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Cloning and Sequencing of Gene Coding for a Periplasmic 5.4 kDa Peptide of the Macronucleus-Specific Symbiont *Holospora obtusa* of the Ciliate *Paramecium caudatum*

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ABSTRACT—We purified a 5.4 kDa peptide which is present in the intermediate and infectious form, but not in the reproductive form of a symbiotic bacterium *Holospora obtusa* of the ciliate *Paramecium caudatum*. Sequencing of its gene revealed that it encodes a peptide composed of 49 amino acids, and that the peptide is preceded by a putative signal peptide of 19 amino acids. We determined the transcription start point for the gene by primer extension analysis, indicating that the transcription starts with a G nucleotide located 33 nucleotides upstream from the translational initiation codon. Northern blot hybridization showed that the gene is highly expressed in the intermediate form, a transitional stage from the reproductive to infectious form of the bacterium. Immunoelectron microscopy with anti-5.4 kDa peptide antiserum revealed that the 5.4 kDa peptide is localized in the periplasm of the infectious form.

INTRODUCTION

The gram-negative bacterium *Holospora obtusa* is a macronucleus-specific symbiont of the ciliate *Paramecium caudatum*. This bacterium shows two distinct morphologies in its life cycle: a reproductive short form of 1.5-2 μm in length and an infectious long form of 13 μm in length (Görtz, 1980; Gromov and Ossipov, 1981). The infectious form consists of three distinct compartments: a periplasmic region, a cytoplasmic region, and a small recognition tip at the end of the periplasmic region (Görtz *et al.*, 1989). The reproductive form multiplies by binary fission in the host macronucleus when the host cell grows vegetatively, but ceases the fission, elongates itself, and differentiates to the infectious form when the host cell starves (Görtz, 1983) or its protein synthesis is inhibited by the emetine administration (Fujishima, 1993). The infectious form is released from the host cell following its cell division (Wiemann, 1989), and infects other host macronucleus via a food vacuole (Ossipov and Podlipaev, 1977; Fujishima and Görtz, 1983). After infection, the bacterium forms constrictions, and divides into several cells of the reproductive form (Görtz *et al.*, 1990). During the differentiation from the reproductive to infectious form, the bacterium changes its protein composition in parallel with its morphological changes (Görtz *et al.*, 1988, 1990; Fujishima *et al.*, 1990). Therefore, it is assumed that the bacterium changes its gene expression during this differentiation. In this study, as an effort to evidence this, we purified a small peptide that is present in the infectious form, but not in the reproductive form, sequenced its gene, and compared its expression in different forms of the bacterium.

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MATERIALS AND METHODS

Strains and culture

Paramecium caudatum strain RB-1 (syngen unknown) used in this study was originally collected in Germany. The *Paramecium* cells were infected newly with *H. obtusa* and cultivated in 1.25% (w/v) fresh lettuce juice in the modified Dryl's solution (Dryl, 1959), in which $KH_2PO_4 \cdot 2H_2O$ was used instead of $NaH_2PO_4 \cdot 2H_2O$, and inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* at 24-26°C (Hiwatashi, 1968).

Isolation of H. obtusa

The intermediate and infectious form of *H. obtusa* were isolated as previously described (Fujishima *et al.*, 1990). In this study, the homogenate of host cells was centrifuged in 70% (v/v) Percoll (Pharmacia) diluted with 10 mM Na,K-phosphate buffer (pH 6.5), followed by a discontinuous density gradient centrifugation in 60, 70, 80, and 90 % (w/v) Percoll. While cells of the infectious form were precipitated, those of the intermediate forms were banded at the interfaces of two different concentrations of Percoll. The reproductive form was isolated according to Freiburg (1985) modified as previously described (Fujishima *et al.*, 1990). In this study, the homogenate of isolated host macronuclei was centrifuged in 45% (v/v) Percoll diluted with 10 mM Na,K-phosphate buffer (pH 6.5). Cells of the reproductive form were isolated as a band formed in the gradient.

