCTGAAGAAAGACAACGAGCTGGGTGTTAATGCTCGTAATGTATTCGGTGTTGGTCTGACTTAT 1968
 K  I  N  L  L  K  K  D  N  E  L  G  V  N  A  R  N  V  F  G  V  G  L  T  Y  346

CAGTTCTAATCACTGCTGATAAACGGTTTAACAAAAAATTAAACACATTTATCTCAGAAAGACCCGTCCGGCGCT 2043
 Q  F  *                                                                       348

GCTTTTTAAATTTTATCTCTCTGTTTTTTCAGGCTTTTTTCTCCTTCGTAAACTACTTCACAAGATTTTATTCTT 2118

TTTGCTACAAGTAGTTGGCAAATGAGTTCCTTAGCGTTACCCTGAGCGGCGTGTAACTTATTTTGGGCTTAATCC 2193
                          AspC
TCCGAGTCTTGGAATCAAAAAAAATGTTTGAAAAAATCACAGCAGCACCGGCAGACCCTATTCTTGGTTTAGCGG 2268
                          M  F  E  K  I  T  A  A  P  A  D  P  I  L  G  L  A

ATAGTTTTAAAGCGGAT·····                                                      2285
D  S  F  K  A  D  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·  ·
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Fig. 2. Nucleotide sequence of *opnP* and partial sequence of *asnS* and *aspC*. The deduced amino acid sequences are shown below the nucleotide sequence. Inverted arrows at the end of *asnS* indicate the putative termination of transcription. The *ompC*-like OmpR binding site is underlined and the −35 and −10 promoter elements are double underlined. The circled nucleotide 'A' denotes the start of transcription as determined by primer extension analysis. The consensus ribosome binding site 'GAGG' is denoted by a single line above the nucleotide sequence. The underlined amino acid sequence indicates the signal peptide. The arrow between Ala at −1 and Ala at +1 shows the site at which the signal peptide is cleaved, and +1 indicates the first amino acid of the mature OpnP protein. The dots at the beginning of *asnS* and at the end of *aspC* represent nucleotide and amino acid sequences that were not completed in this study (see the *Experimental procedures*).

conservation was identical to that found for other members of the OmpF porin family (Jeanteur *et al.*, 1994). The alternating hydrophobic residues in the β-strand region, predicted to face the hydrophobic core of the membrane, were also conserved in OpnP. In marked contrast, the extracellular loop regions located between β-strands 1 and 2, 3 and 4, etc. were very divergent. One exception was the large loop 3 (located between β-strands 5 and 6)

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          Signal Peptide   +1
OpnP  -MKRNILAVVIPALLVAGTANAAEIFNKDGNKLDLYGKVDVRHQFADKRS 28
OmpF  M·····IV·····Y·····V·····AVGL·Y·SKGNG 28
                                  1

OpnP  SE----DGDDSYARIGIKGETQISDQLTGFGRWEYNVKAKGTEAAVAE-- 72
OmpF  ENSYGGN··MT···L·F·····NSD···Y·Q····FQGNNS·G·D·QTG 78
            2              3

OpnP  SSTRLAFAGLKFANYGSLDYGRNYRVNYDVNAWTDVLPIFGGDPMAQTDN 122
OmpF  NK·····Y·DV··F·····G·V··ALGY··M··E····-T·YS·D 127
        4     5

OpnP  FMTGRSTGLLTYRNTDFFGLVDGLNFRLQYQGQNSDRTKNKGRDTER-SN 171
OmpF  ·FV··VG·VART··SN·····AV··L·K·-E·-------··A·R·· 170
          6          7                              

OpnP  GDGYGLSSTYDV-GYGITVGGSYANSARTADQKEKVSDAYGKRAEAWNIG 220
OmpF  ···V·G·IS·EYE·F··-·-·A·GAAD··NL·-·AQPLGN··K··Q·AT· 217
        8        9                          10

OpnP  AKYDANNVYLAAMYGETRNMTRYTRTIADTDATLIANKTQNIELTAQYLF 270
OmpF  L·····I····N·····A·PI·NKFTN·SG--F·····DVL·V···Q· 265
          11                          12

OpnP  SDLGLKPSLAYVQSKGKDLTEGKGFNGDLVKYVSVGTYYYFNKNLSTYVD 320
OmpF  -·F··R··I··TK··A··V-··I·-DV···N·FE··AT·····M····· 312
          13                  14        15

