Survival and transfer ability of phylogenetically diverse bacterial endosymbionts in environmental *Acanthamoeba* isolates

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Summary

Obligate intracellular bacteria are commonly found as endosymbionts of acanthamoebae, however, their survival in and ability to transfer to amoebae are currently uncharacterized. In this study, six bacterial endosymbionts, found in five environmental *Acanthamoeba* isolates (S13, R18, S23, S31, S40) from different locations of Sapporo city, Japan, were characterized. Phylogenetic analysis revealed that three-bacterial endosymbionts (eS31, eS40a, eS23) belonged to α- and β-*Proteobacterium* phyla and the remaining endosymbionts (eR18, eS13, eS40b) belonged to the *Chlamydiales* phylum. The *Acanthamoeba* isolate (S40) contained two phylogenetically different bacterial endosymbionts (eS40a, eS40b). Fluorescent *in situ* hybridization analysis showed that all bacterial endosymbionts were diffusely localized within amoebae. Transmission electron microscopy also showed that the endosymbionts were rod-shaped (eS31, eS40a, eS23) or sphere- or crescent-shaped (eR18, eS13, eS40b). No successful culture of these bacteria was achieved using conventional culture methods, but the viability of endosymbionts was confirmed by live/dead staining and RT-PCR methods. However, endosymbionts (except eR18) derived from original host cells lost the ability to be transferred to another amoeba strain (*Acanthamoebae* ATCC C3). Taken together, our data demonstrate that phylogenetically diverse bacterial endosymbionts found in amoebae are viable and maintain a stable interaction with amoebae.

Introduction

Free-living amoebae, such as *Acanthamoeba*, are readily isolated from a wide range of natural environments, such as soil, river water, domestic tap water, seawater, pond water and dust (Rivera *et al*., 1987; Sawyer, 1989; Ettinger *et al*., 2003; Kilic *et al*., 2004; Gornik K *et al*., 2004; Tsvetkova *et al*., 2004; Lorenzo-Morales *et al*., 2005a; Lorenzo-Morales *et al*., 2005b; Lorenzo-Morales *et al*., 2005c; Lorenzo-Morales *et al*., 2006). *Acanthamoeba* is an opportunistic pathogen, causing keratitis and granulomatous amoebic encephalitis (De Jonckheere, 1991; Marciano-Cabral and Cabral, 2003; Schuster and Visvesvara, 2004; Khan, 2006; Visvesvara *et al*., 2007), and is also an important predator of bacteria. However, some bacteria, such as *Legionella pneumophila* (Rowbotham, 1980), *Mycobacterium* spp. (Cirillo, 1997; Taylor *et al*., 2003), *Listeria monocytogenes* (Ly and Müller, 1990) and *Shigella sonnei* (Jeong *et al*., 2007) have developed strategies to survive in, and multiply within amoebae, although these relationships are only transient (Greub and Raoult, 2004). Free-living amoebae may, therefore, serve as environmental reservoirs of bacteria, referred to as 'Trojan horses', and may be involved in intracellular bacterial replication (Molmeret *et al*., 2005) and in the transmission of pathogenic bacteria to humans (Barker and Brown, 1994). On the other hand, endosymbionts, such as environmental chlamydiae and some bacteria belonging to the *Proteobacterium* phylum are able to maintain stable interactions with host amoebae. The prevalence of symbiotic bacteria found in amoebae is approximately 20-25% of environmental amoeba isolates (Horn and Wagner, 2004). Investigating the stable interaction between endosymbionts and amoebae is of interest to understand the

evolution of symbiosis and to identify differences between the survival mechanisms of endosymbionts and of transients, such as *L. pneumophila*.

In the present study, we have, therefore, attempted to determine the survival and transfer ability of six-bacterial endosymbionts with phylogenetically different lineages found in the five environmental *Acanthamoeba* isolates from different locations of Sapporo city, Japan.