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SDS-polyacrylamide gel electrophoresis (PAGE)

Bacterial pellets of the reproductive, intermediate, and infectious forms of *H. obtusa* were lysed by boiling in Laemmli's lysis buffer (Laemmli, 1970) for 5 min. The lysates were subjected to SDS-PAGE of 16% (w/v) acrylamide gel. To fractionate cell components of the infectious form, the bacteria were sonicated in 50 mM Tris-HCl (pH 6.8) for 5 min on ice and centrifuged at 10,000 × g for 10 min at 4°C. The soluble and insoluble fraction were separately subjected to SDS-PAGE as above.

Native-PAGE and purification of the 5.4 kDa peptide

For native-PAGE (Davis, 1964), cells of the infectious form were lysed by sonication in sample buffer [62.5 mM Tris-HCl (pH 6.8) and 7% (v/v) glycerol] for 5 min on ice and the insoluble materials were removed by centrifugation. The supernatant was subjected to native-PAGE of 20% (w/v) acrylamide gel. Proteins separated on the native-PAGE gel were stained with Coomassie brilliant blue R-250 (CBB), and the band of the 5.4 kDa peptide was excised. The 5.4 kDa peptide was extracted from the gel band, and purified by reverse phase chromatography using a HPLC column CrostPak C18T-5 (JASCO).

Peptide sequencing

To determine the N-terminal amino acid sequence of the 5.4 kDa peptide, the purified peptide was directly analyzed on a PSQ-1 gas-phase peptide sequencer (Shimazu). To determine its internal amino acid sequence, the fragments obtained by digestion of the peptide by Endoproteinase Lys-C (Boehringer Mannheim) were analyzed.

Extraction of genomic DNA and PCR amplification

Genomic DNA of *H. obtusa* was extracted from the infectious form. Cells of the infectious form were lysed by vortexing vigorously with glass beads in lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) SDS and 0.4 mg/ml Proteinase K]. Subsequently, genomic DNA was extracted by the standard method with phenol-chloroform (Sambrook *et al.*, 1989).

A part of the 5.4 kDa peptide gene was amplified by PCR with mixed primers F2 (nt. 139-161, CARAARGTITTYGARGCIGAYGG) and R1 (nt. 233-214, TCRTCYTTIACDATRTCYTT), whose sequences were deduced from the amino acid sequence of the 5.4 kDa peptide. The bases of the primers were denoted according to the IUB codes: A, adenine; C, cytosine; G, guanine; T, thymine; I, inosine; R, A or G; Y, C or T; D, A or G or T.

Cloning and DNA sequencing

The PCR products were directly ligated to the pCR 2.1 vectors, and they were transformed into the One Shot competent cells of *E. coli* INVαF' using the Original TA Cloning Kit (Invitrogen) as described in the instruction manual. Plasmids were extracted from the transformed *E. coli* cells by the alkaline lysis method (Sambrook *et al.*, 1989), and sequenced by the dideoxynucleotide method (Sanger *et al.*, 1977) using the Sequencing PRO (TOYOBO) and [α-^{32}P] dCTP (ICN), as described in the instruction manual.

Cassette PCR amplification

To amplify the upstream sequence of the 5.4 kDa peptide gene, cassette PCRs (Isegawa *et al.*, 1992) were performed using the *Hin*d III Cassette (TaKaRa) ligated to the *Hin*d III digest of the genomic DNA of *H. obtusa* with primers C1 (Cassette primer, GTACATATTGT-CGTTAGAACGCG) and R2 (nt. 215-192, TTCTTATCTTCACTGACT-TCCACT) for first PCR, and with primers T7 (Cassette primer, TAATA-CGACTCACTATAGGGAGA) and R4 (nt. 182-162, TCACTTTTAGAT-TCTGAATCG) for second PCR. PCRs for the downstream amplification were performed in the same way using the *Sau*3A I Cassette (TaKaRa) ligated to the *Bgl* II digest of the genomic DNA with primers C1 and F4 (nt. 161-183, GGCGATTCAGAATCTAAAAG-TGAA), and with primers T7 and F6 (nt. 195-213, TGGAAGTCAGTGA-AGATAAG). The resultant PCR products were cloned and sequenced as described above.

RNA extraction, primer extension analysis and northern blot hybridization

Total RNAs of the reproductive, intermediate, and infectious form of *H. obtusa* were extracted by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) using the TriPure Isolation Reagent (Boehringer Mannheim).

