ゾウリムシ大核内共生細菌に関する
分子細胞生物学的研究

東京大学大学院理学系研究科生物科学専攻

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学位論文

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MOLECULAR AND CELLULAR BIOLOGICAL STUDIES ON THE MACRONUCLEUS-SPECIFIC SYMBIONT *HOLOSPORA OBTUSA* OF THE CILIATE *PARAMECIUM CAUDATUM*

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CONTENTS

GENERAL INTRODUCTION

Many ciliated protozoa harbor bacterial endosymbionts (1, 22), most of which are found in the cytoplasm, whether enclosed by a host membrane or not. In two cases, symbionts are found in the perinuclear space of the macronuclear envelope (4, 21), and in some cases, they are found in the nucleus. Ciliates contain two nuclei in a cell; one is a somatic macronucleus and the other is a germinal micronucleus. Endonuclear symbionts in ciliates are found in either of the two nuclei. All bacterial endonuclear symbionts of the ciliate *Paramecium* belong to the Genus *Holospora* (14, 27). So far, nine species of the symbionts of the Genus *Holospora* have been described (2, 3, 5, 6, 7, 20, 24, 26). These *Holospora* species show host species- and host nucleus-specificity (Table 1).

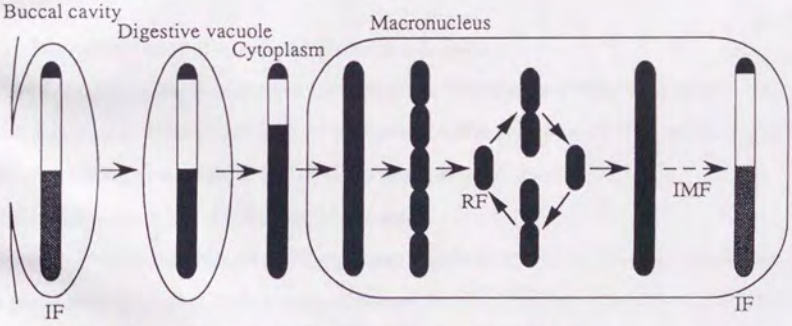
Table 1. Host- and nucleus-specificity of the Genus *Holospora*

Holospora species	Host species	Nucleus	Reference
H. obtusa	*P. caudatum*	Macronucleus	Hafkine, 1890 (20)
H. elegans	*P. caudatum*	Micronucleus	Hafkine, 1890 (20)
H. undulata	*P. caudatum*	Micronucleus	Hafkine, 1890 (20)
H. caryophila	*P. biaurelia*	Macronucleus	Preer, 1969 (26)
	P. novaurelia	Macronucleus	Fokin *et al.*, 1996 (6)
H. acuminata	*P. bursaria*	Micronucleus	Ossipov *et al.*, 1980 (24)
H. curviuscula	*P. bursaria*	Macronucleus	Borchenius *et al.*, 1983 (2)
H. bacillata	*P. woodruffi*	Macronucleus	Fokin, 1989 (3)
	P. calkinsi	Macronucleus	Fokin *et al.*, 1993 (7)
H. recta	*P. caudatum*	Micronucleus	Fokin, 1991 (5)
H. curvata	*P. calkinsi*	Macronucleus	Fokin *et al.*, 1993 (7)

Holospora obtusa is a macronucleus-specific symbiont of the ciliate *Paramecium caudatum* (Fig. 1). This bacterium shows two different morphologies in its life cycle (Fig. 2): a reproductive short form (1.5-2 μm in length) and an infectious long form (11-14 μm in length) (12, 19, 25). The reproductive form is very similar to other gram-negative bacteria in size and morphology, while the infectious form shows a unique cytological structure, which consists of three distinct compartments: a periplasmic region, a cytoplasmic region, and a small recognition tip at the end of the periplasmic region (15). The reproductive form multiplies by binary fission

Figure 1. Photomicrograph of *Holospora obtusa* in the host macronucleus.

Figure 2. Life cycle of *Holospora obtusa*.



IF, infectious form; RF, reproductive form; IMF, intermediate form

in the host macronucleus when the host cell grows vegetatively, but ceases the fission, elongates itself, and differentiates to the infectious form via the intermediate forms when the host cell starves (13) or its protein synthesis is inhibited by the emetine administration (8). The infectious form is released from the host cell following its cell division (28), and infects other host macronucleus by being engulfed into the host's food vacuole, entering its cytoplasm, and

penetrating its macronuclear envelope (9, 10, 18, 23). After infection, the bacterium forms several constrictions along its length, and divides into several cells of the reproductive form (17). During the differentiation from one form to the other, the bacterium changes its protein composition in parallel with its morphological change (11, 16, 17), suggesting that the bacterium changes its gene expression during the differentiation.

In this study, in an effort to elucidate qualitative and quantitative difference in gene expression among the reproductive, intermediate, and infectious form of *H. obtusa*, I took note of two proteins which change in amount during its life cycle. In Part I, I purified a 5.4 kDa peptide which is present in the intermediate and infectious form, but not in the reproductive form. I also cloned, sequenced its gene, and compared its gene expression among the different forms. In Part II, I characterized a 63-kDa protein, or a GroEL homolog of *H. obtusa* which is more abundant in the reproductive form than in the intermediate or infectious form. I also cloned, sequenced its gene, and compared its gene expression.

The content of this thesis will be published as follows:

Part I: Hideo Dohra, Kazuo Yamamoto, Masahiro Fujishima and Hajime Ishikawa. 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*.

Zoological Science Vol. 14, No.1 (1997). In press.

Part II: Hideo Dohra, Masahiro Fujishima and Hajime Ishikawa. Structure and Expression of a *groE* Operon Homolog of the Macronucleus-specific Symbiont *Holospora obtusa* of the Ciliate *Paramecium caudatum*.

Journal of Bacteriology Vol. 179, No. 20 (1997). In press.

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PART I

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*

ABSTRACT

I purified a 5.4 kDa peptide which is present in the intermediate and infectious form, but not in the reproductive form of the macronucleus-specific symbiont *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 composed 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. The transformed cells of *E. coli* producing the recombinant 5.4 kDa peptide could scarcely divide, suggesting that the 5.4 kDa peptide is involved in the inhibition of division of the reproductive form, which switches the differentiation to the infectious form.

INTRODUCTION

The gram-negative bacterium *Holospora obtusa* is a macronucleus-specific symbiont of the ciliate *Paramecium caudatum*. This bacterium shows two different morphologies in its life cycle (12, 18, 23). A reproductive short form is very similar to other gram-negative bacteria in size and morphology, while an infectious long form is longer than the reproductive form, and shows a unique cytological structure (14). These two morphologically distinct forms of the bacterium are also functionally different. The reproductive form represents the reproductive stage in its life cycle, that is, it multiplies by binary fission in the host macronucleus when the host cell grows vegetatively. In contrast, the infectious form is highly infectious and has an function to propagate itself by being released from one host (28) and infecting other (9, 10, 22). The reproductive form differentiates to the infectious form when the host cell starves (13) or its protein synthesis is inhibited by the emetine administration (8). During this differentiation, the bacterium changes its protein composition in parallel with its morphological change (11, 15, 16). Therefore, it is assumed that the bacterium changes its gene expression during this period. In this study, in an effort to evidence this, we purified a small peptide which is present in the intermediate and infectious form, but not in the reproductive form, sequenced its gene, and compared its expression among the different forms of the bacterium.

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 (6), 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 (19).

The transformed cells of *E. coli* strains DH5 and XL1-Blue were cultivated in LB medium (24) in the absence or presence of 2 mM isopropyl-β-D(-)-thiogalactopyranoside (IPTG) at 37 °C.

Isolation of H. obtusa

Cells of the intermediate and infectious form of *H. obtusa* were isolated as previously described (11). 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. Cells of the infectious form precipitated under 90 % Percoll and those of the intermediate forms banded upon 70, 80, and 90 % Percoll were collected. Cells of the reproductive form and the bacteria at various lengths of the elongation process were isolated according to Freiburg (7) modified as previously described (11). 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 Percoll gradient. Isolated cells of the infectious, intermediate, and reproductive form were washed with 10 mM Na, K-phosphate buffer (pH 6.5) to remove Percoll.

SDS-polyacrylamide gel electrophoresis (PAGE)

Isolated cells of the reproductive, intermediate, and infectious form of *H. obtusa* were lysed by boiling in Laemmli's lysis buffer (21) for 5 min. Proteins from 2 x 10^8 cells for the

reproductive form, 1 x 10^7 cells for the intermediate and infectious form of *H. obtusa* were subjected to SDS-PAGE of 16 % (w/v) polyacrylamide gel. These numbers of cells contained nearly the same amount of protein. The proteins separatd on the gel were stained with 0.05 % (w/v) Coomassie brilliant blue R-250 (CBB), 40 % (v/v) methanol and 10 % (v/v) acetic acid.

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 precipitate and supernatant corresponding to 1 x 10^7 cells were boiled in Laemmli's lysis buffer, and separately subjected to SDS-PAGE as an insoluble and soluble fraction, respectively.

Native-PAGE and purification of 5.4 kDa peptide

For native-PAGE (4), 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. Insoluble materials were removed by centrifugation at 10,000 g for 10 min at 4 °C. The supernatant was subjected to native-PAGE of 20% (w/v) polyacrylamide gel. Proteins separated on the native-PAGE gel were stained with 0.05% (w/v) CBB, 50% (v/v) methanol, and 3% (v/v) acetic acid for 10 min, and de-stained with 25% (v/v) methanol and 3% (v/v) acetic acid for 10 min. After the gel was washed 3 times with deionized water for 5 min each, the band of the 5.4 kDa peptide was excised. The 5.4 kDa peptide was eluted from the gel band with 0.1 N NaOH and purified by reverse phase chromatography using a HPLC column CrostPak C18T-5 (JASCO) with a linear gradient of 0-60 % (v/v) acetonitrile containing 0.05 % (v/v) trifluoroacetic acid. The column eluate was monitored at 230 nm, and the fraction containing the 5.4 kDa peptide was collected.

Mass spectroscopy of 5.4 kDa peptide

The purified 5.4 kDa peptide was dissolved in deionized water, mixed with 40 % (v/v) acetonitrile containing 10 mg/ml 3, 5-dimethoxy-4-hydroxycinnamic acid as a matrix, dried, and then analyzed on a Kratos Kompact MALDI-III MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectroscope (Shimazu).

Amino acid sequencing of 5.4 kDa peptide

To determine the N-terminal amino acid sequence of the 5.4 kDa peptide, the purified peptide was directly analysed 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. The purified 5.4 kDa peptide was digested with 2.5 μg of Endoproteinase Lys-C in 25 mM Tris-HCl (pH 8.5) and 1 mM EDTA at 37 °C for 6 hr. The digestion reaction was stopped by adding with a 1/6 volume of acetic acid, and the fragments were purified by reverse phase HPLC as described above, and then analyzed on the peptide sequencer.

Extraction of genomic DNA from H. obtusa

Genomic DNA of *H. obtusa* was extracted from the infectious form as follows. Isolated cells of the infectious form were washed with TE [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA], suspended with lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5 % (w/v) SDS and 0.4 mg/ml Proteinase K], and lysed by vortexing vigorously with glass beads for 20 min. After overnight incubation at 55 °C, the lysate was extracted twice with phenol, and once with phenol-chloroform. Nucleic acids were treated with 20 μg/ml RNase A at 37 °C for 1 hr, and DNA was re-extracted with phenol-chloroform and with chloroform. After ethanol precipitation, DNA was dissolved in TE.

PCR amplification of 5.4 kDa peptide gene

A part of the 5.4 kDa peptide gene was amplified as follows. PCR was performed with 2 μM of a pair of primers F2-R1, whose sequences were deduced from the amino acid sequence of the 5.4 kDa peptide, 1 unit of TaKaRa Ex Taq (TaKaRa), Ex Taq Buffer, 4.5 mM $MgCl_2$, and 15 ng of genomic DNA of *H. obtusa*. After incubation at 94 °C for 5 min, PCR amplification was performed with 30 cycles of 94 °C for 30 sec, 40 °C for 2 min, and 72 °C for 1 min, followed by a 5-min extension cycle at 72 °C.

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 cells of *E. coli* by the alkaline lysis method (24), and sequenced by the dideoxynucleotide method (25) using 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 (20) were performed using the *Hin*d III Cassette (TaKaRa) ligated to the *Hin*d III digest of the genomic DNA of *H. obtusa* with 0.25 μM of a pair of primers C1-R2 for first PCR, and with a pair of primers T7-R4 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 of *H. obtusa* with 0.25 μM of two pairs of primers C1-F4 and T7-F6. The resultant PCR products were cloned and sequenced as described above.

Southern blot hybridization of 5.4 kDa peptide gene

For Southern blot hybridization of the 5.4 kDa peptide gene, 1 μg of genomic DNA from the infectious form of *H. obtusa* was digested with *Bgl* II, *Eco*R I, *Hin*d III, *Bgl* II and *Eco*R I, *Bgl* II and *Hin*d III, and *Eco*R I and *Hin*d III, separated on 0.8 % (w/v) agarose gel, and transferred to Hybond-N nylon membrane (Amersham) in denature solution (1.5 M NaCl and 0.5 M NaOH). The nylon membrane was incubated in hybridization solution [5 x SSC (150 mM NaCl and 15 mM sodium citrate), 0.1 % (w/v) sodium N-lauroyl salcosinate, 0.02 % (w/v) SDS, 50 % (v/v) formamide, and 2 % (w/v) blocking reagent] containing the probe DNA labeled with the DIG DNA labeling mixture (Boehringer Mannheim) by PCR with 0.25 μM of a pair of primers START-STOP at 40 °C for 16 hr, washed twice with 6 x SSC containing 0.1 % (w/v) SDS at room temperature for 5 min each and twice with 0.1 x SSC containing 0.1 % SDS at 68 °C for 15 min each. The hybridized DNAs were detected using the DIG Luminescent Detection Kit (Boehringer Mannheim).

RNA extraction from H. obtusa

Total RNAs of the reproductive, intermediate, and infectious form of *H. obtusa* were extracted by the acid guanidinium thiocyanate-phenol-chloroform method (3) using the TriPure Isolation Reagent (Boehringer Mannheim) with minor modification. Isolated cells of the bacterium were precipitated by centrifugation at 10,000 g for 5 min at 4 °C, lysed by pipetting in 100 μl of boiled denaturing solution [4 M guanidinium thiocyanate, 0.2 M 2-mercaptoethanol, and 0.5 % (w/v) sodium N-lauroyl salcocinate], and added with 900 μl of TriPure Isolation Reagent. Subsequently, RNAs were extracted as described in the instruction manual.

Primer extention analysis

Primer extension analysis was performed as previously described (2). 5'-End of primer R4 was labeled with [γ-^{32}P] ATP (ICN) and 10 units of T4 polynucleotide kinase (TaKaRa) at 37 °C for 1 hr. The labeled primer was hybridized to 2.5 μg of heat-denatured total RNA isolated from the infectious form of *H. obtusa* and extended with M-MuLV reverse transcriptase using the First-Strand cDNA Synthesis Kit (Pharmacia) at 37 °C for 1 hr. The resultant cDNA was precipitated with ethanol, denatured at 65 °C for 5 min, and electrophoresed on 5 % (w/v) polyacrylamide denaturing gel. The gel was dried and autoradiographed at -80 °C. The labeled primer R4 was also used for sequencing of the gene to use as markers for determination of the transcription start point.

Northern blot hybridization of 5.4 kDa peptide mRNA

For northern blot hybridization, 4 μg of total RNAs from the different forms of *H. obtusa* were separated on formamide-1.5 % (w/v) agarose gel and transferred to Hybond-N nylon membrane by capillary transfer. The probe DNA was labeled with [α-^{32}P] dCTP (ICN) by PCR with 0.25 μM of a pair of primers START-STOP, and hybridized to RNAs transferred to the nylon membrane in hybridization solution [5 x SSC, 50 % (v/v) formamide, 10 x Denhart's solution (0.02 % bovine serum albumin, 0.02 % Ficoll 400, and 0.02 % polyvinyl pyrrolidone), 250 μg/ml salmon sperm DNA, and 50 mM Na-phosphate buffer (pH 7.0)] at 42

°C for 16 hr. The nylon membrane was washed twice with 2 x SSC containing 0.1 % SDS at 42 °C for 30 min each and twice with 0.1 x SSC containing 0.1 % SDS at 65 °C for 1 hr each. The Hybridized RNAs were detected by autoradiography at -80 °C.