OpnP  YKINLLKKDNELGVNARNVFGVGLTYQF 348
OmpF  ·I··QIDS··K···GSDDTVA··IV··· 340
                    16
```

Fig. 3. Comparison of the amino acid sequence of OpnP and OmpF. Dots represent identical amino acids and dashes indicate insertions or deletions. The signal peptide is indicated by the line above the amino acid sequence of OpnP. The first amino acid of the mature OpnP and OmpF proteins is denoted by +1. The previously determined β-strands of OmpF (Cowan *et al.*, 1992) are underlined and numbered.

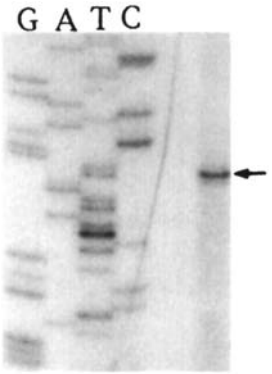

Fig. 4. Primer extension analysis of the transcription initiation site of *opnP*. The order of sequencing lanes is listed above, and the extension product is indicated by the arrow. The sequence ladder and extension product were generated using the non-coding strand of DNA.

which shared 52% amino acid sequence identity with the same region in OmpF. This loop is known to be important in constricting the pore diameter of OmpF. In OmpF, the surface of this constriction zone contains charge polarity because of the positively charged side-chains Lys-16, Arg-42, -82, and -132, and the negatively charged side-chains Asp-113, and Glu-117. In OpnP, these charged amino acids were conserved except that an Ile residue was substituted for Glu-117. This observation is consistent with the somewhat reduced cation selectivity of OpnP compared to OmpF.

Identification of the start of transcription and regulatory sequences of opnP

In order to analyse the regulatory region of *opnP*, the transcription initiation site was determined by primer extension analysis (Fig. 4). The start of transcription mapped to A-766 which is located 102 bp upstream of the AUG start codon (Fig. 2). A putative −35 promoter element, TAGATA, was identified that closely resembled the consensus sequence, TTGACA, of a σ^{70} promoter. A consensus −10 element could also be identified 17 bp downstream of the −35 sequence. In contrast, the −35 and −10 promoter elements of *ompF* contain poorly conserved consensus sequences (Mizuno *et al.*, 1983).

Other aspects of the regulatory regions of *opnP* and *ompF* were found to be divergent. A 20 bp sequence that was identical to the OmpR-binding site of *ompC* (Mizuno *et al.*, 1988) was identified approximately 80 bp upstream of the start of transcription of *opnP* (Fig. 2). In addition, the intrinsic DNA-bending sequence (Mizuno and Mizushima, 1990) and integration host factor (IHF)-binding site (Ramani *et al.*, 1992) found in this region of *ompF* did not exist in the *opnP* regulatory region. Most strikingly, the intergenic region between *asnS* and *opnP* had dramatically diverged from the same upstream region of *ompF*. The intergenic region between *asnS* and *ompF* contains 601 bp. The intergenic region between *asnS* and *opnP* was 313 bp shorter than the equivalent region in *ompF* and the low-affinity OmpR-binding site required for the repression of *ompF* (Huang *et al.*, 1994) was not present in the upstream region of *opnP*. Taken together, these results indicated that the regulatory sequences of *opnP* and *ompF* had significantly diverged. This finding raised the question of whether OpnP could be repressed by growth in high-osmolarity growth media.

Pulse-labelling of OpnP under high-osmolarity conditions

We had previously used Grace's Insect Cell Culture Medium to grow *X. nematophilus* in its natural biological growth environment while characterizing the outer membrane proteins of this bacterium. We found that *X. nematophilus* grew optimally in Grace's medium supplemented with 2.5–5% sucrose. The growth rate was noticeably reduced upon addition of 7.5% sucrose, while the cells began to lyse when 10% sucrose was added to the growth medium. Thus, growth in the presence of 7.5% sucrose represents an osmolarity-stress condition for *X. nematophilus*. To determine whether OpnP was repressed by high osmolarity, cells growing in Grace's medium were shifted to higher osmolarity medium (Grace's medium plus 7.5% sucrose) and pulse-labelled at the indicated time points (Fig. 5). OpnP production appeared to decrease slightly, subsequent to the shift to high-osmolarity conditions (Fig. 5, lanes 2, 3 and 4) but, by 30 min (Fig. 5, lane 5), the amount of OpnP produced was essentially the same as that produced for cells growing in Grace's media without sucrose (lane 1). In marked contrast, OmpF was almost completely repressed within 10 minutes after a shift to high-osmolarity conditions (Forst *et al.*, 1988). These results indicated that, unlike OmpF, OpnP was not repressed by growth in high-osmolarity conditions. Densitometric scanning of the autoradiograph shown in Fig. 5 revealed that OpnP constituted