Primer extension analysis was performed as previously described (Ausubel *et al.*, 1995) using the First-Strand cDNA Synthesis Kit (Pharmacia). The primer R4 was labeled with [γ-^{32}P] ATP (ICN) and T4 polynucleotide kinase (TaKaRa).

For northern blot hybridization, total RNAs from different forms were separated on formamide-agarose gel, transferred to Hybond-N nylon membrane (Amersham), and hybridized to the probe DNA labeled with [α-^{32}P] dCTP (ICN) by PCR with a pair of primers START (nt. 34-53, ATGAATTTTTTGTATTGCTG) and STOP (nt. 240-222, TTAATCTTCATCTTTTACG) in hybridization solution [50% (v/v) formamide, 10 × Denhardt's solution, 5 × SSC, 250 μg/ml salmon sperm DNA, and 50 mM Na-phosphate buffer (pH 7.0)]. The nylon membrane was washed, dried and autoradiographed.

Immunoelectron microscopy

H. obtusa-bearing paramecia were fixed, dehydrated and embedded as previously described (Dohra *et al.*, 1994). Their thin sections were incubated with rabbit anti-5.4 kDa peptide antiserum diluted 100-fold with blocking buffer [PBS (137 mM NaCl, 2.68 mM KCl, 8.1 mM $Na_2HPO_4 \cdot 12H_2O$, 1.47 mM KH_2PO_4, pH 7.2), 0.5% (w/v) BSA, 0.02% (v/v) Tween 20, 1.5% (v/v) normal goat serum, and 0.13% (w/v) NaN_3] and with goat anti-rabbit IgG-gold (BioCell, 10-nm particles) diluted 20-fold with the blocking buffer. Observation was carried out on a JEM-100CX (JEOL) transmission electron microscope. Rabbit anti-5.4 kDa peptide antiserum was produced by injecting synthetic 5.4 kDa peptide as an antigen (Sawady Technology, Inc.).

RESULTS

SDS-PAGE of proteins from H. obtusa

To compare the protein compositions among the different forms, whole cell lysates of *H. obtusa* were subjected to SDS-PAGE (Fig. 1). In spite of the different buoyant density, the intermediate forms banding upon 70, 80 and 90 % (v/v) Percoll (Fig. 1, lanes 2-4) and the infectious form (Fig. 1, lane 5) exhibited similar protein patterns, while that of the reproductive form (Fig. 1, lane 1) was considerably different. In this difference of the protein pattern, we took note of a small peptide indicated by an arrowhead in Fig. 1. This peptide was quite abundant in the infectious and intermediate forms, but not detected at all in the reproductive form. We tentatively designated this 5.4 kDa peptide.

Purification of the 5.4 kDa peptide and its sequencing

When cells of the infectious form of *H. obtusa* were sonicated and centrifuged, the 5.4 kDa peptide was found in the supernatant (Fig. 2, lane 3), but not in the precipitate (Fig. 2, lane 2). We isolated the 5.4 kDa peptide on native-PAGE and further purified it using HPLC by collecting a fraction whose retention time was 36 min. The purified 5.4 kDa peptide was confirmed for its purity by SDS-PAGE (Fig. 2, lane 4), and analyzed by mass spectroscopy, which indicated that its molecular mass was 5,433 Da (data not shown).

Amino acid sequence of the 5.4 kDa peptide was directly

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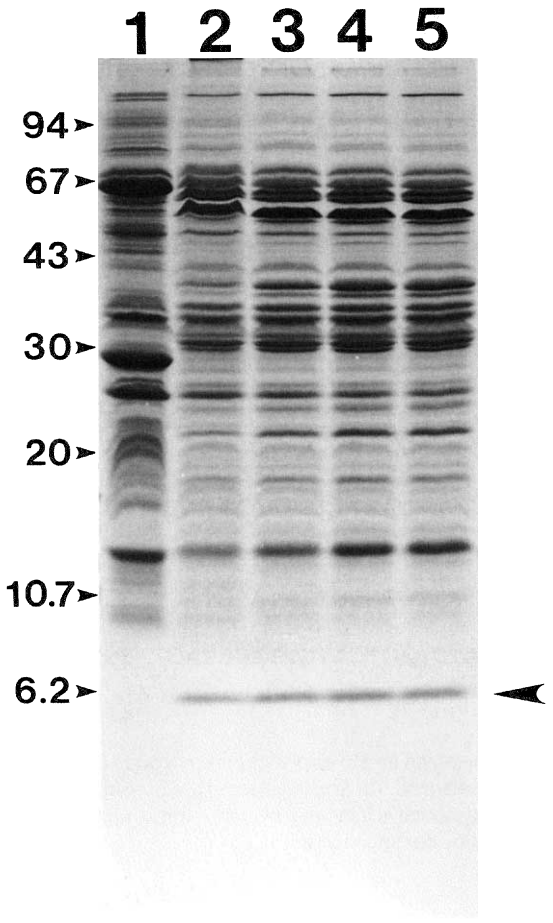