Immunoelectron microscopy of 5.4 kDa peptide

To determine the intracellular localization of the 5.4 kDa peptide, immunoelectron microscopy using anti-5.4 kDa peptide antiserum was performed as previously described (5). *H. obtusa*-bearing paramecia were fixed with 0.05 M Na, K-phosphate buffer (pH 7.2) containing 2.5 % (w/v) paraformaldehyde and 0.5 % (w/v) glutaraldehyde for 1 hr at room temperature, washed 3 times with the same buffer for 20 min each and once with deionized water for 20 min at room temperature. The cells were dehydrated with ethanol series of 30, 50, 70, 90, 100 and 100 % (v/v) for 30 min each, and infiltrated with LR White resin (London Resin) overnight, and then finally embedded in fresh resin for 24 h at 60 °C. Thin sections were cut with a diamond knife on a MT2-B ultra-microtome (Sorvall), and collected on a nickel grid. The thin sections on the grid were treated 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] to eliminate non-specific adsorption of antibodies for 1 hr at room temperature, and incubated with rabbit anti-5.4 kDa peptide antiserum diluted 100-fold with blocking buffer for 2 hr at room temperature. After washed with blocking buffer 3 times for 10 min each, the thin sections were incubated with goat anti-rabbit IgG-gold (BioCell, 10-nm particles) diluted 20-fold with blocking buffer for 2 hr at room temperature, and then washed 5 times with PBS and once with deionized water for 10 min each. The thin sections were dried, stained with saturated uranyl acetate for 15 min, and washed with deionized water. Observation was carried out using a JEM-100CX (JEOL) transmission electron microscope at 80 kV. Rabbit anti-5.4 kDa peptide antiserum was produced by injecting synthetic 5.4 kDa peptide as an antigen (Sawady Technology, Inc.).

Construction of expression vector and Expression of 5.4 kDa peptide in Escherichia coli cell

To predict the function of the 5.4 kDa peptide, its gene was expressed in a transformed cell of *E. coli* as follows. The cording region for the 5.4 kDa peptide and its putative signal peptide was amplified by PCR with a pair of primers START-STOP, which contained a *Bsp*H I and *Hin*d III restriction site at the 5'-end, respectively. The PCR product was cloned using the Original TA Cloning Kit, and the plasmids were extracted as described above, digested with *Bsp*H I and *Hin*d III, and subjected to 0.8 % (w/v) agarose gel electrophoresis. The inserts were cut out, extracted from the gel using the Gene clean II kit (BIO 101), and ligated to *Nco* I and *Hin*d III digests of the pTrc99A expression vector (Pharmacia) at 16 °C for 30 min using the DNA Ligation Kit Ver. 2 (TaKaRa). The resultant plasmide vector containg the insert was tentatively designated pHOB5.4k, which was transformed into competent cells of *E. coli* strains DH5 and XL1-Blue. Production of the 5.4 kDa peptide in the transformed cells was induced by adding 2 mM IPTG. Growth of the transformed cells was monitored by mesuring absorbance of the culture medium at 600 nm.

Extraction of periplasmic proteins with chloroform

Periplasmic proteins were extracted with chloroform from the infectious form of *H. obtusa* and the transformed *E. coli* as described previously (1). Isolated cells of the infectious form were centrifuged at 10,000 g for 10 min, and the supernatant was completely removed. The precipitated cells of the infectious form were added with 20 µl of chloroform, kept at room temperature for 30 min, and mixed with a vortex mixer every 3 min. After adding 200 µl of 20 mM Tris-HCl (pH 8.0), chloroform-extract was centrifuged at 10,000 g for 10 min to remove cell bodies of the infectious form. Proteins extracted with chloroform were precipitated with 0.5 M trichloroacetic acid, washed with diethyl ether, dried, and dissolved in Laemmli's lysis buffer for SDS-PAGE.

Periplasmic proteins of the transformed *E. coli* were extracted as follows. Production of the recombinant 5.4 kDa peptide was induced with 2 mM IPTG when A_{600} of the culture medium was about 0.5. After 2 or 4 hr, the transformed cells of *E. coli* were harvested, and the periplasmic proteins were extracted in the same way as that for the infectious form.

RESULTS

SDS-PAGE of proteins from different forms of H. obtusa

To compare the protein compositions among the reproductive, intermediate, and infectious form of *H. obtusa*, whole cell lysates of the bacterium were subjected to SDS-PAGE of 16 % polyacrylamide gel (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) exhibit similar protein patterns, while that of the reproductive form (Fig. 1, lane 1) was considerably different from those of the intermediate and infectious form. 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 intermediate and infectious form, but not detected at all in the reproductive form. We tentatively designated this 5.4 kDa peptide.

Purification of 5.4 kDa peptide

When cells of the infectious form of *H. obtusa* were sonicated and centrifuged, and the precipitate and supernatant were separately subjected to SDS-PAGE (Fig. 2A), the 5.4 kDa peptide was found in the supernatant (Fig. 2A, lane 3), but not in the precipitate (Fig. 2A, lane 2). I isolated the 5.4 kDa peptide from the supernatant separated on native-PAGE and further purified it using HPLC. Fig. 2B shows the chromatogram when the 5.4 kDa peptide extracted from the gel band was applied on reverse phase HPLC and eluted with acetonitrile gradient. The 5.4 kDa peptide was found in a peak whose retention time was 36 min (an arrow). The purified 5.4 kDa peptide was confirmed for its purity by SDS-PAGE (Fig. 2A, lane 4).

Mass spectroscopy of 5.4 kDa peptide

To estimate molecular mass of the 5.4 kDa peptide, I analyzed the peptide by mass spectroscopy (Fig. 3). An ion with a molecular mass of 5,434 Da, which was indicated by an arrowhead, was detected. Since this ion was thought to be the ionized 5.4 kDa peptide ($[M+H]^+$), molecular mass of this peptide was estimated to be 5,433 Da.

Amino acid sequencing of 5.4 kDa peptide

Amino acid sequence of the 5.4 kDa peptide was directly determined up to 31st residue from the N-terminus (Fig. 4A). For determination of the internal amino acid sequence, four Lys-C fragments from the 5.4 kDa peptide were also sequenced (Fig. 4B). The fragment of 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. Primers used for PCRs and sequencing of the gene in this study were listed up in Table 1.

PCR amplification and nucleotide sequencing

A PCR with a pair of primers F2-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 (Fig. 5). By cloning and sequencing the resultant PCR products, a complete nucleotide sequence of the 5.4 kDa peptide gene, and its 5'- and 3'-flanking region were determined. Figure 6 shows the nucleotide sequence of the 5.4 kDa gene, its 5'- and 3'-flanking regions, and the deduced amino acid sequence encoded by the gene. 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). The 5.4 kDa peptide contained many acidic amino acids, such as a glutamic acid and aspartic acid (19/49 a. a.). Because of this biased amino acid composition, the 5.4 kDa peptide showed an acidic isoelectric point (pI), which was predicted tobe 3.82. By nucleotide sequence analysis, ribosome binding site was found just upstream of the translational initiation codon ATG. The site shared a polypurine sequence TAAGGAG (nt. 20 to 26), known as Shine-Dargarno sequence (27). There was an inverted repeat sequence, 5'-GACTAT-NNNNNNN-ATAGTC-3' (nt. 346 to 364),

downstream of the gene. It possibly served as a transcriptional terminator by forming a stem-loop structure.

Primer extension analysis

The transcription start point for the 5.4 kDa peptide gene was determined by primer extension analysis using ^{32}P-labeled primer which hybridizes near the 5'-end of the 5.4 kDa mRNA. As a result, it was revealed that transcription of the 5.4 kDa peptide gene was initiated at G nucleotide located 33 nucleotides upstream from the translational initiation codon ATG (Fig. 7). Judging from the transcription start point determined by primer extension analysis, the promoter sequence was predicted to be TTTAAT (nt. -34 to -29) for the -35 region and TACAAT (nt. -11 to -6) for the -10 region.

Southern blot hybridization of 5.4 kDa peptide gene

When a PCR-labeled DNA coding for the 5.4 kDa peptide and its putative signal peptide was hybridized to the undigested genomic DNA from the infectious form of *H. obtusa* and *P. caudatum*, and different restriction enzyme digests of the genomic DNA of *H. obtusa* by Southern blot hybridization, the probe DNA was hybridized to the undigested genomic DNA from *H. obtusa* (Fig. 8, lane 1) and its digests (Fig. 8, lanes 2-7), but not to that from *P. caudatum* at all (Fig. 8, lane 8). A single band with size of 1.4, 4.3, 5.6, 1.3, 1.4, and 4.3 kbp was detected in the *Bgl* II, *Eco*R I, *Hin*d III, *Bgl* II and *Eco*R I, *Bgl* II and *Hin*d III, and *Eco*R I and *Hin*d III digest, respectively (Fig. 8, lanes 2-7). These results suggested that the 5.4 kDa peptide gene is a single copy gene and that the 5.4 kDa peptide is encoded by genomic DNA of *H. obtusa*.

Northern blot hybridization of 5.4 kDa peptide mRNA

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 form of *H. obtusa* by Northern blot hybridization, three different sizes of mRNAs, 0.3, 0.7 and 1.4 kb were detected (Fig. 9). 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. 9, lanes 2-4) and infectious form (Fig. 9, lane 5), but not in the reproductive form (Fig. 9, lane 1). In the meantime, 0.7-kb RNA was detected in the intermediate forms banding upon 70 and 80% (v/v) Percoll (Fig. 9, lanes 2 and 3, respectively), and 1.4-kb RNA was detected only in the intermediate form banding upon 70% (v/v) Percoll (Fig. 9, lane 2). However, these RNAs were detected in all the intermediate and infectious form 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 of 5.4 kDa peptide

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 (Fig. 10). Gold particles were detected almost exclusively in the periplasm (P), but only scarcely in the electron-translucent recognition tip (T) and the cytoplasm (C) of the infectious form (Fig. 10A). No significant gold particle was detected in the reproductive forms (Fig. 10B). These results suggested that the 5.4 kDa peptide is localized in the periplasm of the infectious form of *H. obtusa*. A few number of gold particles in the cytoplasm may suggest that the 5.4 kDa peptide which was not cut off the putative signal peptide and not transported into periplasm is present in the cytoplasm.

Expression of 5.4 kDa peptide in transformed E. coli and extraction of periplasmic proteins with chloroform

In an effort to predict the function of the 5.4 kDa peptide, we prepared *E. coli* cells transformed with the plasmide vector pHOB5.4k which contained the coding region for the 5.4 kDa peptide and its putative signal peptide (Fig. 11). When the transformed cells were induced to produce the recombinant 5.4 kDa peptide with IPTG, and their lysate were subjected to SDS-PAGE, the recombinant 5.4 kDa peptide was detected only in a trace amount (data not shown).

So, the periplasmic proteins were extracted with chloroform from the transformed *E. coli* and the infectious form of *H. obtusa*, and subjected to SDS-PAGE (Fig. 12). It is known that chloroform is a reagent which extracts periplasmic proteins of the gram-negative bacterium (1). As expected, the 5.4 kDa peptide of *H. obtusa* was extracted with chloroform from the periplasm of the infectious form (Fig. 12 lane 2). This result is consistent with the intracellular localization observed by immunoelectron microscopy. A peptide with the same molecular mass as that of the 5.4 kDa peptide of *H. obtusa* was extracted with chloroform from the transformed cells of both strain DH5 and XL1-Blue of *E. coli* (Fig. 12 lanes 5-7 and 10-12), suggesting that the mature 5.4 kDa peptide was produced by cutting off the putative signal peptide, and then transported into periplasm even in *E. coll.* The peptide was not produced in the cells of *E. coli* transformed with the pTrc99A vector (Fig. 12, lanes 3 and 8) or in the absense of IPTG (Fig. 12, lane 4 and 9).

I also examined changes in the growth of the transformed *E. coli.* (Fig. 13). The transformed cells of the strain DH5 stopped their growth when production of the recombinant 5.4 kDa peptide was induced with IPTG (Fig. 13A). Meanwhile, the transformed cells of the strain XL1-Blue once decreased their growth from 1 to 3 hr after IPTG administration, but resumed it and grew normally afterward (Fig. 13B). As indicated in Fig. 12, at 4 hr after IPTG administration, transformed DH5 cells contained the same amount of the recombinant 5.4 kDa peptide (Fig. 12 lane 6) as that at 2 hr (Fig. 12 lane 5), while transformed XL1-Blue cells contained smaller amount of the recombinant 5.4 kDa peptide at 4 hr (Fig. 12 lane 11) than at 2 hr (Fig. 12 lane 10). This resumption of the growth in the transformed XL1-Blue cells seemed to be responsible for the decreased amount of the recombinant 5.4 kDa peptide.

DISCUSSION

In this study, I demonstrated that the 5.4 kDa peptide was present in the intermediate and infectious form, but not in the reproductive form of *H. obtusa*. I 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 was reported that protein pattern of this bacterium changes during this differentiation (11, 15, 16), and we reported the two infectious form-specific periplasmic proteins of 39 and 15 kDa, which were recognized by IF-3-1 and IF-3-2 monoclonal antibodies, respectively (5). In the meantime, this is the first report in which a gene encoding a protein appearing during this differentiation was cloned, and its expression was compared among different stages in the life cycle of *H. obtusa*.

Wiemann and Görtz identified five polypeptides extracted by chloroform treatment of the infectious form of *H. obtusa* (29). They reported that one of these polypeptides shows apparent 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.

The function of the 5.4 kDa peptide is not clear yet. However, our observation that the transformed cells of *E. coli* producing the recombinant 5.4 kDa peptide could scarcely divide 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 transformed cells of *E. coli* producing the 5.4 kDa peptide seems to suggest that the 5.4 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 (16), this result also seems to support my 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 (16, 17). 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. Analysis of the amino acid sequence of the 5.4 kDa peptide predicted its pI at 3.82, because it contained many acidic amino acids. This extremely acidic pI may be a clue to know the function of this peptide.

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 (26). 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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FIGURES AND FIGURE LEGENDS

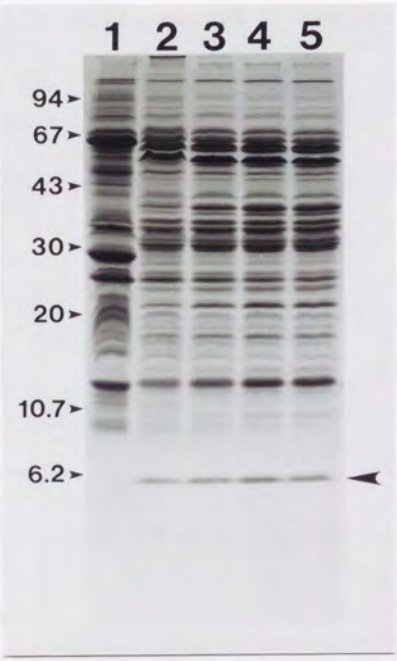

Fig. 1. SDS-PAGE of proteins from different forms of *H. obtusa*. Proteins were separated on 16 % polyacrylamide gel, and stained with CBB. Lane 1, reproductive form (2 x 10^8 cells); lane 2, intermediate form banding upon 70 % Percoll (1 x 10^7 cells); lane 3, intermediate form banding upon 80 % Percoll (1 x 10^7 cells); lane 4, intermediate form banding upon 90% Percoll (1 x 10^7 cells); lane 5, infectious form (1 x 10^7 cells). Numbers of cells indicated in parentheses contained nearly the same amount of protein. An arrowhead (right) indicates the 5.4 kDa peptide. The size of molecular weight markers (left) was given in kDa.