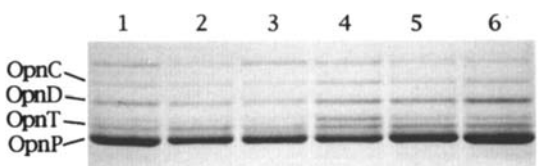

Fig. 5. Pulse-labelling of OpnP in cells grown under high-osmolarity conditions. Cells were grown in Grace's Insect Cell Culture Medium (lane 1) and pulse labelled with radioactive methionine. Cells were then shifted to Grace's medium containing 7.5% sucrose, and pulse-labelled at 1 min (lane 2), 5 min (lane 3), 15 min (lane 4), 30 min (lane 5) and 60 min (lane 6) after addition of sucrose. Outer membrane proteins were separated by SDS–PAGE and processed by autoradiography. In *E. coli*, both OmpF and OmpC are produced in cells grown in Grace's media (data not shown).

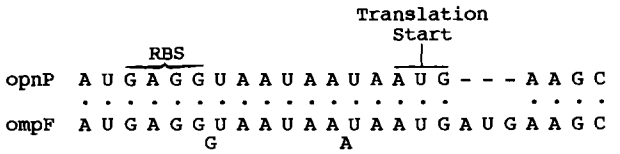

Fig. 6. Comparison of the *ompF* mRNA sequence of *E. coli* that binds to *micF* RNA with the equivalent sequence of *X. nematophilus opnP*. The 24 out of 26 contiguous nucleotides of *ompF* mRNA that base pair with *micF* RNA of *E. coli* are shown. The nucleotides 'G' and 'A' below the sequence of *ompF* do not base pair with *micF* RNA, and these nucleotides are not present in *opnP*. The AUG start site of translation is shown.

approximately 60% of the total outer membrane proteins of *X. nematophilus* (see the *Experimental procedures*).

De novo *synthesis of OpnP at elevated temperatures*

Further analysis of the regulatory region of *opnP* revealed that a putative *micF* RNA-binding site was present in the region of the mRNA of *opnP* encompassed by the ribosome-binding site and start of translation (Fig. 6). In *E. coli*, the antisense *micF* RNA molecule hydridizes to this region of *ompF* mRNA (Andersen and Delihas, 1990). Twenty one out of 26 positions of the *micF* RNA-binding region of *E. coli ompF* mRNA are conserved in the mRNA of *opnP*. Under elevated temperature conditions, formation of the *micF–ompF* RNA:RNA hybrid caused the levels of *ompF* mRNA to be reduced, with a concomittant decrease of OmpF production (Andersen *et al.*, 1989). To determine whether changes in growth temperature had an affect on OpnP production, cells were pulse-labelled at various growth temperatures (Fig. 7). We have found that *X. nematophilus* grows well between 19–30°C while the growth rate decreases as the temperature is elevated above 30°C. Cells were grown in Grace's medium at 26°C, 30°C and 34°C, and proteins were radiolabelled during exponential growth. The results shown in Fig. 7 indicated that at increased temperatures (30°C and 34°C) there was a noticeable reduction in the *de novo* synthesis of OpnP. The reduction of OpnP synthesis was not a result of an overall decrease in outer membrane protein synthesis since OpnD and OpnT were synthesized at the same or elevated levels (Fig. 7, lanes 2 and 3) as that found in cells grown at 26°C (Fig. 7, lane 1). Results from the densitometric scanning of the autoradiograph shown in Fig. 7 indicated that the level of OpnP produced in cells grown at 34°C was reduced approximately 25% relative to that produced in cells grown at 26°C (see the *Experimental procedures*).

Discussion

We have shown that the structural and functional aspects of OpnP, the major protein in the outer membrane of *X. nematophilus*, were closely similar to those of OmpF of *E. coli*. In contrast, the regulatory sequences that control the production of OpnP had diverged from the upstream regulatory sequences of *ompF*. OpnP, unlike OmpF, was not repressed by high-osmolarity growth conditions. As the *ompF* gene had not previously been sequenced in a bacterium other than *E. coli*, our results provide novel information concerning the structure and regulation of *ompF*-like genes in diverse Gram-negative bacteria.

Using planar lipid-bilayer analysis, OpnP was found to form relatively non-specific, water-filled channels. The OpnP 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 OpnP channel was slightly cation selective. OpnP was isolated as a stable monomer, while OmpF is functional as a trimer. The subunit:subunit interactions of the putative OpnP 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 OpnP trimer. Each monomer of the OmpF trimer contains a non-specific 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 OpnP 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, 1991).