Fig. 1. SDS-PAGE of proteins from different forms of *H. obtusa*. Proteins were separated on 16% (w/v) polyacrylamide gel, and stained with CBB. Lane 1, reproductive form (2×10^7 bacterial cells); lane 2, intermediate form banding upon 70% (w/v) Percoll (1×10^6 bacterial cells); lane 3, intermediate form banding upon 80% Percoll (1×10^6 bacterial cells); lane 4, intermediate form banding upon 90% Percoll (1×10^6 bacterial cells); lane 5, infectious form (1×10^6 bacterial cells). Bacterial cells indicated in parentheses contained nearly the same amount of protein. An arrowhead indicates the 5.4 kDa peptide. The size of molecular weight markers is given in kDa. Note that the 5.4 kDa peptide is present in the intermediate and infectious form, but not in the reproductive form.

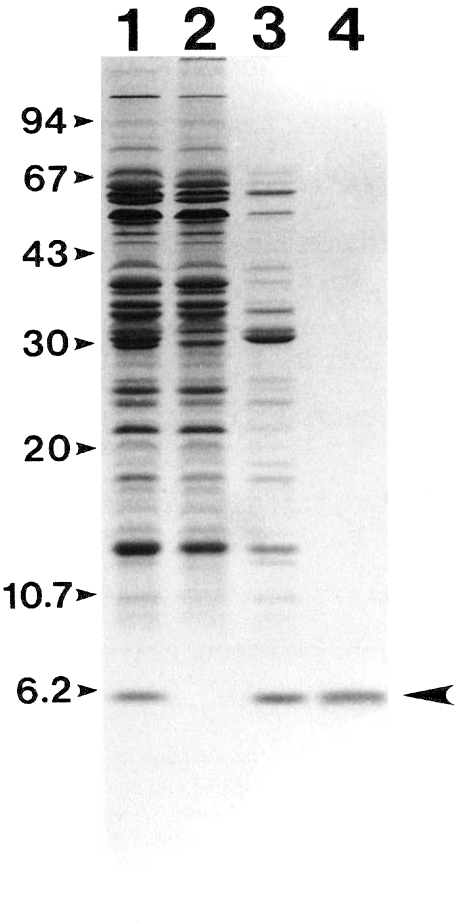


Fig. 2. SDS-PAGE of a whole cell lysate, an insoluble and soluble fraction of the infectious form of *H. obtusa*, and the purified 5.4 kDa peptide. Proteins were separated on 16% (w/v) polyacrylamide gel, and stained with CBB. Cells of the infectious form of *H. obtusa* were sonicated and centrifuged, and the precipitate and supernatant were used as an insoluble and soluble fraction, respectively. Lane 1, a whole cell lysate (1×10^6 bacterial cells); lane 2, an insoluble fraction (corresponding to 1×10^6 bacterial cells); lane 3, a soluble fraction (corresponding to 1×10^6 bacterial cells); lane 4, the purified 5.4 kDa peptide.

determined up to the 31st residue from the N-terminus (data not shown). For determination of the internal amino acid sequence, four Lys-C fragments from the 5.4 kDa peptide were also sequenced (data not shown). Among them, a fragment with retention time of 18 min on HPLC was estimated to be the C-terminal fragment of the 5.4 kDa peptide, because its C-terminal amino acid residue was not lysine. Oligonucleotides deduced from these amino acid sequences were synthesized and used as primers for PCR to amplify a part of the 5.4 kDa peptide gene.