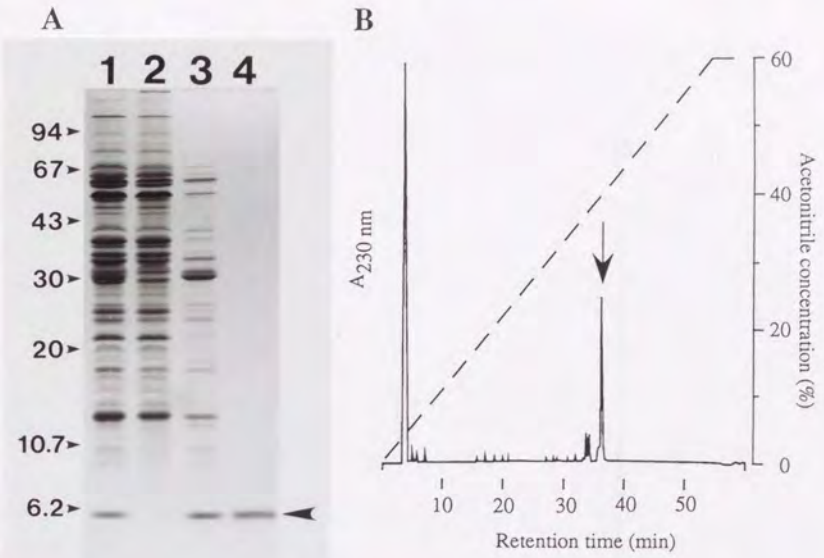

Fig. 2. Purification of the 5.4 kDa peptide. A, SDS-PAGE of the 5.4 kDa peptide in purification step. Cells of the infectious form of *H. obtusa* were sonicated and centrifuged, and the precipitate and supernatant were used as an insoluble and soluble fraction, respectively. Proteins from whole cells, the insoluble and soluble fraction of the infectious form, and the purified 5.4 kDa peptide were separated on 16% (w/v) polyacrylamide gel, and stained with CBB. Lane 1, whole cell lysate (1×10^7 cells); lane 2, insoluble fraction (corresponding to 1×10^7 cells); lane 3, soluble fraction (corresponding to 1×10^7 cells); lane 4, purified 5.4 kDa peptide. An arrowhead in the panel A indicates the 5.4 kDa peptide. B, reversephase HPLC chromatogram of the 5.4 kDa peptide. The 5.4 kDa peptide isolated from the gel band was subjected to reverse phase HPLC and eluted by acetonitrile gradient. An arrow in the panel B indicates a peak containing the 5.4 kDa peptide.

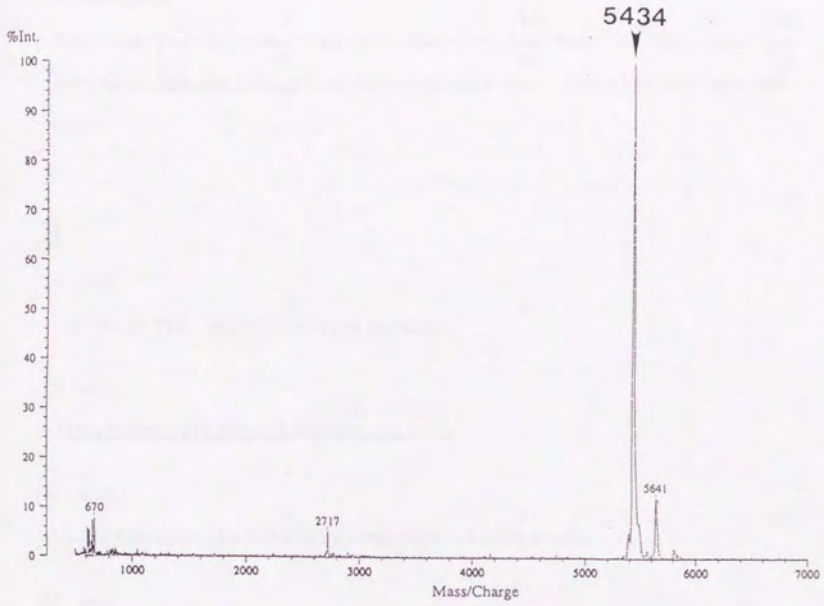

Fig. 3. Mass spectrum of the 5.4 kDa peptide. The purified 5.4 kDa peptide was analyzed by mass spectroscopy to estimate its molecular mass. An arrowhead indicates an ionized 5.4 kDa peptide ($[M+H]^+$), whose molecular mass was 5,434 Da. Therefore, molecular mass of the 5.4 kDa peptide was estimated to be 5,433 Da.

A

N-terminus

```
                    5                   10                  15
 Ser-Asp-Val-Ser-Asp-Lys-Glu-Ile-Asp-Asp-Glu-Val-Ser-Ser-Val
                   20                  25                  30
-Leu-Gln-Lys-Val-Phe-Glu-Ala-Asp-Gly-Asp-Ser-Glu-Ser-Lys-Ser

-Glu-
```

B

16 min

```
                    5                   10
  X -Glu-Val-Ser-Glu-Asp-Lys-Lys
```

18 min

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                         5                   10
-(Lys)-Asp-Ile-Val-Lys-Asp-Glu- X
```

20 min

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                    5                   10                  15
 Val-Phe-Glu-Ala-Asp-Gly-Asp-Ser-Glu-Ser-Lys
```