OpnP and OmpF were highly conserved at the amino acid level. The transmembrane β strands and the internal Loop 3 contained more than 60% amino acid identity, while the M9 sequence and the external loop regions are more divergent. These data clearly indicate that OpnP belongs to the OmpF family of porin proteins (Jeanteur *et al.*, 1994). It has been noted that the sequence PEFGGD within loop 3 is highly conserved in this family of porins. In OpnP, this sequence was also conserved (PIFGGD) with the exception that Ile (I-111) replaced Glu (E) of

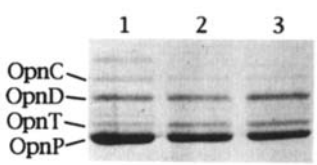

Fig. 7. Pulse-labelling of OpnP in cells grown at elevated temperatures. Cells were grown at 26°C (lane 1), 30°C (lane 2), and 34°C (lane 3) and radiolabelled during exponential growth.

OmpF. That differences in the sequence of loop 3 can significantly affect pore selectivity was demonstrated by the observation that substitution of Lys-125 of PhoE for the equivalent residue, Gly-131, of OmpF strongly contributed to the anion selectivity of the PhoE pore (Cowan *et al.*, 1992). In OpnP, Arg-97, Ile-111 and Asn-122 were substituted for Gly, Glu, and Asp, respectively, in OmpF, resulting in a net charge increase of +3 in loop 3 of OpnP. More detailed analysis of the OpnP pore may reveal important functional differences between this porin and OmpF.

Comparison of the upstream regulatory regions revealed that the absence of a repression site in *opnP* may account for the lack of repression of this gene by high-osmolarity conditions. The intergenic region between *asnS* and *opnP/ompF* was 313 bp shorter in *X. nematophilus* and lacked the low-affinity OmpR-binding site found between −380 and −360 of *ompF*. We show that OpnP was produced at high levels, irrespective of the osmolarity of the growth media. Three tandemly repeated 10 bp OmpR-binding sites have been identified in the −100/−45 region of *ompF* (Rampersaud *et al.*, 1994). DNA-bending sequences and IHF-binding sites are also present in this region. The *ompC* regulatory region contains tandem 20 bp OmpR-binding sites separated by a 10 bp spacer, and lacks the DNA-bending sequence and IHF-binding sites. The central *ompC* consensus binding site was identified as TGAAAC (Mizuno and Mizushima, 1990). In *opnP*, an OmpR-binding sequence (−103/−84) was found to be identical to the 20 bp OmpR-binding sequence of *ompC*, and a second consensus OmpR-binding site (TGTTAC) was identified 10 bp downstream of the −103/−84 sequence. The DNA-bending sequence and IHF-binding sites were not present in the regulatory region of *opnP*. These findings indicate that although *opnP* and *ompF* map to the same chromosomal location and are highly conserved structurally, the regulatory region of *opnP* was more closely similar to *ompC*. It was previously found that the OmpR proteins of *X. nematophilus* and *E. coli* shared 78% amino acid identity and the EnvZ protein of *X. nematophilus* was able to complement an *envZ*$^-$ strain of *E. coli* (Tabatabai and Forst, 1995). This information provided support for the conclusion that *opnP, ompF* and *ompC* are regulated by the same two-component signal-transduction pathway. These results also indicate that the expression of homologous porin genes of distinct bacterial species may be controlled differently, depending on the organization of the upstream regulatory regions of the respective genes. In a symbiotic bacterium such as *X. nematophilus*, which apparently does not exist as a free-living organism and therefore may not experience extremes in osmolarity, the repression of the major outer membrane porin may not be an essential adaptive response. As *X. nematophilus* inhabit the constant environments of the non-feeding, infective, juvenile-nematode gut and larval haemolymph, constitutive production of a porin that is optimally adapted to this unique ecological niche may circumvent the requirement to repress a major porin protein when variable and stressful conditions are encountered.