PCR amplification and nucleotide sequencing

A PCR with a pair of primer F2 and R1 amplified a fragment of about 100 bp, which roughly corresponded to the expected size calculated from the molecular mass of the 5.4 kDa peptide. This fragment was cloned, sequenced, and extended toward the both sides to cover the entire gene by cassette PCR. By cloning and sequencing the resultant PCR products, a complete nucleotide sequence of the 5.4 kDa peptide gene was determined. Figure 3 shows the nucleotide sequence of the 5.4 kDa peptide gene and its flanking regions, and the deduced amino acid sequence encoded by the gene.

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Fig. 3. Nucleotide sequence of the 5.4 kDa peptide gene and its flanking regions, and deduced amino acid sequence encoded by the gene. The putative promoter region (-35 and -10) and the Shine-Dalgarno (SD) sequence were boxed. An arrow indicates the transcription start point (tsp) determined by primer extension analysis. Amino acid sequence of the 5.4 kDa peptide is indicated by bold letters (a.a. 20-68). The 5.4 kDa peptide is preceded by a putative signal peptide (a.a. 1-19), which is indicated by common letters.

It was revealed that the 5.4 kDa peptide is composed of 49 amino acids (a.a. 20-68) with a predicted molecular mass of 5,434 Da, which was almost consistent with the molecular mass estimated by mass spectroscopy. Sequencing of the gene also revealed that the 5.4 kDa peptide was preceded by a putative signal peptide composed of 19 amino acids (a.a. 1-19).

Primer extension analysis

The transcription start point for the 5.4 kDa peptide gene was determined by primer extension analysis using a ^{32}P-labeled primer which was hybridized with the sequence near the 5'-end of the 5.4 kDa mRNA. As a result, it turned out that transcription of the 5.4 kDa peptide gene starts with a G nucleotide located 33 nucleotides upstream from the translational initiation codon ATG (Fig. 4). Judging from the transcription start point thus determined, the promoter sequence was predicted to be TTTAAT (-34 to -29) for the -35 region and TACAAT (-11 to -6) for the -10 region.

Northern blot hybridization

When a PCR-labeled DNA coding for the 5.4 kDa peptide and its putative signal peptide was hybridized to total RNAs from the reproductive, intermediate and infectious forms of *H. obtusa* by northern blotting, three different sizes of RNA, 0.3, 0.7 and 1.4 kb were detected (Fig. 5). A total amount of the hybridized RNAs was highest in the intermediate form banding upon 70% (v/v) Percoll, and decreased as the buoyant density of the bacterium increased. The 0.3 kb RNA which was most abundantly expressed was detected in all the intermediate (Fig. 5, lanes 2-4) and infectious form (Fig. 5, lane 5), but not in the reproductive form (Fig. 5, lane 1). In the meantime, 0.7 kb RNA was detected in the intermediate forms banding upon 70 and 80% (v/v) Percoll (Fig. 5, lanes 2 and 3, respectively), and 1.4 kb RNA was detected only in the intermediate form banding upon 70% (v/v) Percoll (Fig. 5, lane 2). However, these RNAs were detected in all the intermediate and infectious forms upon overexposure (data not shown). Judging from the size of these RNAs, the 0.3 kb RNA seemed to be the transcript from the G nucleotide determined by primer extension analysis. We did not further characterize the 0.7 and 1.4 kb RNAs.

Immunoelectron microscopy

To determine the intracellular localization of the 5.4 kDa peptide, we performed immunoelectron microscopic observation using rabbit anti-5.4 kDa peptide antiserum and anti-rabbit IgG-gold. As a result, gold particles were detected almost exclusively in the periplasm (P), but only scarcely in the electron-translucent recognition tip (T) and in the cytoplasm (C) of the infectious form (Fig. 6A). No significant amount of gold particle was detected in the reproductive form (Fig. 6B). These results suggested that the 5.4 kDa peptide is localized in the periplasm of the infectious form of *H. obtusa*.