26 min

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                    5                   10                  15
 Glu-Ile-Asp-Asp-Glu-Val-Ser-Ser-Val-Leu-Gln-Lys
```

Fig. 4. Amino acid sequence of the N-terminus and the Lys-C fragments from the 5.4 kDa peptide. The purified 5.4 kDa peptide was directly analyzed to determine its amino acid sequence from the N-terminus. The Lys-C fragments from the 5.4 kDa peptide were analyzed to determine its internal amino acid sequences. A, amino acid sequence of the 5.4 kDa peptide from the N-terminus. B, amino acid sequences of four Lys-C fragments with given retension time on HPLC. Oligonucleotides deduced from underlined amino acid sequences were used as primers for PCR.

F2 (nt. 139-161)	5'-CAR-AAR-GTI-TTY-GAR-GCI-GAY-GG-3'
F4 (nt. 161-183)	5'-GGC-GAT-TCA-GAA-TCT-AAA-AGT-GAA-3'
F6 (nt. 195-213)	5'-TGG-AAG-TCA-GTG-AAG-ATA-AG-3'
R1 (nt. 233-214)	5'-TCR-TCY-TTI-ACD-ATR-TCY-TT-3'
R2 (nt. 215-192)	5'-TTC-TTA-TCT-TCA-CTG-ACT-TCC-ACT-3'
R4 (nt. 182-162)	5'-TCA-CTT-TTA-GAT-TCT-GAA-TCG-3'
C1 (Cassette primer)	5'-GTA-CAT-ATT-GTC-GTT-AGA-ACG-CG-3'
T7 (Cassette primer)	5'-TAA-TAC-GAC-TCA-CTA-TAG-GGA-GA-3'
START (nt. 34-53)	5'-ATG-AAT-TTT-TTG-TAT-TGC-TG-3'
STOP (nt. 240-222)	5'-TTA-ATC-TTC-ATC-TTT-TAC-G-3'

Table 1. Oligonucleotide primers used in this study. Primers F2 and R1 were used for ordinary PCR. Primers F4, F6, R2, R4, C1, and T7 were used for cassette PCRs. Primers START and STOP were used for labeling of probes for Southern and Northern blot hybridization and amplification of a segment coding for the 5.4 kDa peptide and its putative signal peptide for insertion into expression vector. 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, not C.

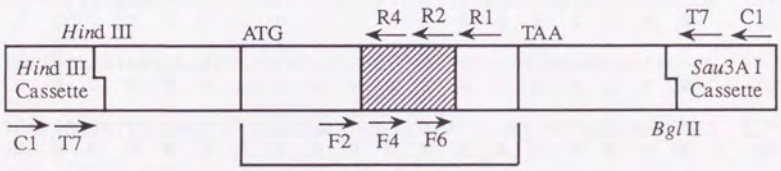

Fig. 5. Strategy for PCR amplification of coding region for the 5.4 kDa peptide and its flanking regions. A shaded part of the coding region for the 5.4 kDa peptide was amplified by PCR with a pair of primers F2-R1, sequenced, and extended toward the both sides by cassette PCRs with two pairs of primers C1-R2 and T7-R4 for upstream amplification, and with two pairs of primers C1-F4 and T7-F6 for downstream amplification. Thus, the nucleotide sequence of the coding region for the 5.4 kDa peptide and its flanking regions were determined.

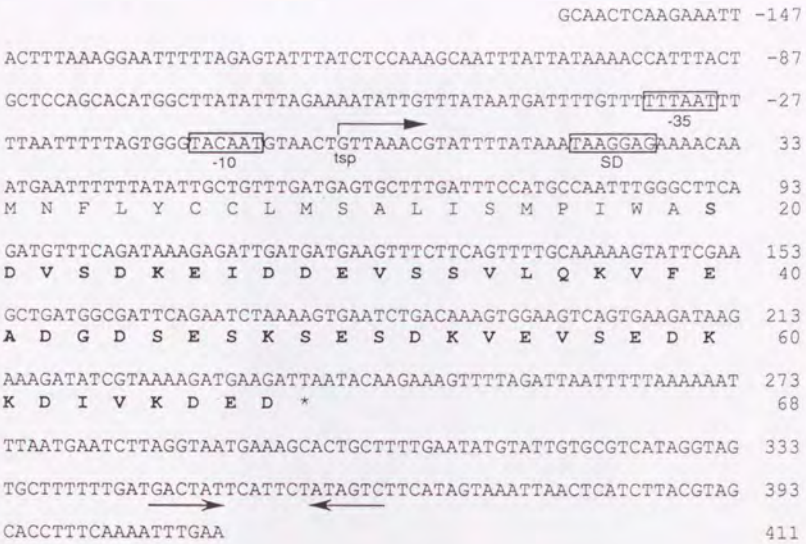

Fig. 6. Nucleotide sequence of the 5.4 kDa peptide gene and its flanking regions, and deduced amino acid sequence encoded by the gene. The putative promoter region (-35 and -10) and the Shine-Dalgarno (SD) sequence were boxed. An arrow indicates the transcription start point (tsp) determined by primer extension analysis. An asterisk indicates the translational termination codon. Facing arrows indicate the inverted repeat sequence, which was the putative transcriptional termination sequence. 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.

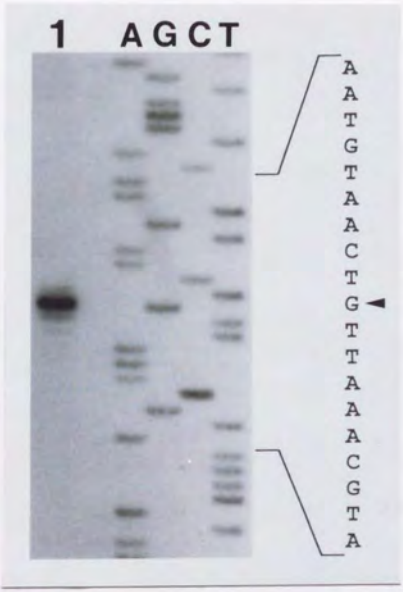

Fig. 7. Primer extension analysis to determine the transcription start point for the 5.4 kDa peptide gene. The labeled primer was hybridized to 2.5 μg of total RNA isolated from the infectious form and extended with M-MuLV reverse transcriptase. The resultant cDNA was denatured and electrophoresed on 5% 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.

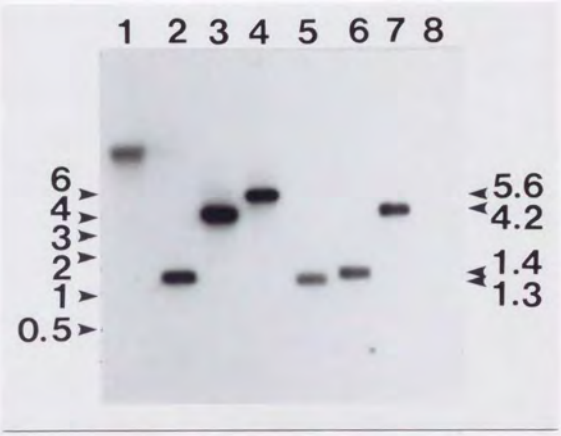

Fig. 8. Southern blot hybridization of the 5.4 kDa peptide gene. Genomic DNA from the infectious form of *H. obtusa* was digested with different restriction enzymes, sepsrated on 0.8 % agarose gel, transferred to nylon membrane, and hybridized to the PCR-labeled probe. The hybridized DNAs were detected using the DIG Luminescent Detection Kit. Lane 1, undigested genomic DNA of *H. obtusa*; lane 2, *Bgl* II digest; lane 3, *Eco*R I digest, lane 4, *Hin*d III digest; lane 5, *Bgl* II-*Eco*R I digest; lane 6, *Bgl* II-*Hin*d III digest; lane 7, *Eco*R I-*Hin*d III digest; lane 8, undigested genomic DNA of *P. caudatum*. The sizes of DNA markers (left) and detected DNA (right) were given in kbp.

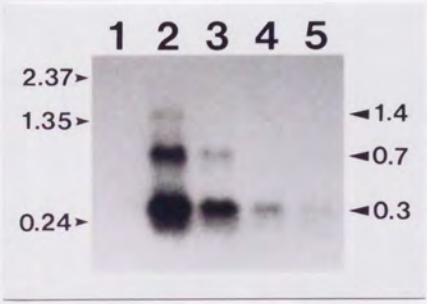

Fig. 9. Northern blot hybridization of the 5.4 kDa peptide mRNA. Four μg of total RNAs from the different forms of *H. obtusa* were separated on formamide-1.5% agarose gel, transferred to nylon membrane, and hybridized to the PCR-labeled probe. The hybridized RNAs were detected by autoradiography. Lane 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 detected RNA (right) were given in kb.

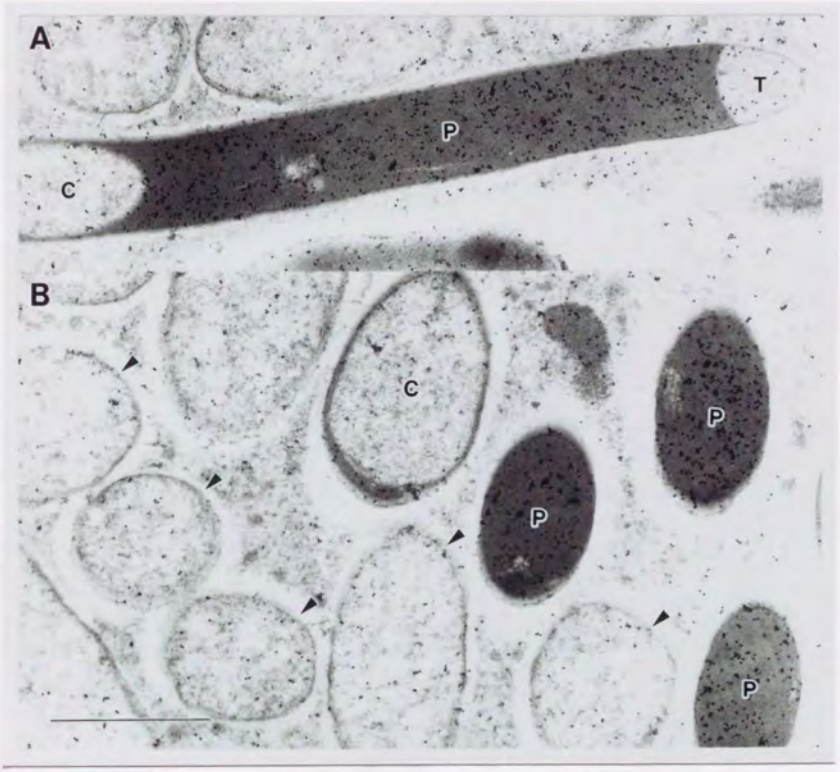

Fig. 10. Immunoelectron micrographs of *H. obtusa* in the macronucleus of *P. caudatum*. Thin sections were treated with rabbit anti-5.4 kDa peptide antiserum and goat anti-rabbit IgG-gold (10-nm), and stained with uranyl acetate. A, a longitudinal section of the infectious form. B, a transversal section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

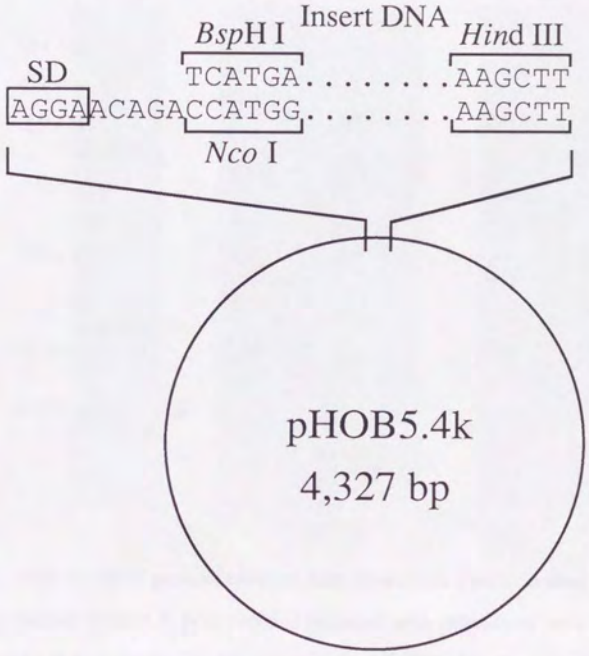

Fig. 11. Construction of plasmid vector pHOB5.4k. A segment coding for the 5.4 kDa peptide and its putative signal peptide was amplified by PCR and inserted into the pTrc99A expression vector, which was tentatively designated pHOB5.4k.

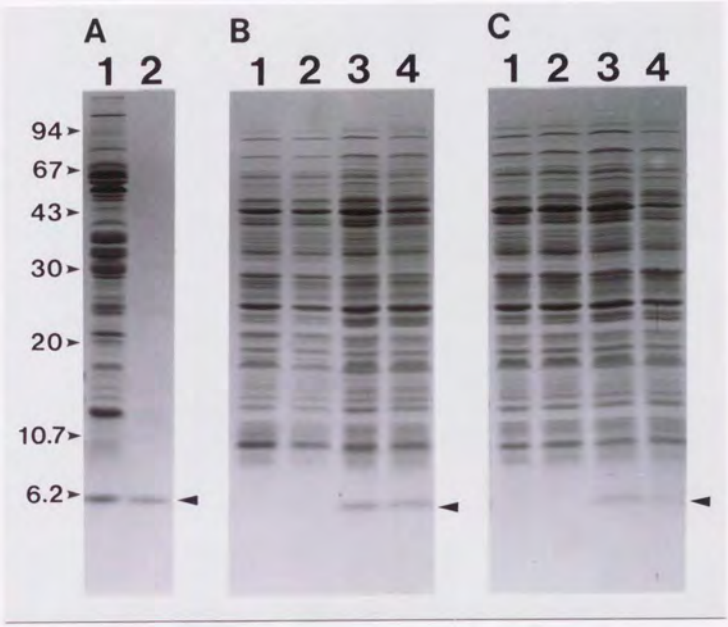

Fig. 12. SDS-PAGE of proteins extacted with chloroform from infectious form of *H. obtusa* and transformed cells of *E. coli*. Proteins extracted with chloroform were subjected to SDS-PAGE of 16 % polyacrylamide gel, and stained with CBB. A, infectious form. Lane 1, whole cell lysate (1 x 10^7 cells); lane 2, periplasmic proteins (corresponding to 5 x 10^7 cells). B, *E. coli* strain DH5. C, *E. coli* strain XL1-Blue. Lane 1, transformed with the pTrc99A vector; lanes 2-4, transformed with the pHOB5.4k; lane 2, without IPTG; lanes 3 and 4, with 2 mM IPTG; lane 3, at 2 hr after IPTG administration; lane 4, at 4 hr. An arrowhead in the panel A indicates the 5.4 kDa peptide of *H. obtusa*, and arrowheads in the panel B and C indicate the recombinant 5.4 kDa peptide.

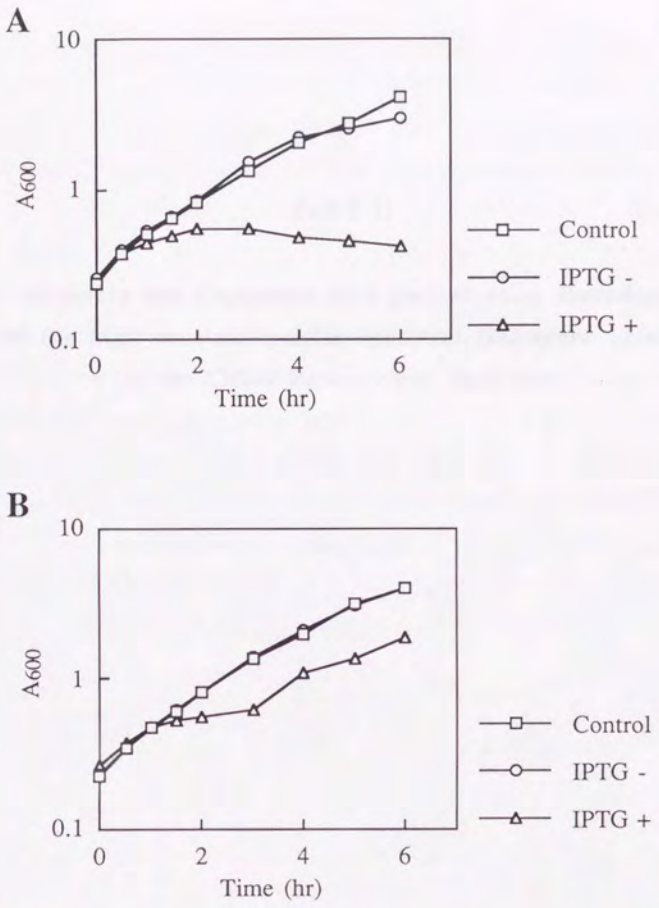

Fig. 13. Growth curve of transformed cell of *E. coli*. A, strain DH 5. B, strain XL1-Blue. The cells of *E. coli* transformed with the pTrc99A vector (open square) and with pHOB5.4k were cultivated in the absence (open circle) or presence (open triangle) of 2 mM IPTG. Growth of the transformed cells of *E. coli* was monitored by mesuring absorbance of the culture medium at 600 nm.

PART II

Structure and Expression of a *groE* Operon Homolog of the Macronucleus-specific Symbiont *Holospora obtusa* of the Ciliate *Paramecium caudatum*

ABSTRACT

The macronucleus-specific symbiont *Holospora obtusa* of the ciliate *Paramecium caudatum* contained a 63-kDa protein immunologically related to the GroEL protein, or Hsp60 of *Escherichia coli*. This protein was most abundantly synthesized in the reproductive form, but not in the infectious form in the host macronucleus. Heat shock treatment *in vitro* of isolated cells of the reproductive and infectious form of the bacterium induced the synthesis of the 63-kDa protein. Cloning and sequencing of a gene for the 63-kDa protein suggested that the protein is 55.2 % identical to GroEL of *E. coli* in amino acid sequence, and that the gene is preceded by an ORF which encodes a protein 39.6 % identical to GroES of *E. coli*. Northern blot hybridization showed that the *groEL* gene is expressed highly in the reproductive form, but only in a trace amount in the intermediate and infectious form. Immunoelectron microscopy revealed that the GroEL homolog is localized in the cytoplasm of the reproductive and infectious form. Phylogenetic analysis on the GroEL proteins of different bacteria suggested that *H. obtusa* belongs to the α-subdivision of the Phylum *Proteobacteria*, and is modelately related to the intracellular parasitic bacteria *Rickettsia*, *Ehrlichia*, and *Cowdria*.

INTRODUCTION

The gram-negative bacterium *Holospora obtusa* is a macronucleus-specific symbiont of the ciliate *Paramecium caudatum*. This bacterium shows two different morphologies in its life cycle: a reproductive short form and an infectious long form (18, 20, 40). The reproductive form contains a large amount of a 63-kDa protein which is cross-reacted with a monoclonal antibody against GroEL homolog from X-bacteria, the symbionts of *Amoeba proteus* (6, 13).

Homologs of GroEL protein, or Hsp60 are ubiquitous in eubacteria and eukaryotic organelles (25). In the past several years, *groEL* genes from many eubacteria have been cloned, sequenced, and characterized, which suggested that structure of GroEL homologs is highly conservative throughout many organisms. It has been reported that aphid symbionts harbored by the mycetocyte synthesize selectively symbionin, a GroEL homolog (22). In the soybean bacteroid *Bradyrhizobium*, the amount of GroEL homolog at the symbiotic phase is 7 times as large as that at the free-living one (6). Large amounts of GroEL homologs are also synthesized by the intracellular parasitic bacteria, such as *Mycobacterium* (34), *Chlamydia* (35), *Coxiella* (50), and *Rickettsia* (48).

In this study, in an effort to characterize a GroEL homolog of *H. obtusa*, I examined its synthesis *in vivo*, *in vitro*, and under the heat shock conditions. For further characterization, I cloned its gene by PCR using primers with nucleotide sequences deduced from amino acid sequence estimated for the GroEL homolog, and determined nucleotide sequence of the gene. Also, I compared the expression of this gene among the different forms of *H. obtusa*.

It was reported that the phylogenetic tree based on 16S rRNA sequences shows a moderate relationship between *H. obtusa* and the intracellular parasite *Rickettsia* (2). However, given the limitations in assumptions on which present phylogenetic methods are based, a phylogenetic tree based on a single macromolecule may not necessarily be reliable (24, 50). Therefore, I reexamined relationship of *H. obtusa* to other eubacteria by constructing a phylogenetic tree based on GroEL proteins.

MATERIALS AND METHODS

Strains and culture

Cells of *Paramecium caudatum* used for protein synthesis experiments were of a strain FK-1 (syngen unknown), which was originally collected in Yamaguchi, Japan and for cloning and sequencing of a gene coding for the 63-kDa peptide were of a strain RB-1 (syngen unknown), which was originally collected in Germany. The cells of *Paramecium* were infected newly with *Holospora obtusa* and cultivated as described in the Methods section of Part I. Non-pathogenic cells of *Klebsiella pneumoniae* were cultivated on a slunt of LB medium at 24-26 °C and used as a prey bacterium of *P. caudatum* and for protein synthesis experiments.

Antiserum against symbionin

Symbionin, a GroEL homolog of the symbiont of the pea aphid *Acyrthosiphon pisum*, was purified from tissues of the insects (21). Antiserum against this protein was prepared in a male Japan White Rabbit as described by Hara *et al.* (23).

Isolation of H. obtusa

Cells of the reproductive, intermediate, and infectious form of *H. obtusa* were isolated as described in the Methods section of Part I.

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting were basically performed as previously described (7). Proteins from isolated cells of the reproductive, intermediate, and infectious form of *H. obtusa* were subjected to SDS-PAGE of 12.5 % (w/v) polyacrylamide gel. After SDS-PAGE, one set was stained with CBB and the other set was transferred to Immobilon-P PVDF membrane (Millipore) in the transfer buffer [48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20 % (v/v) methanol (pH 9.2)] at 1 mA/cm^2 for 2 h. The PVDF membrane was incubated with anti-symbionin antiserum diluted 10,000-fold with TTBS [100 mM Tris-HCl (pH 7.5), 0.9 % (w/v) NaCl and 0.15 % (v/v) Tween 20] containing 5 % (w/v) skim milk overnight. After washed

with TTBS, the 63-kDa protein on the PVDF membrane was detected using the Vectastain ABC-AP kit and Alkaline phosphatase substrate kit IV (Vector Laboratories).

In vivo and in vitro protein synthesis of H. obtusa

To analyze *in vivo* protein synthesis of *H. obtusa*, proteins synthesized by *H. obtusa* in the host macronucleus were labeled with Tran ^{35}S-label (ICN). The reproductive and infectious form-bearing paramecia were concentrated to 1 x 10^5 and to 2 x 10^4 cells/ml, added with 42 and 100 μCi/ml Tran ^{35}S-label, respectively, and cultivated at 25 °C for 3 hr. Labeling was stopped by washing with modified Dryl's solution (8), and cells of the reproductive and infectious form were isolated and subjected to SDS-PAGE as described above. Proteins separated on the gel were stained with CBB, and those synthesized newly were detected by fluorography using the Enlightning (NEN Research Products).

To analyze *in vitro* protein synthesis of *H. obtusa*, proteins synthesized by isolated cells of the reproductive and infectious form were labeled with 40 μCi/ml Tran ^{35}S-label in 10 mM Na, K-phoaphate buffer (pH 6.5) containing 1 mg/ml NH_4Cl at 25 °C for 1 hr. Labeling was stopped by washing with 10 mM Tris-HCl (pH6.8), and cells of the bacterium were subjected to SDS-PAGE and fluorography.

Heat shock treatment of H. obtusa

Isolated cells of the reproductive and infectious form of *H. obtusa* were suspended in 10 mM Na, K-phosphate buffer (pH6.5) containing 1 mg/ml NH_4Cl, added with 200 μCi/ml Tran ^{35}S-label (ICN), and incubated at 25, 30, 33, 36, 39, 42 and 45 °C for 20 min. Labeling of proteins was stopped by adding with excessive amounts of cold methionine and cysteine. After centrifugation at 10,000 g for 10 min at 4 °C, precipitated cells were lysed by boiling in Laemmli's lysis buffer. Proteins from 3 x 10^6 cells of the reproductive and 2 x 10^5 cells of the infectious form were subjected to SDS-PAGE and stained with CBB. Proteins synthesized during heat shock treatment were detected by fluorography.

Preparation of host macronuclear extract and its effect on protein synthesis of H. obtusa

Macronuclei of *P. caudatum* were isolated from cells at the log and stationary phase as follows. Cells were harvested and lysed with 0.25 M TSCM-NPS buffer [10 mM Tris-HCl (pH 8.0), 0.25 M sucrose, 3 mM $CaCl_2$, 8 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 0.01 % (w/v) spermidine, and 0.2 % (v/v) Nonidet P-40]. The lysate was overlaid on 1.6 M TSCM-PS buffer [10 mM Tris-HCl (pH 8.0), 1.6 M sucrose, 3 mM $CaCl_2$, 8 mM $MgCl_2$, 0.1 mM PMSF, and 0.01 % (w/v) spermidine] and centrifuged at 500 g for 10 min at 4 °C. The macronuclei precipitated at the bottom of the tube were suspended with 10 mM Na, K-phosphate buffer (pH 6.5) containing 1 mg/ml NH_4Cl to 5 x 10^6 nuclei/ml, homogenized with Teflon homogenizer, and centrifuged at 10,000 g for 10 min at 4 °C to remove insoluble materials. The supernatant was used as a macronuclear extract to examine its effects on *in vitro* protein synthesis of *H. obtusa*.

Cells of the reproductive and infectious form of *H. obtusa*, and *Klebsiella pneumoniae* were suspended with 10 mM Na, K-phosphate buffer (pH 6.5) containing 1 mg/ml NH_4Cl or with the host macronuclear extract corresponding to 1 x 10^5 nuclei, preincubated at 25 °C for 30 min, added with 40 μCi/ml Tran ^{35}S-label (ICN), and incubated at 25 °C for 1 hr. After centrifugaation at 10,000 g for 5 min at 4 °C, proteins from precipitated cells were subjected to SDS-PAGE and stained with CBB, and proteins synthesized during incubation in the presence of the macronuclear extract were analyzed by fluorography.

Amino acid sequencing of 63-kDa protein of H. obtusa

To determine the N-terminal amino acid sequence of the 63-kDa protein of *H. obtusa*, proteins from 2 x 10^8 cells of the reproductive form were subjected to SDS-PAGE of 12.5 % (w/v) polyacrylamide gel, transferred to Immobilon-P^{SQ} PVDF membrane (Millipore), and stained with Immobilon-CD Stain (Millipore). The band of the 63-kDa protein was excised, washed with deionized water, and then directly analyzed on a PPSQ-21 gas-phase protein sequencer (Shimazu). To determine the internal amino acid sequence of the 63-kDa protein, proteins from 2 x 10^9 cells of the reproductive form were subjected to SDS-PAGE, stained with CBB, and the band of the 63-kDa protein was excised. The 63-kDa protein was extracted from the homogenate of the gel band in 50 mM Tris-HCl (pH 8.8) containing 0.05 % (w/v) SDS, and