Results

Phylogenetic analysis of bacterial endosymbionts found in environmental amoeba isolates

Six bacterial endosymbionts, found in five environmental *Acanthamoeba* isolates (amoeba strain names: S13, R18, S23, S31, S40) from different locations of Sapporo city, Japan, were characterized. As shown in Fig. 1A, phylogenetic analysis of *rRNA* by PCR indicated that three bacterial endosymbionts (designated as eS31, eS40a, eS23) present in amoebae (S31, S40, S23) were not from the order *Chlamydiales* and that the remaining endosymbionts (designated as eR18, eS13, eS40b) present in amoebae (R18, S13, S40) were of the order *Chlamydiales*. Interestingly, the *Acanthamoeba* isolate (S40) contained two different bacterial endosymbionts (eS40a and eS40b). Phylogenetic analysis of nearly full-length 16S rRNA sequence revealed that two bacterial endosymbionts (eS31, eS40a) located to an unknown cluster and one (eS23) located to the Procabacter cluster

within the α- and β-classes of Proteobacterium, respectively (Fig. 1B). The remaining endosymbionts were located to the Protochlamydia cluster (eR18) and the Neochlamydia-like cluster (eS13, eS40b) of the order Chlamydiales (Fig. 1C).

Morphological characterization of bacterial endosymbionts

To demonstrate the intracellular localization of the bacterial endosymbionts within their amoeba host cells, FISH, with specific probes, was performed. FISH staining showed that all bacterial endosymbionts were diffusely spread throughout amoebae (Fig. 2A). No obvious differences in the distribution of endosymbionts in amoebae were observed, regardless of the culture duration or amoeba concentration (data not shown). Transmission electron microscopy (TEM) showed that the endosymbionts were rod-shaped (eS23, eS31, eS40a) and sphere- or crescent-shaped bacteria (eS13, eR18, eS40b) (Fig. 2B), similar to *P. acanthamoebae*, which is a representative environmental chlamydia and forms crescent bodies (Fig. 2B, f) (Greub and Raoult, 2002). Most bacteria were found in inclusion-like vacuoles surrounded by plasma membrane (Fig. 2Bb is a representative image), but some endosymbionts were freely present in the amoeba's cytoplasm [Fig.2Ba, e (arrowhead)].

Ability of bacterial endosymbionts to survive in amoebae

The viability of endosymbionts drawn from amoebae was confirmed by live/dead staining. As shown in Fig. 3A, most endosymbionts (eS13, eR18, eS23 and eS40ab) maintained viability immediately after being drawn from amoebae, but their viability

gradually decreased during incubation for 24 h under amoeba-free conditions. Interestingly, the eS31 endosymbiont rapidly lost viability after being drawn from amoebae. The bacterial endosymbionts from frozen bacterial stocks, and those drawn from frozen amoebae stocks also maintained their viability after thawing (data not shown). The viability of bacterial endosymbionts in amoebae were also confirmed by RT-PCR, using markers of bacterial or *Chlamydiales* 16S rRNA gene transcription levels (Fig. 3B); each endosymbiont transcript was consistently observed, and there were no differences between the transcription levels throughout the culture periods. In addition, amoebae cultures were simultaneously infected with each of the bacterial endosymbionts and the cultures remained stable over several months (data not shown).

Ability of bacterial endosymbionts to transfer between amoebae

We determined the ability of bacterial endosymbionts, drawn from an original host, to transfer to a new amoeba host. As shown in Fig. 4B, most endosymbionts (eS13, eS23, eS31 and eS40ab) derived from original host cells completely lost the ability to transfer to another amoeba strain (*Acanthamoebae* ATCC C3). Interestingly, culture of eR18 produced an increased number of amoeba cells containing DAPI stained small particles (Fig. 4A) and increased AIU assay viable counts (Fig. 4C), relative to the other endosymbionts, suggesting that eR18 maintained the ability to transfer to other amoeba host cells. TEM also confirmed the attachment of eR18 to amoebae and of replicated bacteria inside amoebae (Fig. 4D). In contrast, it was confirmed that eS13, which had lost the ability to transfer, was digested inside amoebae and that no replication of the bacteria

occurred (Fig. 4D).