It is known that chloroform is a reagent that extracts

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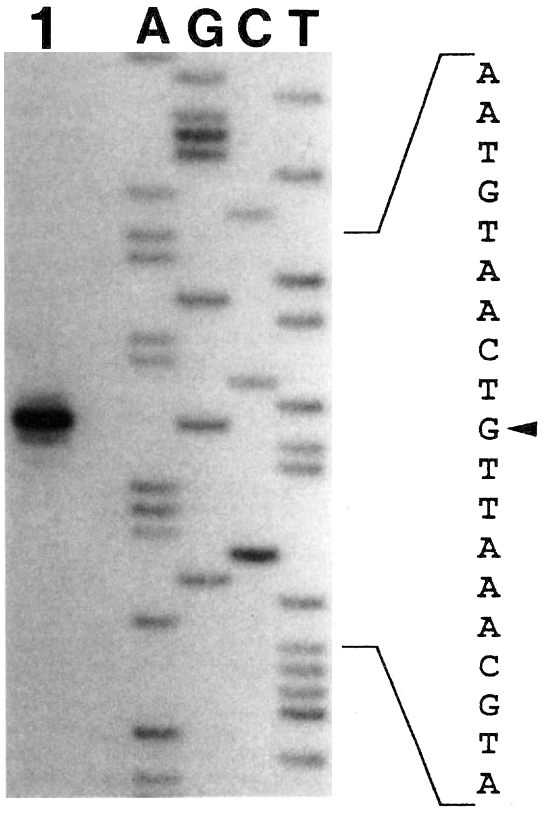


Fig. 4. Primer extension analysis to determine the transcription start point for the 5.4 kDa peptide gene. The labeled primer was hybridized to 2.5 μg of total RNA isolated from the infectious form and extended with M-MuLV reverse transcriptase. The resultant cDNA was denatured and electrophoresed on 5% (w/v) polyacrylamide denaturing gel. An arrowhead indicates the transcription start point, G nucleotide. Lane 1, cDNA produced by primer extension; lane A, G, C and T, sequencing ladder using the same primer R4 as in lane 1.

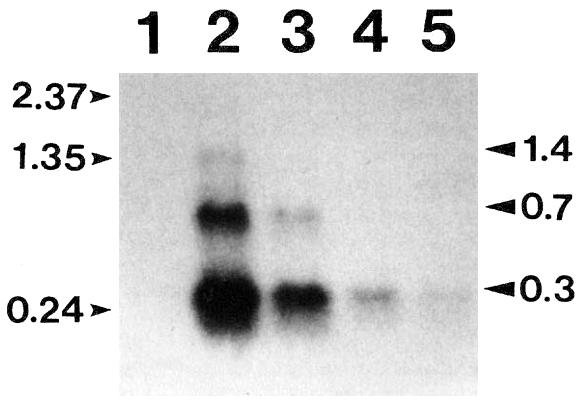


Fig. 5. Northern blot hybridization of the 5.4 kDa peptide mRNA. Four μg of total RNAs from the different forms of *H. obtusa* were separated on formamide-1.5% (w/v) agarose gel, transferred to nylon membrane, and hybridized to the PCR-labeled probe. Lane 1, reproductive form; lane 2, intermediate form banding upon 70% (v/v) Percoll; lane 3, intermediate form banding upon 80% Percoll; lane 4, intermediate form banding upon 90% Percoll; lane 5, infectious form. The sizes of RNA markers (left) and the 5.4 kDa mRNA (right) were shown in kb. Three different sizes (0.3, 0.7 and 1.4 kb) of RNAs were detected in the intermediate and infectious form, but not in the reproductive form.

bacterial periplasmic proteins (Ames *et al.*, 1984). We found that the 5.4 kDa peptide is also extracted from the infectious form with chloroform (data not shown). The intracellular localization of the 5.4 kDa peptide observed by immunoelectron microscopy conformed to these results.

DISCUSSION

In this study, we showed that the 5.4 kDa peptide was present in the intermediate and infectious form, but not in the reproductive form of *H. obtusa*. We also showed that the 5.4 kDa peptide gene was highly expressed in the intermediate form, a transitional stage from the reproductive to infectious form. It has been reported that protein pattern of this bacterium changes during this differentiation (Görtz *et al.*, 1988, 1990; Fujishima *et al.*, 1990), and we have reported the two infectious form-specific periplasmic proteins of 39 and 15 kDa, which are recognized by IF-3-1 and IF-3-2 monoclonal antibodies, respectively (Dohra *et al.*, 1994). However, this is the first report that a gene encoding a protein which appears during this differentiation was cloned and that its expression was compared among different stages of the life cycle of *H. obtusa*.