digested with 2.5 μg of Endoproteinase Lys-C (Boehringer Mannheim) in 25 mM Tris-HCl (pH 8.5) and 1 mM EDTA at 37 °C for 16 hr. The reaction was stopped by adding with 1/6 volume of acetic acid, and the Lys-C fragments were purified by reverse phase HPLC as described in the Methods section of Part I. The purified Lys-C fragments were dissolved with 10 % (v/v) acetonitrile containing 0.05 % (v/v) trifluoroacetic acid, dried on Immobilon-CD PVDF membrane (Millipore), and then analyzed on the protein sequencer.

Extraction of genomic DNA from H. obtusa

Genomic DNA of *H. obtusa* was extracted from the infectious form as described in the Methods section of Part I, and used for PCR amplifications and Southern blot hybridization.

PCR amplification and nucleotide sequencing of a gene coding for 63-kDa protein

Two parts of the gene coding for the 63-kDa protein were amplified by PCR with 2 μM of two pairs of mixed primers NF-34R and 22F-26R, whose sequences were deduced from the amino acid sequences of N-terminus, Lys-C fragments with retention time of 34, 22, and 26 min of the 63-kDa protein, respectively. After incubation at 94 °C for 5 min, PCR amplification was performed with 30 cycles of 94 °C for 30 sec, 40 °C for 2 min, and 72 °C for 1 min, followed by a 5-min extension time at 72 °C. The resultant PCR products were cloned and sequenced as described in the Methods section of Part I. The fragment obtained by PCR with a pair of primers F3-R3, whose sequences were determined from the NF-34R and 22F-26R fragment, respectively, were cloned, sequenced, and extended toward the both sides by the cassette PCRs (26). Cassette PCRs were performed using the *Sau*3A I Cassette (TaKaRa) ligated to *Bgl* II digest of the genomic DNA of *H. obtusa* with two pairs of primers C1-R1 and T7-R2 for upstream amplification, and with two pairs of primers C1-F1 and T7-F2 for downstream amplification. The resultant PCR products were cloned and sequenced as described in the Methods section of Part I. Thus, the nucleotide sequence of the coding region for the 63-kDa protein and its 5'- and 3'-flanking regions were determined.

Southern blot hybridization of groEL gene

Southern blot hybridization of the *groEL* gene was basically performed as described in the Methods section of Part I. One μg of the genomic DNA from the infectious form of *H. obtusa* was digested, separated, transferred, and then hybridized to the probe DNA labeled by PCR with 0.25 μM of a pair of primers F5-R6. The hybridized DNAs were detected using the DIG Luminescent Detection Kit (Boehringer Mannheim).

RNA extraction and Northern blot hybridization

RNA extraction and Northern blot hybridization were basically performed as described in the Methods section of Part I. For Northern blot hybridization, 5 μg of total RNAs extracted from the reproductive, intermediate, and infectious form of *H. obtusa* were separated, transferred, and hybridized to the probe DNA labeled by PCR with a pair of primers F5-R6, and the hybridized RNAs were detected by autoradiography.

Immunoelectron microscopy of 5.4 kDa peptide

To determine the intracellular localization of the GroEL homolog of *H. obtusa*, I performed immunoelectron microscopy using antiserum against symbionin, a GroEL homolog of the symbiont of the pea aphid *Acyrthosiphon pisum* as previously described (7) and in the Methods section of Part I. Thin sections on a grid were incubated with rabbit anti-symbionin antiserum diluted 200-fold and goat anti-rabbit IgG-gold diluted 20-fold. Observation was carried out using a JEM-100CX (JEOL) transmission electron microscope.

Phylogenetic analysis based on GroEL amino acid sequences

The deduced amino acid sequence of the GroEL homolog of *H. obtusa* was aligned with those of 21 species of other bacteria. The phylogenetic tree was constructed by the neighbor-joining distance method (42), which has been shown to be relatively consistent compared to other methods, even in the presence of unequal rates of evolution, using the Clustal W 1.6. The confidence limits for the phylogenetic tree were placed by the bootstrap procedure (10). Bacteria used as sources of GroEL homolog were *Rickettsia tsutsugamusi* (48), *Ehrlichia chaffeensis* (49), *Cowdria ruminantium* (29), *Bradyrhizobium japonicum* (12), *Rhizobium meliloti* (41),

Bartonella bacilliformis (unpublished), *Brucella abortus* (17), *Agrobacterium tumefaciens* (44), *Zymomonas mobilis* (3), *Neisseria gonorrhoeae* (unpublished), *Coxiella burnetii* (51), symbiont of *Amoeba proteus* (1), symbiont of *Acyrthosiphon pisum* (39), *Escherichia coli* (25), *Salmonella typhi* (30), *Pseudomonas aeruginosa* (28), *Helicobacter pylori* (31), *Chlamydia trachomatis* (35), *Borrelia burgdorferi* (45), *Bacillus subtilis* (43), and *Mycobacterium leprae* (34).

RESULTS

SDS-PAGE and immunoblotting of 63-kDa protein

To compare the amounts of the 63-kDa protein of *H. obtusa* among the reproductive, intermediate, and infectious form, proteins from different forms were subjected to SDS-PAGE, transferred to PVDF membrane, and probed with anti-symbionin antiserum. As a result, the 63-kDa protein, whose apparent molecular mass was estimated to be 63 kDa by SDS-PAGE, was detected in all the forms (Fig. 1B). Protein amounts from the same number of the different forms were quite different between the reproductive form (Fig 1A, lane 1) and the intermediate and infectious form (Fig 1A, lanes 2-5), because the intermediate and infectious form is about 15-20 times as large as the reproductive form. Nevertheless, amounts of the 63-kDa protein detected with the antiserum were nearly same among the different forms (Fig 1B, lanes 1-5), indicating that the amount of the 63-kDa protein per cell of different forms is roughly constant. Therefore, the amount of the 63-kDa protein per unit of total protein should be largest by far in the reproductive form.

In vivo and in vitro protein synthesis of H. obtusa

To compare the amounts of the 63-kDa protein synthesized *in vivo* and *in vitro* by the reproductive and infectious form of *H. obtusa*, proteins were labeled *in vivo* and *in vitro* with Tran ^{35}S-label and detected by fluorography. As a result, patterns of protein synthesis of the reproductive (Fig. 2A, lane 2) and infectious form (Fig. 2B, lane 2) in the host macronucleus were roughly consistent with the protein patterns detected with CBB (Figs. 2A and 2B, lane 1), suggesting that the reproductive and infectious form synthsized constitutive proteins in the host macronucleus. However, isolated cells of the bacterium showed different patterns of protein synthesis due to the form (Figs. 2A and 2B, lane 3). While the reproductive form selectively synthesized the 63-kDa protein *in vivo* (Fig. 2A, an arrowhead), it synthesized other proteins as well *in vitro*. Meanwhile, the infectious form only scarcely synthesized this protein both *in vivo* and *in vitro* (Fig. 2B, an arrowhead). Since the 63-kDa protein of *H. obtusa* is immunologically related to GroEL, a stress protein, these results may suggest that the reproductive form is under

the stress conditions in the host macronucleus, and the infectious form is under the conditions released from the stress. These protein syntheses were inhibited with chloramphenicol (Figs. 2A and 2B, lane 5), but not with cycloheximide (Figs. 2A and 2B, lane 4).

Heat shock response of isolated H. obtusa

In considering that the 63-kDa protein of *H. obtusa* is immunologically related to GroEL, a heat shock protein of *E. coli*, we examined changes in amount of the 63-kDa protein synthesized by isolated cells of the reproductive and infectious form under the heat shock conditions. In the reproductive form, patterns of protein synthesis did not change much when the temperature shifted up from 25 to 33 °C (Fig. 3A, lanes 1-3). Synthesis of the 63-kDa protein increased at 36 °C as well as other heat shock proteins (Fig. 3A, lane 4), and gradually decreased over 39 °C (Fig. 3A, lanes 5-7). The infectious form scarcely synthesized the 63-kDa protein at 25 °C (Fig. 3B, lane 1), while, unlike in the case of the reproductive form, its synthesis gradually increased from 30 °C (Fig. 3B, lanes 2 and 3), reached the maximum at 36 °C (Fig. 3B, lane 4), and then gradually decreased over 39°C (Fig. 3B, lanes 5-7). Protein synthesis was scarcely detected at 45 °C (Figs. 3A and 3B, lane 7). It was noted that the patterns of protein synthesis in the reproductive and infectious form over 39 °C were very similar to each other, while those below 36 °C were quite different. Under the strong stress conditions, the stress response of *H. obtusa* may become similar even in different forms of the bacterium. Heat shock response of isolated *H. obtusa* was different from stress response in the host macronucleus in the following points: (1) the reproductive and infectious form of *H. obtusa* scarcely synthesized other heat shock proteins in the host macronucleus; (2) the infectious form scarcely synthesized the 63-kDa protein in the host macronucleus, whereas its synthesis was induced by heat shock treatment. These results suggested that the stress response of *H. obtusa* in the host macronucleus occurs in a different way from the heat shock response of isolated *H. obtusa*.

Effects of Paramecium macronuclear extract on in vitro protein synthesis

The 63-kDa protein of *H. obtusa* was more abundant and highly synthesized in the reproductive form than in the intermediate or infectious form in the host macronucleus (Figs. 1

and 2). Synthesis of the 63-kDa protein were induced by heat shock treatment of isolated cells of the reproductive and infectious form (Fig. 3). To examine whether an stressor inducing the synthesis of the 63-kDa protein was present in the host macronucleus, I examined effects of host macronuclear extract on the *in vitro* protein synthesis of *H. obtusa*. When isolated cells of the reproductive and infectious form of *H. obtusa*, and *Klebsiella pneumoniae* were cultivated in the presence of macronuclear extracts from cells of *Paramecium* at the log and stationary phase, the *in vitro* protein syntheses of the reproductive and infectious form of *H. obtusa* were inhibited. The inhibitory effect was stronger in the infectious form (Fig. 4B, lanes 2 and 3) than in the reproductive one (Fig. 4A, lanes 2 and 3). There is no significant difference of the effect between host macronuclear extracts from the host cells at the log and stationary phase. The inhibitory effect of the macronuclear extract on the *in vitro* protein synthesis was lost by heat treatment at 100 °C, but not by dialysis (data not shown), suggesting that the substance(s) which inhibited the protein synthesis was(were) heat-unstable and undialyzable. Synthesis of the 63-kDa protein was induced neither in the reproductive nor in the infectious form by the macronuclear extracts from cells at both the log and stationary phase. In contrast, the *in vitro* protein synthesis of *K. pneumoniae*, the prey bacterium of *Paramecium*, was increased in the presence of the *Paramecium* macronuclear extracts. The effect of the macronuclear extract from cells of *Paramecium* at the log phase (Fig. 4C, lane 2) was stronger than that from those at the stationary phase (Fig. 4C, lane 3). It was noted that the responses to the same macronuclear extract were different between the symbiotic bacterium *H. obtusa* and the free-living bacterium *K. pneumoniae*. It was conceivable that *P. caudatum* inhibits the protein synthesis of *H. obtusa* to prevent its excessive multiplication lest the symbiotic relationship with *H. obtusa* should be destroyed.

Determination of amino acid sequence of 63-kDa protein

To determine amino acid sequence of the 63-kDa protein of *H. obtusa*, this protein transferred to PVDF membrane was directly analyzed up to 34th amino acid residue from the N-terminus (Fig. 5A). The N-terminal amino acid was not methionine but serine, which was deduced to be a second amino acid by sequencing of the gene (Fig. 7). Therefore, the N-

terminal amino acid, methionine was cut off from the 63-kDa protein posttranslationally. To determine the internal amino acid sequence of the 63-kDa protein, four Lys-C fragments, whose retention times on HPLC were 22, 26, 33 and 34 min were also sequenced (Fig. 5B). By homology analysis, amino acid sequence of the N-terminus and the Lys-C fragments showed moderate similarities to those of GroEL homologs of other bacteria. Two fragments with retention times of 33 and 34 min were found to be connected by alignment with GroEL of *E. coli*. Oligonucleotides deduced from these amino acid sequences were synthesized and used as primers for PCR to amplify two parts of its gene. Primers used for PCRs and sequencing of the gene in this study were listed up in Table 1.

PCR amplification and nucleotide sequencing of gene coding for 63-kDa protein

PCRs with two pairs of primers NF-34R and 22F-26R amplified two fragments, whose sizes roughly corresponded to the expected sizes calculated from the alignment with the GroEL of *E. coli*. These fragments were cloned and sequenced, and oligonucleotides which annealed with the nucleotide sequence thus determined were synthesized and used for ordinary PCR as primers. PCR with a pair of primers F3-R3 amplified a fragment, which was cloned, sequenced, and extended toward the both sides by cassette PCR to cover the entire gene. As a result, an entire nucleotide sequence of the gene coding for the 63-kDa protein and its 5'- and 3'-flanking regions were determined (Fig. 7). It turned out that the gene encodes a protein composed of 554 amino acids with a predicted molecular mass of 59.2 kDa. Sequencing of the gene also revealed that it is preceded by an open reading frame (ORF) which encodes a protein composed of 96 amino acids with a predicted molecular mass of 10.4 kDa. By homology analysis, the 63-kDa protein was found to be a GroEL homolog of *H. obtusa*, and the protein encoded by the preceding ORF was found to be a GroES homolog of *H. obtusa*. The amino acid sequence deduced from the nucleotide sequence of the gene was completely consistent with those determined by the peptide sequencing. By sequence analysis, ribosome binding sites were found just upstream of the translational initiation codons for both the GroES and GroEL homolog. The sites shared a polypurine sequence, known as the Shine-Dalgarno sequence (46), GGAGG (nt. 296 to 300) for the GroES homolog, and AAGGAGG (nt. 597 to 603) for the

GroEL homolog. There were inverted repeat sequences upstream of the *groES* gene and downstream of the *groEL* gene. The former was an imperfect inverted repeat, 5'-TTAGCACTC-NNNNNNNNN-GACTGCTAA-3' (nt. 243 to 269), containing a consensus sequence (GCACTC-N9-GAGTGC) which has been found upstream of heat shock genes in some species. The latter one, 5'-CATGTTT-NNNNNNNNN-AAACATG-3' (nt. 2432 to 2454), was a putative transcriptional termination sequence, which seemed to form a stem-loop structure and to serve as a transcriptional terminator. Judging from the structural feature of the two ORFs and the result that there was no transcription start point between the *groES* and *groEL* gene (data not shown), it was thought that the *groES* and *groEL* gene of *H. obtusa* together form a single operon, just as the *groE* operon of *E. coli*, and that their transcription occurs continuously.

Homology analysis of GroEL and GroES homolog of H. obtusa

For comparison of the deduced amino acid sequences of the GroEL and GroES homolog of *H. obtusa* with those of GroEL and GroES of *E. coli*, they were aligned in Fig. 8 and 9, respectively. Among 554 amino acid residues of the GroEL homolog of *H. obtusa*, 306 were identical (55.2 %) and 396 were identical or analogous (71.5 %) to those of *E. coli* (Fig. 8). Among 96 residues of the GroES homolog of *H. obtusa*, 38 were identical (39.6 %) and 59 were identical or analogous (61.5 %) to those of *E. coli* (Fig. 9).

Southern blot hybridization of groEL gene

When a PCR-labeled DNA coding for the GroEL homolog was hybridized to the undigested genomic DNA of *H. obtusa* and *P. caudatum*, and different restriction enzyme digests of the genomic DNA of *H. obtusa* by Southern blot hybridization, the probe DNA was hybridized to the undigested genomic DNA of *H. obtusa* (Fig. 10, lane 1) and its digests (Fig. 10, lanes 2-7), but not to that of *P. caudatum* at all (Fig. 10, lane 8). Single bands of DNA with size of about 3.4, 10, 0.8, 3.4, 0.8, and 0.8 kbp was detected in the *Bgl* II, *Eco*R I, *Hin*d III, *Bgl* II and *Eco*R I, *Bgl* II and *Hin*d III, and *Eco*R I and *Hin*d III digests, respectively. These results suggested that the *groEL* gene of *H. obtusa* was a single copy gene and that the GroEL homolog was encoded by the genomic DNA of *H. obtusa*.