OpnP production was reduced at temperatures at or above 30°C. In *E. coli*, elevated levels of *micF* RNA were shown to be essential for the reduction of OmpF in cells grown at 42°C. The putative *micF* RNA-binding site identified in *opnP* mRNA contained 21 of the 24 nucleotides involved in the formation of the *micF/ompF* hybrid (Schmidt *et al.*, 1995). These findings suggested that *X. nematophilus* may possess a gene encoding *micF* RNA and that the *micF* RNA molecule is involved in the thermal regulation of OpnP production. The *micF* RNA gene has recently been shown to be phylogenetically conserved in several enteric bacteria and there is a particularly high degree of sequence conservation in the *micF* RNA-binding site (Esterling and Delihas, 1994). The sequences of the *micF* RNA molecules that bind to *ompF* RNA were shown to be 87% identical, while the region that follows the binding site contained a low level of nucleotide identity (32%). The *ompF* gene had previously only been sequenced in *E. coli*. In this regard, identification and analysis of the *micF* RNA gene in *X. nematophilus* could provide additional information concerning the role of antisense RNA regulation of *ompF*-like genes in Gram-negative bacteria.

Experimental procedures

Bacterial strain and growth media

The AN6/1 strain of *X. nematophilus* was used in this study (Leisman *et al.*, 1995). The growth media employed was Grace's Insect Cell Culture Medium (Gibco BRL). *X. nematophilus* was maintained and grown as described previously (Leisman *et al.*, 1995).

Purification of OpnP

Stationary-phase cells of *X. nematophilus* grown at 30°C were broken by passage through a French Press, and outer membrane proteins were obtained as described by Leisman *et al.* (1995). The membrane pellets were incubated overnight at 37°C with TES (50 mM Tris-HCl (pH 7.2), 5 mM EDTA, 1% SDS) and centrifuged (for 14 min at 353 000 × *g*). OpnP was solubulized by overnight incubation in TES containing 400 mM NaCl. OpnP was then chromatographed on a column (1.6 × 84 cm) of Sephacryl S-200 using, as the elution buffer, TES containing 400 mM NaCl.

Characterization of pore-forming ability

The pore-forming properties of OpnP were examined using a planar lipid-bilayer model membrane system as previously

described (Benz and Hancock, 1981; Benz *et al.*, 1985). As controls, several other porins including porins from *P. aeruginosa* (OprP), *Treponema pallidum*, *Helicobacter pylori*, and *Borrelia burgdorferi*, were tested. In all instances, lipid bilayers were made from 1.5% (w/v) oxidized cholesterol in *n*-decane. Single-channel-conductance measurements were obtained after forming a lipid bilayer across a 0.2 mm^2 hole separating two compartments of a Teflon chamber which contained an aqueous salt solution. 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. Stepwise conductance increases were recorded and measured for each different salt solution used. Zero-current membrane-potential experiments were performed in chambers with a 2.0 mm^2 hole separating compartments, each of which contained 6 ml of 0.1 M KCl. Next, 50 ng of protein was then 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 ml of 3.0 M KCl was added to one side of the membrane, while 100 ml of 0.1 M KCl was added to the other. Eight such additions subsequently were made, and differences in potential caused by preferential diffusion of one ion species, creating a potential that opposed the concentration gradient, were measured after each addition.

Enzymes, reagents, chemicals and kits

All restriction enzymes were purchased from Promega except *Hin*dIII which was purchased from United States Biochemical. The *X. nematophilus* chromosomal DNA library (Tabatabai and Forst, 1995) was made in Promega Protoclone Lambda λgt10 using the Packagene System (Promega). *opnP*-containing DNA fragments were purified using the Gene Clean II Kit from Bio 101. Nucleotide sequence analysis was carried out using a Sequenase Version 2.0 Kit from United States Biochemical. DNA was amplified using a Cetus Polymerase Chain Reaction (PCR) Kit from Perkin Elmer Cetus. Cycle sequencing was carried out using the Cycle Sequencing Kit from Pharmacia Biotech.

Cloning and DNA sequence analysis