Wiemann and Görtz (1991) identified five polypeptides extracted by chloroform treatment of the infectious form of *H. obtusa*. They reported that one of these polypeptides is with molecular mass of 11,500-M_r on Tricine SDS-PAGE, and has an unusually low affinity for nitrocellulose membrane. The 5.4 kDa peptide described in this report resembled the 11,500-M_r polypeptide in the following points: (1) it is extracted by chloroform treatment; (2) when subjected to Tricine SDS-PAGE, it shows a molecular mass of 11 kDa; (3) it has a low affinity for PVDF membrane. These similarities suggested that the 5.4 kDa peptide and the 11,500-M_r polypeptide may be identical.

E. coli cells transformed with a vector inserted with a segment coding for the 5.4 kDa peptide and its putative signal peptide produced a peptide with the same molecular mass as that of the 5.4 kDa peptide, and the peptide was extracted from the *E. coli* periplasm by chloroform treatment (data not shown). These results suggested that the mature 5.4 kDa peptide is produced by cutting off the putative signal peptide and then transported into the periplasm even in a *E. coli* cell.

The function of the 5.4 kDa peptide is not clear yet. However, our observation that the *E. coli* cell transformed with the 5.4 kDa peptide gene could scarcely divide (data not shown) may become a clue to know the function of this peptide. When the reproductive form differentiates to the infectious one, the bacterium ceases binary fission, elongates itself, and develops its periplasmic space. During this differentiation process, the 5.4 kDa peptide is produced and accumulates in the periplasm. Inhibition of division of the *E. coli* cell which is producing the 5.4 kDa peptide seems to suggest that the 5.4

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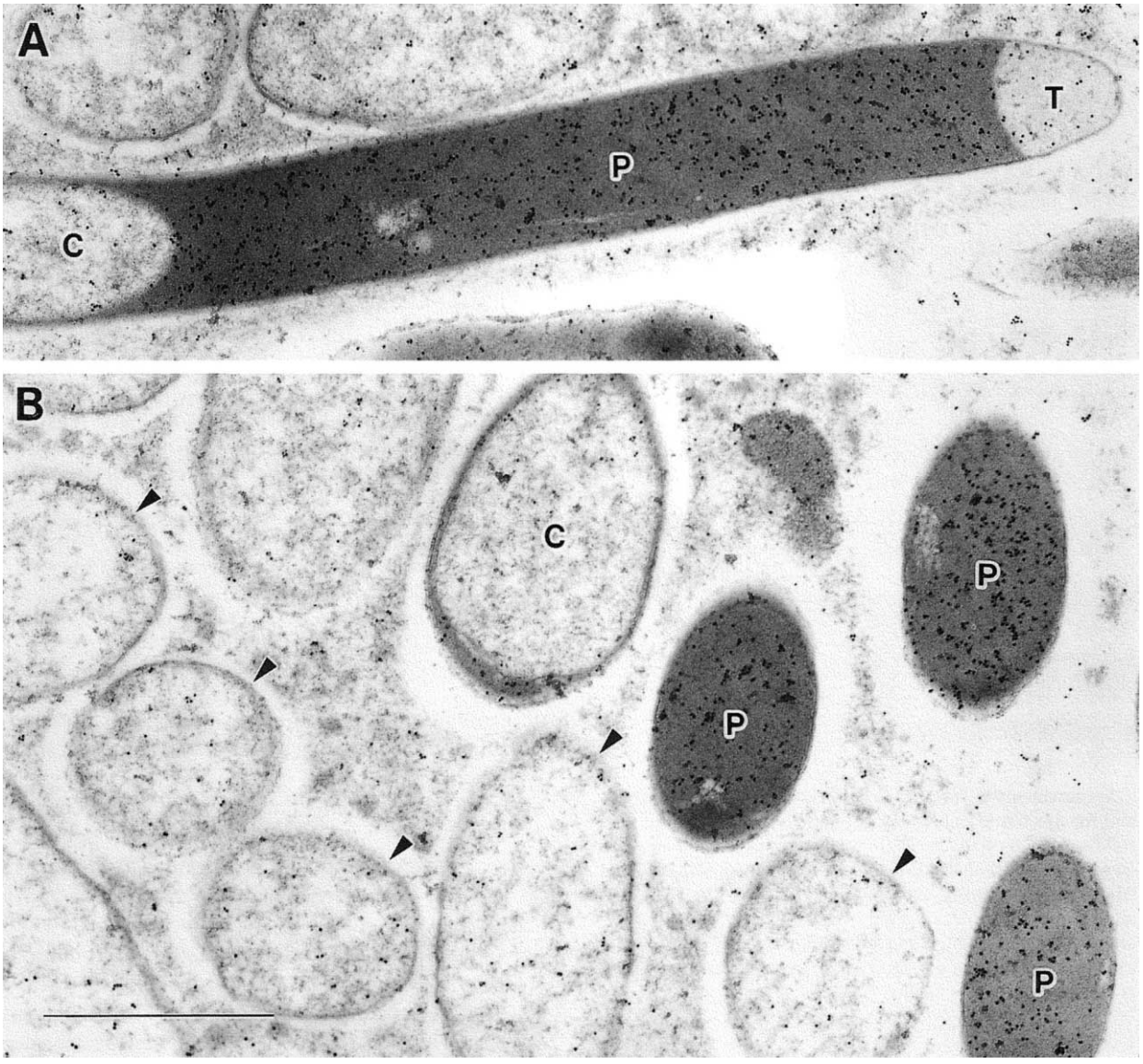