Northern blot hybridization of groEL mRNA

To compare expression of the *groEL* gene among the reproductive, intermediate, and infectious form of *H. obtusa*, a PCR-labeled DNA coding for the GroEL homolog was hybridized to total RNAs from these forms. As a result, RNA with a size of 2.3 kb was detected in all the forms, but the amounts detected were quite various (Fig. 11). The hybridized RNA was highest in amount in the reproductive form (Fig. 11, lane 1), while it was only in a trace amount in the intermediate and infectious form (Fig. 11, lanes 2-5). It was noted that the detected RNA amount decreases as the buoyant density of the bacterium increases. These results suggested that the *groEL* gene is highly expressed in the reproductive form, and that its expression is dramatically decreased during the differentiation from the reproductive to infectious form. Immunoblotting of the GroEL homolog showed that its amount per cell is nearly same among the different forms, suggesting that the GroEL homolog is neither synthesized nor degraded during the differentiation from the reproductive to infectious form, which will account for the fact that a cell contains the same amount of the GroEL homolog irrespective of its form..

Immunoelectron microscopy of GroEL homolog

To determine the intracellular localization of the GroEL homolog of *H. obtusa*, I performed immunoelectron microscopic observation using rabbit anti-symbionin antiserum and anti-rabbit IgG-gold (Figs. 12 and 13). Gold particles were detected almost exclusively in the cytoplasm (C) of both the reproductive (Fig. 12A) and infectious form (Fig. 12B), and only barely detected both in the electron-translucent recognition tip (T) and the periplasm (P) of the infectious form (Figs. 12B and 13). Gold particles were also detected in the cytoplasmic extrusion into periplasmic space (Fig. 13B). These results suggested that the GroEL homolog of *H. obtusa* is localized in the cytoplasm of both the reproductive and infectious form of *H. obtusa*.

Phylogenetic analysis based on GroEL amino acid sequences

Phylogenetic analysis based on 16S rRNA sequence showed that *H. obtusa* belongs to α-subdivision of the Phylum *Proteobacteria,* and that it shows moderate relationship to an intracellular parasite *Rickettsia* (2). The GroEL, a heat shock protein of *E. coli*, is efficient as an evolutionary chronometer (50), because it is highly conserved and ubiquitously distributed among eubacteria (52). To confirm the evolutionary relationship of *H. obtusa* to other eubacteria, I depicted a phylogenetic tree based on their GroEL homolog amino acid sequences (Fig. 14). This unrooted tree suggested that *H. obtusa* belongs to α-subdivision of the Phylum *Proteobacteria,* and is related to *Rickettsia*, *Ehrlichia*, and *Cowdria*, just as demonstrated by the phylogenetic tree based on 16S rRNA sequences. It has also been reported that *H. obtusa* is more closely related to *Caedibacter caryophila*, a killing endosymbiont of *P. caudatum* than to *Rickettsia* (47). In this study, however, the relationship between *H. obtusa* and *C. caryophila* could not be inferred based on the GroEL homolog sequences, because GroEL homolog of *C. caryophila* has not been sequenced yet.

DISCUSSION

In this study, I characterized the 63-kDa protein of the endosymbiont *H. obtusa*, which is immunologically related to GroEL of *E. coli* by cloning and sequencing its gene and the flanking regions. Sequence analysis revealed the presence of two ORFs which encode proteins homologous to GroES and GroEL, indicating that the 63-kDa protein is a GroEL homolog of *H. obtusa*. The coding regions for the GroES and GroEL were separated by a 13-base intergenic sequence, whose size is very short compared with those of other bacteria reported so far. Although the genome size of *H. obtusa* is not clear yet, this short intergenic sequence between the *groES* and *groEL* gene may reflect a small size of this genome.

Although synthesis of the GroEL homolog of *H. obtusa* increased upon heat shock, a consensus sequence of the heat shock promoter was not identified in the region upstream of the *groES* gene. Instead, I found that an imperfect inverted repeat (5'-TTAGCACTC-NNNNNNNNN-GACTGCTAA-3') is present in that region. This inverted repeat contained a consensus sequence (5'-GCACTC-N9-GAGTGC-3'), which has been found previously in the region upstream of heat shock genes of some species of gram-positive bacteria (9, 34, 36, 37), *Cyanobacteria* (5), and *Proteobacteria* (11). This sequence has been proposed to be functionally significant for transcriptional regulation by either acting as a recognition site for DNA or RNA binding proteins, or forming secondary structure in DNA or RNA (36, 43). Probably, this sequence is also involved in the regulation of the expression of the *groESL* gene in *H. obtusa*. Primer extension analysis failed to determine transcription start point for the *groESL* gene (data not shown), probably because the *groESL* mRNA formed a secondary structure due to this inverted repeat.

The GroEL homolog was very abundant in *H. obtusa*, especially in the reproductive form, while the GroES homolog could be detected neither in the reproductive nor in the infectious form by immunoblotting or immunoelectron microscopy (data not shown). According to the endosymbiosis theory (32, 33), an endosymbiont is taken to be an intermediate between a free-living bacterium and cell organelle. It has been reported that in *E. coli* synthetic level of GroES is nearly same as that of GroEL (38), while GroES is not detected immunologically in

mitochondria. It has been also reported that GroES is much smaller in amount in primary symbionts than in secondary ones of the pea aphid *Acyrthosiphon pisum* (15). These results may suggest that the amount of GroES decreases as endosymbiosis proceeds. Interestingly, the similarity between GroES proteins of *H. obtusa* and *E. coli* were lower than that between GroEL proteins. This lower similarity in GroES proteins may occur for the same reason why the GroES decreases in endosymbionts. One possibility is that GroES may not be as essential as GroEL homolog in endosymbionts, consequently GroES tends to change its amino acid sequence and decrease in amount. The result that there was no transcriptional start point between the *groES* and *groEL* gene based on primer extension analysis (data not shown) suggested that the *groES* and *groEL* gene form an operon and are transcribed as a unit. Therefore, quantitative difference between the GroES and GroEL homolog may be caused by translational or post-translational regulation. By analysis of the nucleotide sequence of the *groESL* gene, the Shine-Dalgarno (SD) sequences for the *groES* and *groEL* gene were found just upstream of their translational initiation codons. The SD sequence is known to affect translation of proteins (27), and the spacing between the SD sequence and the initiation codon strongly affects translational efficiency (4). In the *groESL* gene of *H. obtusa*, the SD sequence for the *groEL* gene was more conservative than that for the *groES* gene, and the spacing between the SD sequence and the initiation codon were 6 nucleotides for the *groEL* gene and 5 nucleotides for the *groES* gene. Therefore, quantitative difference between the GroES and GroEL homolog in *H. obtusa* is possibly due to the difference of translational efficiency caused by these differences of the SD sequence and the spacing between the SD sequence and the initiation codon.

By Southern blot hybridization, no other genes which were so homologous as to be detected under the washing conditions as described in the Methods section were found, suggesting that only one copy of the *groEL* gene is present in *H. obtusa*. This result was consistent with previous reports for most eubacteria (52), although some interesting exceptions were reported for *Streptomyces* (9), *Cyanobacterium* (5), *Rhizobium* (41), and *Bradyrhizobium* (12), in which two or more *groEL*-homologous genes were detected.

Immunoelectron microscopy showed that the GroEL homolog of *H. obtusa* is localized in the cytoplasm of the reproductive and infectious form. In an obligate intracellular parasite

Mycobacterium, GroEL homolog were originally defined as a major antigen associated with a cell wall (16). Preliminary subfractionation of a GroEL homolog from the Q-fever rickettsia *Coxilla burnetii* has shown that it is enriched in a peptidoglycan fraction (51). The GroEL homologs from these parasitic bacteria contain a repetitive Gly-Gly-Met motif at their C-terminus, which perhaps serves as an anchor to the membrane or peptidoglycan by utilizing its hydrophobicity, thereby the GroEL homologs are thought to associate with a cell wall. Although the GroEL homolog of *H. obtusa* also contained a similar repetitive Gly-Gly-Gly-Met motif, its intracellular localization was unlike those of *Mycobacterium* and *Coxiella*. In view of this difference in localization, a functional role of the GroEL homolog of *H. obtusa* may be different from that of GroEL homologs of these parasitic bacteria.

In this study, SDS-PAGE and immunoblotting showed that the GroEL homolog was the most abundant protein in the reproductive form, and that its amount per protein content was more in the reproductive form than in the intermediate or infectous form. It is possible that the reproductive form imports proteins from the host macronucleus to multiply by binary fission, and requires a large amount of the GroEL homolog as molecular chaperone for folding and assembly of imported proteins. Northern blot hybridization also showed that the *groESL* gene is highly expressed in the reproductive form, and that its expression dramatically decreases during the differentiation from the reproductive to the infectious form. However, it is not known in which stage during this process its expression is actually augmented. It is of great interest how expression of the *groESL* gene is regulated in a different stage of the life cycle of *H. obtusa*. One possibility is that host macronuclear materials differentially regulate its expression. It is plausible that the macronucleus of the host cell at the log phase contains materials which induce the expression of the *groESL* gene. However, synthesis of the GroEL homolog *in vitro* were not induced in the presence of macronuclear extracts from host cells at either the log or stationary phase, not supporting this explanation. Another possibility is that the difference of the gene expression is due to qualitative differences between the reproductive and infectious form, such as cell wall structure, size of periplasmic space, and composition of σ-factors. The reproductive form changes its cell wall into rigid one (14) and develops a large periplasmic space (19) during the differentiation to the infectious form. It is possible that because of these two peri-cytoplasmic

structures, the infectious form shows different responses from the reproductive form to the external environment, and thus shows different gene expression and protein synthesis even under the same environmental conditions. The composition of σ-factors, one of the components of RNA polymerase, is possibly different in between the reproductive and infectious form. This difference must be directly related to the difference of gene expression observed between the reproductive and infectious form.

It has been reported that aphid symbionts selectively synthesize only symbionin, a GroEL homolog in a mycetocyte (22). In the soybean bacteroid *Bradyrhizobium*, the amount of the GroEL homolog in the symbiotic phase was 7 times larger than that in the free-living one (6). Large amounts of GroEL homologs are also synthesized by the intracellular parasitic bacteria, such as *Mycobacterium* (34), *Chlamydia* (35), *Coxiella* (51), and *Rickettsia* (48). *H. obtusa* is similar to *Bradyrhizobium* in that the amount of the GroEL homolog fluctuates in its life cycle. Judging from these observations that the intacellular symbionts and parasites contains large amounts of the GroEL homolog, and the fact that GroEL is a stress protein, the host cell cytoplasm and nucleus are hostile environments to these microorganisms, and they are under stress conditions. This stressor present in the host is not identified yet, and functions of the GroEL homologs in endosymbionts and parasites are still a matter of speculation. One possibility is that these GroEL homologs serve as molecular chaperones in transporting and folding of proteins imported from the host cell. Therefore, the GroEL homolog and the stressor must be important molecules in the symbiotic systems in general.

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FIGURES AND FIGURE LEGENDS

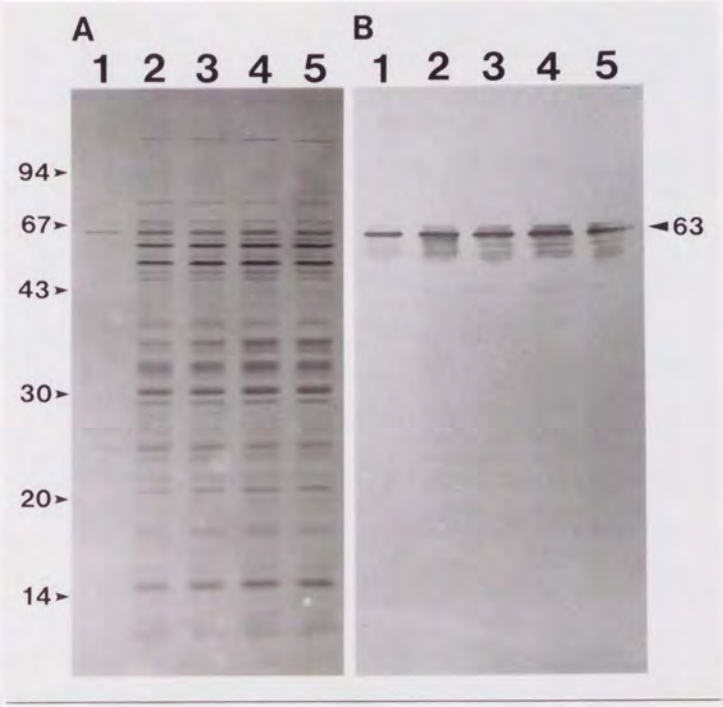

Fig. 1. SDS-PAGE and immunoblotting of the 63-kDa protein. Proteins from the different forms of *H. obtusa* (2 x 10^6 cells) were subjected to SDS-PAGE of 12.5 % polyacrylamide gel, transferred to PVDF membrane, and probed with anti-symbionin antiserum. A, CBB-staining. B, immunoblotting using anti-symbionin antiserum. Lane 1, reproductive form; lane 2, intermediate form banding upon 70 % Percoll; lane 3, intermediate form banding upon 80 % Percoll; lane 4, intermediate form banding upon 90 % Percoll; lane 5, infectious form. An arrowhead indicates a band of the 63-kDa protein of *H. obtusa*. Sizes of molecular weight markers (left) and the 63-kDa protein (right) were given in kDa. Note that the amount of proteins from the reproductive form was smaller than those from the intermediate and infectious form because the reproductive form is smaller than the intermediate and infectious form.

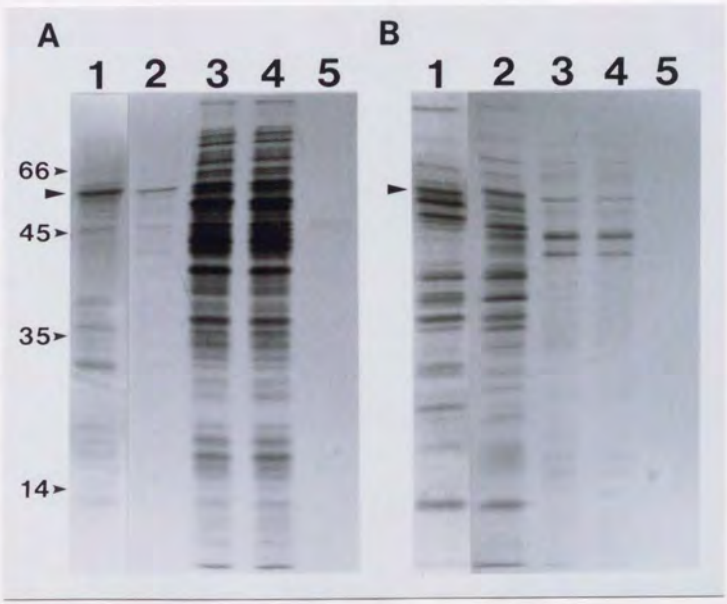

Fig. 2. *In vivo* and *in vitro* protein synthesis and antibiotic sensitivity in isolated *H. obtusa*. For analysis of proteins synthesized *in vivo*, *H. obtusa*-bearing paramecia were incubated in culture medium containing Tran ^{35}S-label, the symbionts were isolated and subjected to SDS-PAGE and fluorography. For analysis of proteins synthesized *in vitro*, isolated symbionts were incubated in 10 mM Na, K-phosphate buffer (pH 6.5) containing Tran ^{35}S-label in the presence or absence of antibiotics, and subjected to SDS-PAGE and fluorography. A, reproductive form (5 x 10^6 cells). B, infectious form (2 x 10^6 cells). Lane 1, CBB-staining; lane 2, proteins synthesized *in vivo*; lanes 3-5, proteins synthesized *in vitro*; lane 3, without antibiotics; lane 4, with 20 μg/ml cycloheximide; lane 5, with 20 μg/ml chloramphenicol. Arrowheads in the panel A and B indicate the 63-kDa protein of the reproductive and infectious form, respectively.

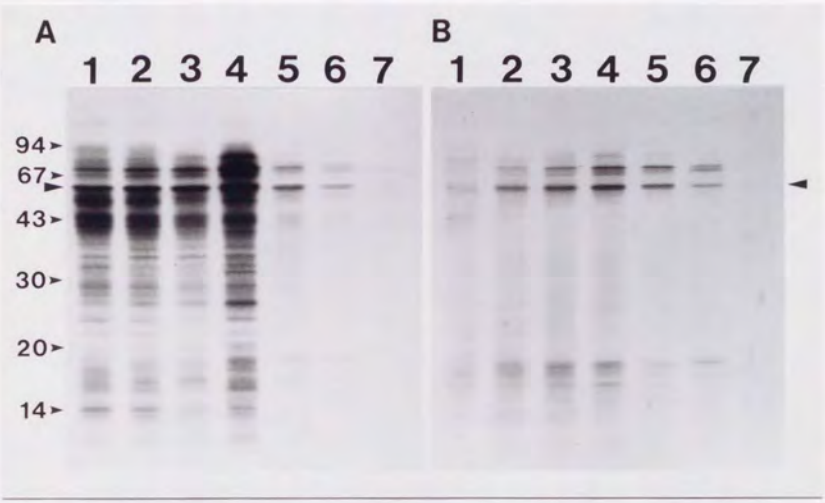

Fig. 3. Heat shock response of isolated *H. obtusa*. Isolated cells of the reproductive and infectious form of *H. obtusa* were incubated in 10 mM Na, K-phospate buffer (pH 6.5) containing Tran ^{35}S-label at different temperatures, and proteins synthesized during heat shock treatment were analyzed by SDS-PAGE and fluorography. A, reproductive form (1.5 x 10^7 cells). B, infectious form (1 x 10^6 cells). Lane 1, 25 °C; lane 2, 30 °C; lane 3, 33 °C; lane 4, 36 °C; lane 5, 39 °C; lane 6, 42 °C; lane 7, 45 °C. Arrowheads in the panel A and B indicate the 63-kDa protein of the reproductive and infectious form, respectively.

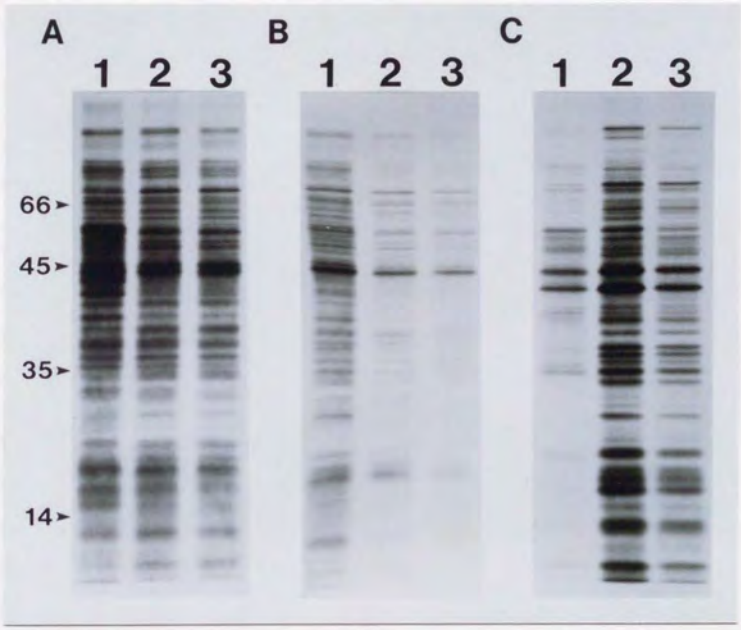

Fig. 4. Effects of host macronuclear extract on *in vitro* protein synthesis of *H. obtusa*. Isolated cells of *H. obtusa* and *Klebsiella pneumoniae* were incubated in 10 mM Na, K-phospate buffer (pH 6.5) containing Tran ^{35}S-label in the presence or absence of macronuclear extract from *P. caudatum*. A, reproductive form (1 x 10^6 cells). B, infectious form (1 x 10^6 cells). C, *K. pneumoniae* (1 x 10^6 cells). Lane 1, without macronuclear extract; lane 2, with macronuclear extract from *P. caudatum* at the log phase; lane 3, with macronuclear extract from *P. caudatum* at the stationary phase.

A