An *E. coli. ompF* probe was used to screen our λgt10 library. A positive clone containing a 5.5 kb *Eco*RI insert was identified and used for subcloning of *opnP*. A 3.2 kb *opnP*-containing *Eco*RI–*Hin*dIII chromosomal fragment obtained from the above clone was digested with *Bgl*II and the resulting *Eco*RI–*Bgl*II and *Bgl*II–*Hin*dIII fragments were subcloned into both M13mp18 and M13mp19. The nucleotide sequence of the region located upstream of the *Bgl*II site of *opnP* was obtained from the M13mp18 and M13mp19 clones, using the universal primer and two other internal primers. The nucleotide sequence downstream of the *Bgl*II site was obtained from a M13mp19 clone, containing the *Bgl*II–*Hin*dIII chromosomal fragment, using the universal primer and five consecutive internal primers. We were unable to obtain a clone containing the reverse strand of DNA by this method. To obtain double-stranded DNA to confirm this sequence, a 1.5 kb PCR fragment, containing the sequence between nucleotides 802–2318, was amplified directly from a clone containing chromosomal *opnP* using 10 pmol of primers and 5 ng of the *opnP* clone. Reactions were carried out in a Perkin Elmer Cetus 960 Thermocycler as previously described (Waukau and Forst, 1992). The PCR DNA was digested with Sau3A1 and the resulting fragments were subcloned into M13mp18 and M13mp19. Several M13 clones were sequenced using the universal primer and several internal primers. As shown in Fig. 2 we report the nucleotide sequence up to bp 2285. The nucleotide sequence downstream of this position was obtained, but was not confirmed by sequence analysis of both strands, and, therefore, was not included in the present study. In addition, direct sequencing (Waukau and Forst, 1992) of a 1.28 kb PCR fragment including the sequence between bp 802–2088, and Cycle (Pharmacia Biotech) sequencing of a 0.9 kb fragment including the sequence between bp 802–1734, was performed to resolve sequence ambiguities.

Primer extension reactions

X. nematophilus AN6/1 was grown in Grace's Insect Cell Culture Medium with 30 mM trehalose (Sigma), and total RNA was extracted with TRIzol reagent (Gibco BRL) according to manufacturer's protocol. Primer extension analysis was carried out using a modification of the method previously described (Saffarini and Nealson, 1993), using an oligonucleotide complimentary to nucleotides 810–899 of the *opnP* coding strand. The primer extension reaction was performed by annealing 4 ng of oligonucleotide to 7 μg of total RNA. Avian myeloblastosis virus reverse transcriptase (5 U; Seikagaku America, Inc.), 10 mCi of [α-^{32}P]-ATP (NEN–DuPont), and 2 mM of each dCTP, dGTP, and dTTP were added, and the extension reaction was carried out at 48°C for 30 min. The resulting DNA–RNA hybrids were denatured at 90°C before being run on a 6% acrylamide gel. The same oligonucleotide primer was used to sequence M13-clone DNA containing this region of *opnP*.

Radiolabelling of outer membrane proteins

AN6/1 was grown to mid-log phase in 40 mls of Grace's Insect Cell Culture Medium. At t_0, a aliquot (1/6 volume) was removed and pulse-labelled, and sucrose was added to the remaining volume to a final concentration of 7.5%. Aliquots (1/6 volume) were pulse-labelled at indicated time points. For pulse-label experiments, 40 mCi of ^{35}S protein labelling mix (NEN–DuPont) was added to the cells and incubated at 30°C for 2 min, at which time unlabelled L-methionine was added to a final concentration of 5 mM. To terminate the incorporation of radiolabel, sodium azide was added to a final concentration of 20 mg ml^{-1}. The cells were then pelleted at 4°C and washed once with 1 ml of Grace's medium. For the temperature pulse-label experiments, the cells were grown in 10 ml of Grace's Insect Cell Culture Medium with 30 mM trehalose, at the indicated conditions, until mid-log phase and then 10 ml of cells were labelled as described above. The radiolabelled outer membrane proteins were prepared and electrophoresed as described previously (Leisman *et al.*, 1995). SDS–PAGE gels were dried and subjected to

autoradiography. Results from pulse-label experiments are representative of duplicate experiments.

Densitometric scanning of radiolabelled proteins

Autoradiographs of the dried, labelled protein gels were scanned on a GS 300 Transmittance/Reflectance Scanning Densitometer (Hoefer Scientific Instruments). Scan data was analysed with the GS370 1–D Data System, Version 2.0 (Hoefer Scientific Instruments). The seven major outer membrane protein bands were scanned and OpnP was expressed as a percentage of the total outer membrane proteins. Lanes were scanned twice each and the results averaged. At 26°C OpnP represented 63% of the total outer mebrane protein and this amount decreased to 58% and 50% at 30°C and 34°C, respectively.

Nucleotide sequence accession number

The sequence of *opnP* is available from GenBank under accession number L40919.

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