Discussion

The first report describing the presence of bacterial endosymbionts in free-living amoebae was by Jeon and Lorch (1967). Subsequently, many free-living amoebae, such as *Naegleria* and *Acanthamoeba* were also found to possess bacterial endosymbionts (Phillips 1974; Proca-Ciobanu *et al*., 1975; Hall and Voelz 1985; Rowbotham 1980; Drozanski *et al*., 1991; Fritsche *et al*., 1993; Yagita *et al*., 1995), indicating that endosymbiosis occurs ubiquitously among a wide range of free-living amoebae. Likewise, endosymbionts were reported in several other species, such as *Paramecium* (Preer *et al*., 1974; Gortz 1982) and *Giardia lamblia* (Radulescu *et al*., 1988). Endosymbionts of amoeba, such as environmental chlamydiae and some bacteria belonging to the *Proteobacterium* phylum, are able to maintain a stable interaction with host amoebae and the prevalence of symbiotic bacteria found in amoeba is approximately 20-25% of environmental amoeba isolates (Fritsche *et al*., 2000; Horn and Wagner, 2004; Thomas *et al*., 2006; Heinz *et al*., 2007; Schmitz-Esser *et al*., 2008). However, the mechanism, by which this stable interaction is maintained remains unknown. The goal of this study is, therefore, to clarify the survival and transfer abilities of bacterial endosymbionts with phylogenetic diversity in free-living amoebae.

We determined the genotypes of the amoeba isolates because the genus *Acanthamoeba*

has been classified into 15 different genotypes (T1-T15) (Khan 2006), although 90% of *Acanthamoeba* isolates belong to the T4 genotype, which is the most likely genotype to express a strong virulence against human infection (Khan 2006). The 41 different amoeba strains were isolated from geographically different locations. The genotypes of amoebae isolates were T2 [1 strain (2%)], T4 [29 strains (66%)], T6 [3 strains (75%)], and T13 [8 strains (20%)] (data not shown). Subsequently, six bacterial endosymbionts were found in five environmental *Acanthamoeba* strains (strain names: S13, R18, S23, S31, S40), all of which were genotype T4 (S13, R18, S23, S40) and T6 (S31). However, whether the presence of bacterial endosymbionts is correlated with amoeba host genotype remains to be determined.

To identify the bacterial endosymbionts found in the five amoeba isolates, their near-full length 16S rRNA gene sequences were amplified, sequenced and analyzed according to sequence similarity, as described previously (Everett *et al*., 1999). Three-bacterial endosymbionts, eS31, eS40a, and eS23, were identified as *Trojanella*-like endosymbiont (closely related to endosymbiont of *Acanthamoeba* sp. UWC9), *Caedibacter*-like endosymbiont (closely related to endosymbiont of *Acanthamoeba* sp. HN-3), and *Procabactor*-like endosymbiont (closely related to endosymbiont of '*Candidatus* Procabacter sp.' OEW1), respectively. The remaining endosymbionts, eR18, eS13, and eS40b, were also identified to *Protochlamydia* sp. (closely related to endosymbiont of *Protochlamydia amoebophila* UWE25), *Neochlamydia*-like endosymbiont (closely related to endosymbiont of *Acanthamoeba* sp. UWC22), and *Neochlamydia*-like endosymbiont (closely related to endosymbiont of *Acanthamoeba* sp.