```
N-terminus
                    5                   10                  15
 Ser-Val-Lys-Gln-Ile-Ala-Phe-Gly-Ser-Lys-Val-Gly-Glu-Ser-Leu
                   20                  25                  30
-Leu-Asn-Gly-Val-Ile-Lys-Leu-Ala-Asn- X -Val-Gln-Val-Thr-Leu
                   35
-Gly-Pro-Asn-Gly-
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B

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22 min
                    5                   10                  15
 Ile-Arg-Ser-Glu-Ile-Gln-Glu-Ala-Thr-Ser-Asp-Tyr-Asp-Lys

26 min
                    5                   10                  15
 Ala-Ser-Asp-Val-Asn-Val-Gly-Tyr-Asp-Ala-Arg-His-Asp-Gln-Tyr
                   20
-Val-Asp-Met-Ile-Lys

33 and 34 min
                    5                   10                  15
 Thr-Glu-Leu-Asp-Val-Val-Pro-Gly-Met-Gln-Phe-Asp-Arg-Gly-Tyr
                   20                  25                  30
-Ile-Ser-Pro-Tyr-Phe-Ile-Thr-Arg-Gln-Asp-Lys-Gly-Ile-Ala-Glu
                   35                  40
-Leu-Glu-Arg-Ser-Tyr-Ile-Leu-Leu-Tyr-Asp-Gly-Lys
```

Fig. 5. Amino acid sequence of the N-terminus and the Lys-C fragments from the 63-kDa protein. The 63-kDa protein was separated by SDS-PAGE, transferred to PVDF membrane, and directly analyzed to determine its amino acid sequence from N-terminus. Lys-C fragments from the 63-kDa protein were analyzed to determine the internal amino acid sequence. A, amino acid sequence of the N-terminus of the 63-kDa protein. B, amino acid sequences of four Lys-C fragments with given retension times on HPLC. Two fragments with retension times of 33 and 34 min were found to be connected by alignment with GroEL of *E. coli*. Oligonucleotides deduced from underlined amino acid sequences were used as primers for PCRs.

NF (nt. 616-635)	5'-GTN-AAR-CAR-ATH-GCN-TTY-GG-3'
34R (nt. 1253-1234)	5'-TCN-GCD-ATN-CCY-TTR-TCY-YG-3'
22F (nt. 1675-1697)	5'-GAR-GCI-ACN-WSN-GAY-TAY-GAY-AA-3'
26R (nt. 2102-2080)	5'-TTD-ATC-ATR-TCN-ACR-TAY-TGR-TC-3'
F1 (nt. 1982-2005)	5'-GTT-ATC-AAA-TTG-CTA-GCA-ATG-CTG-3'
F2 (nt. 2016-2035)	5'-TGG-AGT-CGT-AGT-AGC-TGA-GG-3'
F3 (nt. 644-667)	TAG-GGG-AAT-TCT-TGC-TCA-ATG-GAG-3'
F4 (nt. 2213-2237)	5'-AAG-AAA-AGC-CGA-ATA-TGG-GCG-GTG-3'
F5 (nt. 1361-1384)	5'-TTG-CCG-AAG-ACG-TAG-AGG-GTG-AGG-3'
F6 (nt. 842-869)	5'-CGT-TCA-AAA-ACT-GCA-GAT-ATG-GTA-GGA-G-3'
F7 (nt. 773-799)	5'-CCG-TAG-CAA-AAC-ATG-TAG-AGC-TAG-AGG-3'
R1 (nt. 712-691)	5'-CGT-TTG-GCC-CTA-ATG-TAA-CCT-G-3'
R2 (nt. 682-657)	5'-TAG-CAA-GCT-TAA-TTA-CTC-CAT-TGA-GC-3'
R3 (nt. 2057-2038)	5'-TTC-ACA-TCA-GAA-GCT-TTC-AG-3'
R4 (nt. 2586-2559)	5'-TTA-CTT-CAT-CTA-AAA-AGC-GCA-TTT-CCT-G-3'
R5 (nt. 1416-1390)	5'-TCT-TAG-TTT-ATT-GAC-TAC-CAA-CAT-CCG-3'
R6 (nt. 1758-1734)	5'-TCC-AAC-ACG-AAT-CAC-TGC-GAC-TCC-G-3'
R7 (nt. 1210-1184)	5'-TGT-ATC-CGC-GAT-CAA-ATT-GCA-TAC-CCG-3'
R8 (nt. 291-263)	5'-TTG-ATG-AAT-CGA-TAT-AGA-AAA-ATT-AGC-AG-3'
R9 (nt. 1054-1027)	5'-CGA-CTT-GTG-AAA-TTT-TCT-GAT-AGT-CTC-C-3'
C1 (Cassette primer)	5'-GTA-CAT-ATT-GTC-GTT-AGA-ACG-CG-3'
T7 (Cassette primer)	5'-TAA-TAC-GAC-TCA-CTA-TAG-GGA-GA-3'

Table 1. Oligonucleotide primers used in this study. Primers NF, 34R, 22F, 26R, F3, and R3 were used for ordinary PCRs. Primers F1, F2, R1, R2, C1, and T7 were used for cassette PCRs. The other primers were used for sequencing of the gene. Primers F5 and R6 were also used for labeling of probes for Southern and Northern blot hybridization. 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; S, C or G; W, A or T; Y, C or T; D, not C; H, not G; N, any.

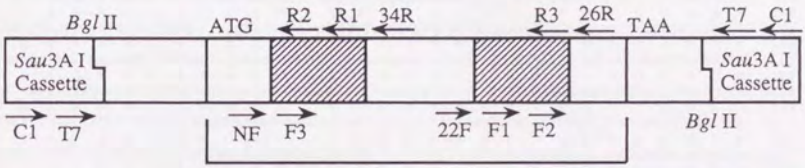

Fig. 6. Strategy for PCR amplification of the coding region for the 63-kDa protein and its flanking regions. Two shaded parts of the coding region for the 63-kDa protein were amplified by PCRs with two pairs of primers NF-34R and 22F-26R, and then sequenced. The fragment obtained by PCR with a pair of primers F3-R3 was sequenced and extended toward the both sides by cassette PCRs with two pairs of primers C1-R1 and T7-R2 for upstream amplification, and with two pairs of primers C1-F1 and T7-F2 for downstream amplification. Thus, the nucleotide sequence of the coding region for the 63-kDa protein and its flanking regions were determined.

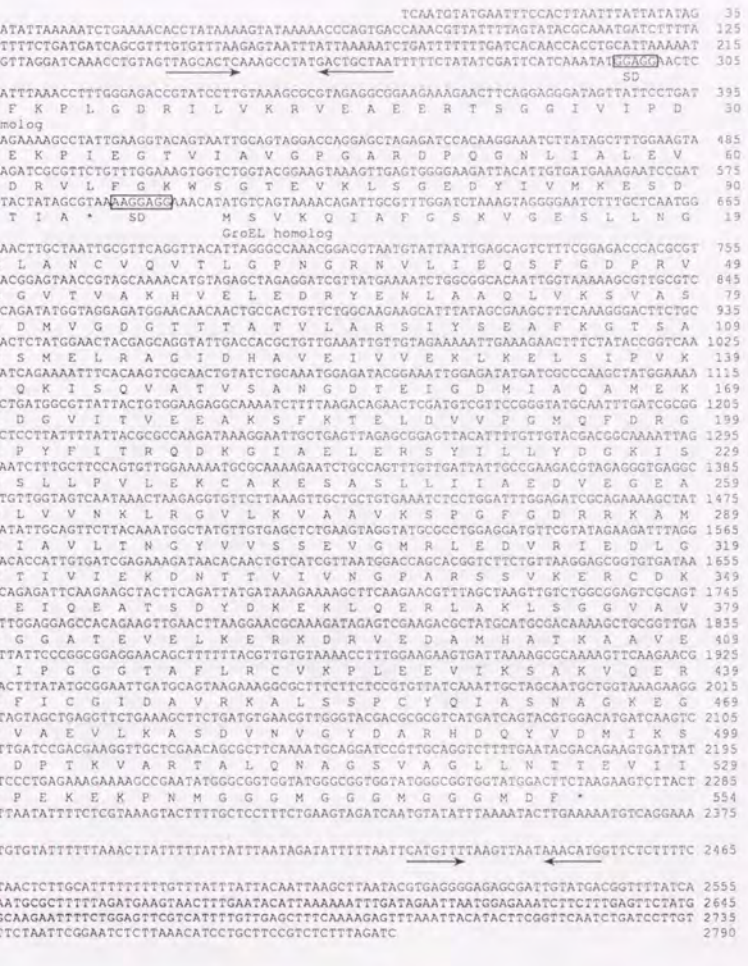

Fig. 7. Nucleotide sequence of *groESL* gene, its flanking regions and deduced amino acid sequence encoded by the gene. The putative Shine-Dalgarno (SD) sequence were boxed. Asterisks indicate translational termination codon. The inverted repeat sequences upstream of *groES* gene and downstream of *groEL* gene were indicated by facing arrows.

```
Identity = 306/554 (55.2 %)  Analogy = 396/554 (71.5 %)

                10        20        30        40        50        60
E. coli         MAAKDVKFGNDARVKMLRGVNVLADAVKVTLGPKGRNVVLDKSFGAPTITKDGVSVAREI
                *. * . **      .* **  ** .*.***** ****....*** * .*****.**. .
H. obtusa       MSVKQIAFGSKVGESLLNGVIKLANCVQVTLGPNGRNVLIEQSFGDPRVTKDGVTVAKHV

                70        80        90        100       110       120
                ELEDKFENMGAQMVKEVASKANDAAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGI
                ****..**. **.** ****. *  ***********..* .*  *  .**** *.*. **
                ELEDRYENLAAQLVKSVASKTADMVGDGTTTATVLARSIYSEAFKGTSAGMNSMELRAGI

                130       140       150       160       170       180
                DKAVTAAVEELKALSVPCS-DSKAIAQVGTISANSDETVGKLIAEAMDKVGKEGVITVED
                *.**   ** ** **.*   * . *.** *.*** *  .* .**.**.*** .******.
                DHAVEIVVEKLKELSIPVKGDYQKISQVATVSANGDTEIGDMIAQAMEKVGSDGVITVEE

                190       200       210       220       230       240
                GTGLQDELDVVEGMQFDRGYLSPYFINKPETGAVELESPFILLADKKISNIREMLPVLEA
                . ***** ********.*****.. . *  ***  .*** * ***  . .*****
                AKSFKTELDVVPGMQFDRGYISPYFITRQDKGIAELERSYILLYDGKISSAQSLLPVLEK

                250       260       270       280       290       300
                VAKAGKPLLIIAEDVEGEALATAVVNTIRGIVKVAAVKAPGFGDRRKAMLQDIATLTGGT
                 **    **************   *** .**..******.*********** *** **.*
                CAKESASLLIIAEDVEGEALRMLVVNKLRGVLKVAAVKSPGFGDRRKAMLGDIAVLTNGY

                310       320       330       340       350       360
                VISEEIGMELEKATLEDLGQAKRVVINKDTTTIIDGVGEEAAIQGRVAQIRQQIEEATSD
                *.* *.** **   .****.*  .** **.**.* .     .... *  .** .*.*****
                VVSSEVGMRLEDVRIEDLGRADTIVIEKDNTTVIVNGPARSSVKERCDKIRSEIQEATSD

                370       380       390       400       410       420
                YDREKLQERVAKLAGGVAVIKVGAATEVEMKEKKARVEDALHATRAAVEEGVVAGGGVAL
                **.******.***.******.** *****.**.* *****.***.******.. *** *
                YDKEKLQERLAKLSGGVAVIRVGGATEVELKERKDRVEDAMHATKAAVEEGIIPGGGTAF

                430       440       450       460       470       480
                IRVASKLADLRGQNEDQN------VGIKVALRAMEAPLRQIVLNCGEEP-SVVANTVKGG
                .*    * ..      *        **    .*. .*  **  *.* *   ***  .*
                LRCVKPLEEVIKSAKVQERGRDFICGIDAVRKALSSPCYQIASNAGKEGGVVVAEVLKAS

                490       500       510       520       530       540
                DGNYGYNAATEEYGNMIDMGILDPTKVTRSALQYAASVAGLMITTECMVTDLPKNDAADL
                * * ** *  ..*  **  **.*****.*.*** * *****. *** ... .*  .
                DVNVGYDARHDQYVDMIKSGIIDPTKVARTALQNAGSVAGLLNTTEVIIAQVPEKEKPNM

                550       560
                GAAGGMGGMGGMGGMM--
                *  ****  ***** *
                G--GGMG--GGMGGGMDF
```

Fig. 8. Comparison of deduced amino acid sequence of GroEL proteins of *E. coli* and *H. obtusa*. Amino acid sequence of the GroEL homolog of *H. obtusa* was aligned with that of *E. coli* using the Clustal W 1.6. Asterisks represent conserved amino acid residues and dots represent analogous amino acid substitutions between GroEL and its homolog of *H. obtusa*.