Fig. 6. Immunoelectron micrographs of *H. obtusa* in the macronucleus of *P. caudatum*. Thin sections were treated with rabbit anti-5.4 kDa peptide antiserum and goat anti-rabbit IgG-gold (10-nm), and stained with uranyl acetate. (**A**) A longitudinal section of the infectious form. (**B**) A transversal section of the reproductive and infectious forms. Abbreviations: C, cytoplasm; P, periplasm; T, electron-translucent recognition tip. Arrowheads in **B** indicate reproductive forms. Bar represents 1 μm. Note that the 5.4 kDa peptide is localized in the periplasm of the infectious form.

kDa peptide is involved in the inhibition of division of the reproductive form which switches the cell differentiation to the infectious form. The 5.4 kDa peptide disappeared within at least 3 hr after the bacterium infected the host macronucleus (data not shown). Since the bacterium undergoes multiple division after infection (Görtz *et al.*, 1990), this result also seems to support our hypothesis. Another possibility is that the 5.4 kDa peptide may function in the infection process because the amount of electron-dense materials in the periplasm decreases within 5 min after being ingested into a phagosome (Görtz and Wiemann, 1989; Görtz *et al.*, 1990). The infectious form ingested into the host digestive vacuole escapes from the vacuole into the cytoplasm, moves to the target host macronucleus and penetrates the nuclear envelope. During this process the 5.4 kDa peptide disappears, though not known in which stage.

The 5.4 kDa peptide did not show amino acid sequence similarity with any known peptide in the SwissProt, PIR and PRF databases. It is also unclear whether the other species of the *Holospora* genus have the 5.4 kDa peptide. Although protein patterns of the infectious forms of *H. obtusa* and *H. elegans* are considerably different, their structural features, developmental cycles, and mode of infection are similar to each other (Schmidt *et al.*, 1987). Thus homologs of the 5.4 kDa peptide may be present widely in *Holospora* species if these peptides share a function essential to their life cycle.

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H. obtusa changes its protein pattern in parallel with the morphological change in accordance with the growth of the host cell (Görtz, 1983; Görtz *et al.*, 1988, 1990; Fujishima *et al.*, 1990). This suggests that the growth conditions of the host cell influence the gene expression of *H. obtusa*. Therefore, the 5.4 kDa peptide would be used as a good marker molecule to study the host-symbiont interactions at the gene expression level. Control mechanisms over the gene expression of *H. obtusa* by the host would be clarified by studying the expression mechanism of the 5.4 kDa peptide gene.

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