TUME1), respectively. The phylogenies of the bacterial endosymbionts identified in this study were closely related to those of previous studies (Fritsche *et al*., 1999; Horn *et al*., 1999; Fritsche *et al*., 2000; Horn and Wagner, 2004). Interestingly, the phylogenic affiliation of two different bacterial endosymbionts (eS40a, eS40b) found in the S40 isolate was also consistent with a previous report (Heinz *et al*., 2007). This suggests that the lineage of bacteria found in amoebae may be limited and that the evolution of bacterial endosymbiosis in amoeba hosts may involve only specific bacteria-amoeba combinations.

To define the intracellular localization and the ultrastructures of the bacterial endosymbionts within *Acanthamoeba* isolates, FISH and TEM analyses were performed. Symbiont-specific probes for FISH analysis were selected from previous reports (Amann *et al*., 1997; Fritsche *et al*., 2000; Schmitz-Esser *et al*., 2008). The FISH staining clearly indicated that all bacterial endosymbionts were diffusely localized within amoebae and that no obvious differences in intracellular localization were found, regardless of the culture period or amoeba cell concentration. Thus, these findings suggest that the bacteria-amoebae interactions were stably maintained. The ultrastructural TEM analysis showed that the endosymbionts were rod-shaped (eS31, eS40a, eS23) and sphere- or crescent-shaped bacteria (eR18, eS13, eS40b). Endosymbionts (eR18, eS13, eS40b) of the *Chlamydiales* phylum showed a typical crescent body morphological feature, indicating elementary bodies in the developmental stage. The bacterial endosymbionts were occasionally located directly in the amoeba cell cytoplasm, not enclosed in vacuoles but surrounded by a space, implying a stable association in the amoebae.

On the other hand, whether viability of bacterial endosymbionts in amoebae is continually maintained is a critical point for understanding the interaction between endosymbionts and amoebae. The ability to survive and to transfer was, therefore, assessed by live/dead staining and RT-PCR. The viabilities of bacterial endosymbionts (eS13, eR18, eS23, eS40ab) under amoeba-free conditions gradually decreased, and the eS31 endosymbiont lost viability immediately after being drawn from amoebae. Although the reason underlying the loss of viability is unknown, these results suggest that the interactions between bacteria and amoebae are stably maintained and that the survival of bacterial endosymbionts are dependent on amoebae, possibly for the acquirement of energy and for protection against environmental stresses. Our phylogenic analysis confirmed that endosymbiont eS31 was most related to *Trojanella* sp., which belongs to the family *Rhodospirillaceae*, which are phototrophic bacteria (Breznak *et al*., 1978). Unique properties, such as this may be associated with the rapid loss of eS31 viability immediately after being drawn from amoebae. The viability of endosymbionts in amoebae were also confirmed by RT-PCR as using a marker of bacterial or *Chlamydiales* 16S rRNA gene transcription levels. Each of the endosymbiont transcripts was continually observed, and there were no differences between the transcription levels throughout the culture periods. These results clearly indicate that the bacterial endosymbionts found in amoebae are persistently viable, suggesting that bacteria found in amoebae preserve the possibility of transfer to new amoebae hosts.

Finally, we investigated the ability of bacterial endosymbionts isolated from original amoebae isolates to transfer to new host amoeba. Contrary to our expectation, most

endosymbionts (eS31, eS40ab, eS23, eS13) derived from original host cells completely lost the ability to transfer to another amoeba strain (*Acanthamoeba* ATCC C3). In contrast, one of the endosymbionts, eR18, was able to transfer to *Acanthamoeba* ATCC C3. The endosymbionts (eS13, eS40b) were assigned to a cluster in the *Parachlamydiaceae* family, belonging to the order *Chlamydiales* (eR18 also belongs to the order *Chlamydiales*), but the individual features of bacterial endosymbionts relevant to the ability to survive and transfer were obviously highly diverse. Thus, these findings suggest that bacterial endosymbiont traits may develop individually, regardless of bacterial group, enabling adaptation to ever-changing amoeba cellular conditions. Although the mechanisms contributing to this diversity remain unknown, it seems likely to involve differences of sensitivity against several proteases which *Acanthamoeba* secrete in large amounts and the secretion of endosymbiont effector proteins that improve cellular conditions.