```
Identity = 38/96 (39.6 %)  Analogy = 59/96 (61.5 %)

                10        20        30        40        50
E. coli    MN-IRPLHDRVIVKRKEVETKSAGGIVLTGSAAAKSTRGEVLAVGNGRIL
           *   .** **..*** * * ...****.  .*  *   * *.*** *
H. obtusa MTKFKPLGDRILVKRVEAEERTSGGIVIPDTAKEKPIEGTVIAVGPGARD

                60        70        80        90       100
          ENGEVKPLDVKVGDIVIFNDGYGVKSEKIDNEEVLIMSESDILAIVEA
           .* .  *.** ** *.*.   *    *. .*. ..* ***.   . *
          PQGNLIALEVKQGDRVLFGKWSGT-EVKLSGEDYIVMKESDVFGTI-A
```

Fig. 9. Comparison of deduced amino acid sequence of GroES proteins of *E. coli* and *H. obtusa*. Amino acid sequence of the GroES homolog of *H. obtusa* was aligned with that of *E. coli* using the Clustal W 1.6. Asterisks represent conserved amino acid residues and dots represent analogous amino acid substitutions between GroES and its homolog of *H. obtusa*.

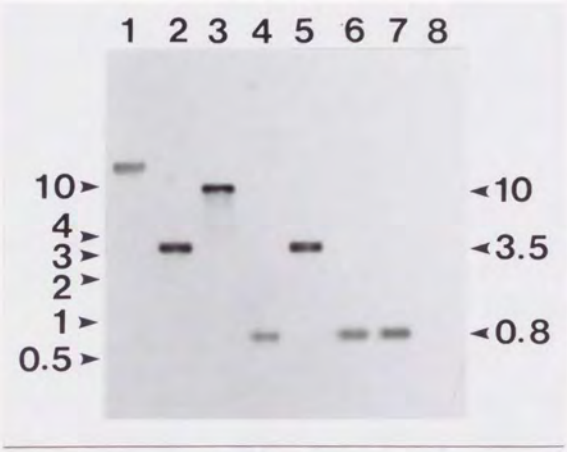

Fig. 10. Southern blot hybridization of the *groEL* gene. Genomic DNA from the infectious form of *H. obtusa* was digested with different restriction enzymes, separated on 0.8 % agarose gel, transferred to nylon membrane, and hybridized to the PCR-labeled probe. The hybridized DNAs were detected using the DIG Luminescent Detection Kit. Lane 1, undigested genomic DNA of *H. obtusa*; lane 2, *Bgl* II digest; lane 3, *Eco*R I digest, lane 4, *Hin*d III digest; lane 5, *Bgl* II-*Eco*R I digest; lane 6, *Bgl* II-*Hin*d III digest; lane 7, *Eco*R I-*Hin*d III digest; lane 8, undigested genomic DNA of *P. caudatum*. Sizes of DNA markers (left) and detected DNA (right) were given in kbp.

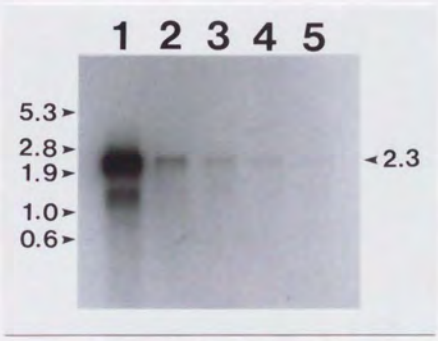

Fig. 11. Northern blot hybridization of the *groE* mRNA. Five μg of total RNAs from different forms of *H. obtusa* were separated on formamide-1.5 % agarose gel, transferred to nylon membrane, and hybridized to the PCR-labeled probe. The hybridized RNAs were detected by autoradiography. Lane 1, reproductive form; lane 2, intermediate form banding upon 70 % Percoll; lane 3, intermediate form banding upon 80 % Percoll; lane 4, intermediate form banding upon 90 % Percoll; lane 5, infectious form. Sizes of RNA markers (left) and detected RNA (right) were given in kb.

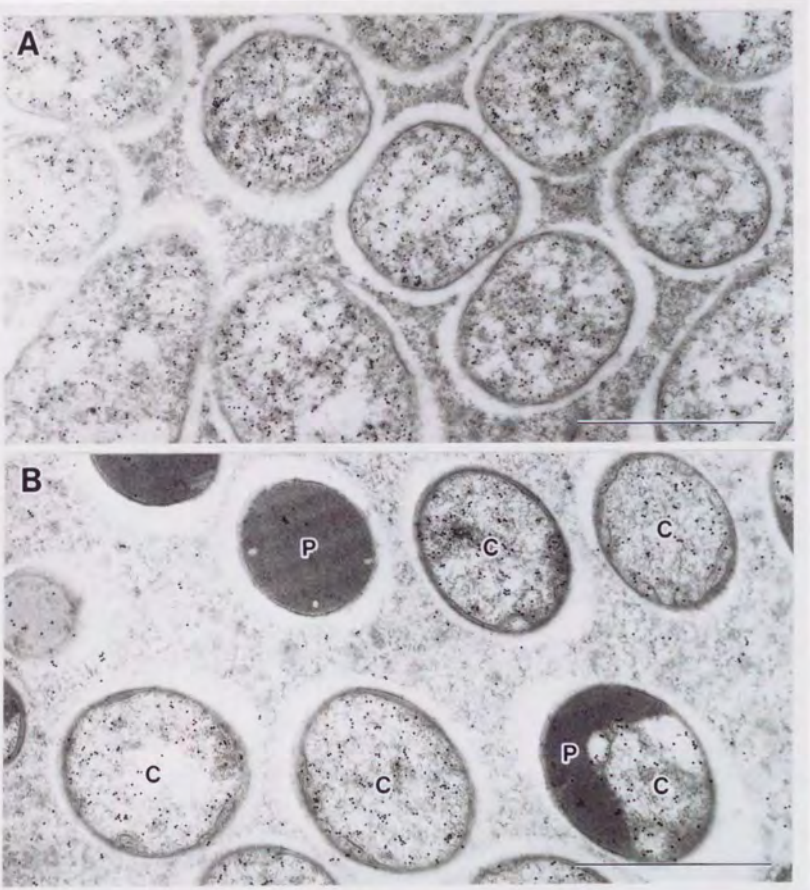

Fig. 12. Immunoelectron micrographs of *H. obtusa* in the macronucleus of *P. caudatum*. Thin sections were treated with rabbit anti-symbionin antiserum and goat anti-rabbit IgG-gold (10-nm), and stained with uranyl acetate. A, transversal section of the reproductive form. B, transversal section of the infectious form. Abbreviations in the panel B: C, cytoplasm; P, periplasm. Bars represent 1 μm.

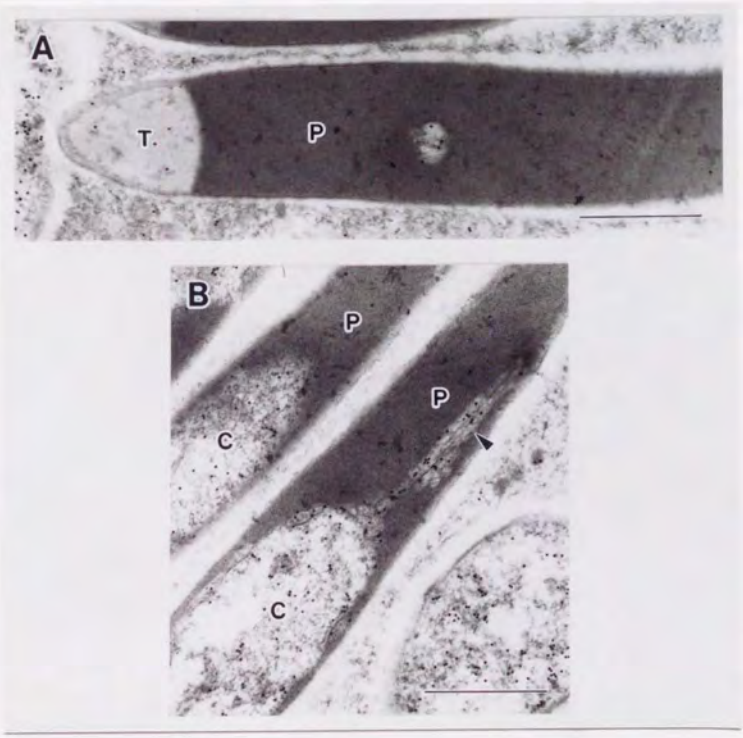

Fig. 13. Immunoelectron micrographs of the infectious form of *H. obtusa* in the macronucleus of *P. caudatum*. Thin sections were treated with rabbit anti-symbionin antiserum and goat anti-rabbit IgG-gold (10-nm), and stained with uranyl acetate. A, transversal section of periplasmic region and electron-translucent recognition tip of the infectious form. B, transversal section of boader area between cytoplasmic and periplasmic region of the infectious form. Abbreviations: C, cytoplasm; P, periplasm; T, electron-translucent recognition tip. An arrowhead in the panel B indicates cytoplasmic extrusion into periplasmic region. Bars represent 0.5 μm.

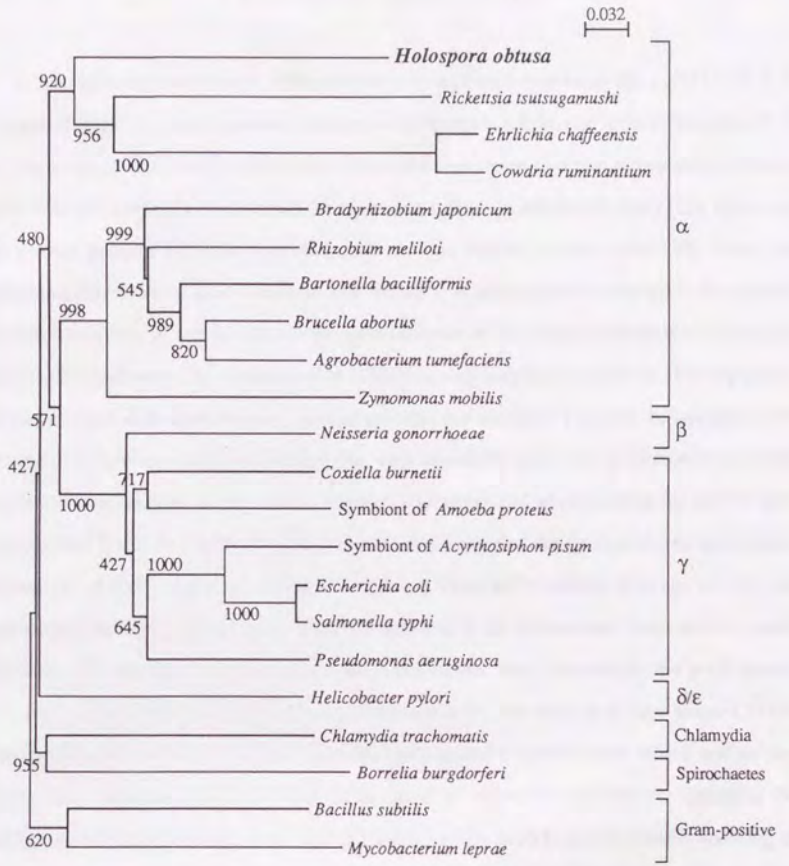

Fig. 14. Phylogenetic tree showing evolutionary relationships of *H. obtusa* based on GroEL amino acid sequences. The unrooted tree was constructed by the neighbor-joining distance method. The numbers at the end of branch indicate the number of times that the adjacent two groups occurred, as obtained by the bootstrap procedure from 1,000 trials. Greek letters indicate the corresponding subdivision of the Phylum *Proteobacteria*. Bar represents 0.032 substitutions/site.

GENERAL DISCUSSION

It has been reported that *H. obtusa* shows two distinct forms in its life cycle (4, 5, 6, 9, 11), and changes its protein patterns during the differentiation from one form to the other (3, 7, 8). The intracellular parasitic bacterium *Chlamydia* also shows two morphologically different types of a cell: a reproductive reticulate body and an infectious elementary body (12). However, the protein patterns of these types of a cell are very similar to each other (10). Thus, the differentiation of *H. obtusa* is very unique in that it accompanies the change in the protein pattern. Therefore, it is assumed that the differentiation of *H. obtusa* is controled by the gene expression which seems to be regulated in a different way specific to each form. The regulation mechanisms of differentiation and gene expression are not clear yet. The endosymbiont *H. obtusa* is a very interesting bacterium that may hopefully lend clue to approach for these regulation mechanisms. In this study, I cloned and sequenced genes coding for the 5.4 kDa peptide (Part I) and the GroEL homolog (Part II), and I clarified the qualitative and quantitative differences of their expression among the different forms of *H. obtusa*. It turned out that the gene coding for the 5.4 kDa peptide is highly expressed in the intermediate forms and in a small amount in the infectious form, but not in the reproductive form. Meanwhile, the *groE* operon homolog was highly expressed in the reproductive form, but only in a trace amount in the intermediate and infectious form. However, the regulation mechanisms which control the expression of these genes in the different forms of *H. obtusa* is not clear yet. Studying the regulation mechanisms of the genes coding for the 5.4 kDa peptide and the GroEL homolog of *H. obtusa* will unveil the regulation mechanisms of the gene expression and the cell differentiation in general.

Unfortunately, attempts to cultivate *H. obtusa* outside its host cell have not been successful. When the reproductive and infectious form of the bacterium were cultivated in the presence of the host macronuclear extract, they did not show a pattern of protein synthesis similar to that observed while in the macronucleus. In the presence of the extract, the reproductive form neither divided nor differentiated to the infectious form, and *vice versa*. It has been reported that an intracellular parasite *Rickettsia* could grow not only in the cytoplasm, but also in the nucleus of

infected cells (1, 2). Like *H. obtusa*, *Rickettsia*, except *Rochalimaea quintana*, could not be cultivated in a cell-free medium, either. Weiss speculated that it is because of *Rickettsia*'s physiological dependencies on the host cell, such as its dependency on ATP and key metabolites, and its highly sensitive regulatory mechanisms (13). The reason why *H. obtusa* could not be cultivated outside the host cell may be accounted for in a similar way. *H. obtusa* also depends on the host in that it changes its morphology in accordance with the growth of the host cell (3, 5, 7, 8). That is, whether the reproductive form of *H. obtusa* multiplies by binary fission or differentiates to the infectious form depends on factors present in the host macronucleus. It is of interest what are the factors, and how the they switch between the multiplication and differentiation of *H. obtusa*. One possibility is that the macronucleus of the host cell at the log phase contains a material which is needed for the reproductive form to multiply, but when the host cell reaches the stationary phase and the material decreases in the macronucleus, the reproductive form stops multiplication and differentiates to the infectious form. Another possibility is that the macronucleus of the host cell at the log phase contains a material which is needed for the reproductive form to multiply, while the macronucleus of the cell at the stationary phase contains a material which allows the reproductive form by stopping its multiplication to differentiate to the infectious form. During the differentiation of *H. obtusa*, it changes the protein pattern, implying that it also changes the gene expression pattern. Therefore, it is hypothesized that the materials in host macronucleus control the gene expression of *H. obtusa*, which, in turn, changes morphology of the symbiotic bacterium. To study the host-symbiont interactions at the gene expression level, the 5.4 kDa peptide and the GroEL homolog would be used as a good marker molecule. Control mechanisms over the gene expression of *H. obtusa* by the host will be clarified by studying the expression mechanism of the genes coding for the 5.4 kDa peptide and the GroEL homolog.

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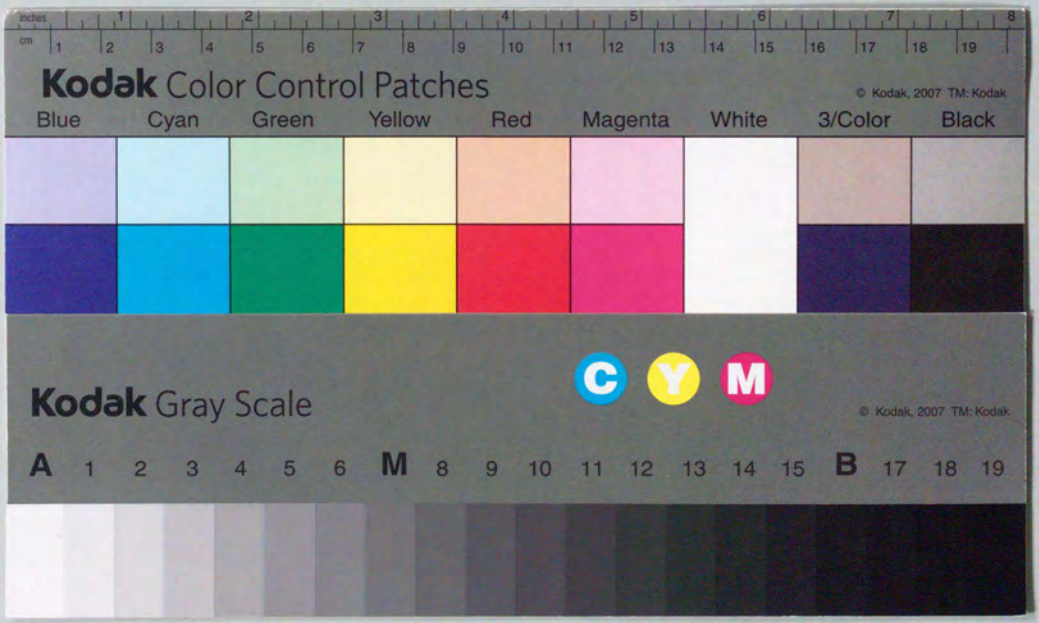
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