Bacterial endosymbionts in free-living amoebae have been known for a long time (Jeon and Lorch, 1967; Proca-Ciobanu *et al*., 1975; Hall and Voelz 1985; Rowotham 1980; Drozanski *et al*., 1991; Fritsche *et al*., 1993; Yagita *et al*., 1995), but have only recently been characterized using molecular tools, such as phylogenetic analysis. In the present study, we have demonstrated that phylogenetically diversity bacterial endosymbionts found in amoebae are viable and are able to develop stable interactions with amoeba. Further analysis of these stable symbiotic relationships could provide new insights into the evolution of bacterial endosymbionts in amoebae.

Experimental procedures

Acanthamoeba strains

Forty one environmental amoeba strains were isolated from 66 samples (41 soil samples, 19 river water samples, 4 lake water samples, and 2 pond water samples) collected from geographically different places in Hokkaido, Japan. In brief, a few drops of Page's modified Neff's amoeba saline (PAS) (Page, 1988) were added to the samples [1 g of soil or the micro particles trapped on 0.45 μm filters (Millipore, Milford, MA) from 500 ml of each water sample] and then placed on the center of non-nutrient agar (NNA) plates that were covered with heat-inactivated *Escherichia coli* (a stock collection in our laboratory) as a food source. Plates were incubated at 30°C for 2 weeks according to previously described procedures (Page, 1988; Schuster, 2002). At 3-4 days after this incubation, the filters on the NNA plates were carefully removed. *Acanthamoeba* isolates on the plates were then identified according to morphological observation of amoeba cysts (Page, 1988; Visvesvara, 1993). Isolates were continuously cultured with axenic solution (PYG broth) containing peptone, yeast extract, glucose at 30°C (Page, 1988; Schuster, 2002). *A. castellanii* C3 (ATCC 50739^{TM}), purchased from the American Type Culture Collection, was also used for this study.

Bacteria stock and determination of infective progeny

The *Acanthamoeba* isolate harboring bacterial endosymbionts was harvested and

disrupted by freeze-thawing. After centrifugation at 180 x g for 5 min to remove cell debris, the bacteria were concentrated by high-speed centrifugation at 2,000 x g for 30 min. The bacterial pellet was resuspended in sucrose-phosphate-glutamic acid (SPG) buffer containing 0.2 M sucrose, 3.8 mM KH_2PO_4, 6.7 mM Na_2HPO_4 and 5 mM L-glutamic acid (pH 7.4), and then stored at -80°C until use. The number of infective progeny for eR18 endosymbiont identified to be *Chlamydiales* was determined by amoeba- infectious units (AIU) assay, as described previously (Matsuo *et al*., 2008). The number of other endosymbionts identified to be *Chlamydiales* was determined using a standard turbidimetrical assay with *E. coli*. We also confirmed that all bacterial endosymbionts were unable to be cultured on conventional agar plates (5% horse blood agar, chocolate agar, B-CYE agar) under aerobic, microaerophilic, or anaerobic atmospheres at 15 or 30°C (data not shown). *Parachlamydia acanthamoebae* Bn_9 (ATCC VR-1476) was purchased from American Type Culture Collection and the bacteria were propagated using an amoeba cell culture system, according to methods described previously (Matsuo *et al*., 2008).

Live/dead staining

Bacteria, immediately after being drawn from amoebae and stored in SPG solution, were gently washed four times with PAS. The bacteria were then stained with SYTO10 [a green fluorescent stain for living cells (the green color is converted to white in the photo images)] and ethidium homodimer-2 [a red fluorescent stain for dead cells (the red color is converted to black in the photo images)] for 15 min using a LIVE/DEAD reduced

biohazard viability/cytotoxicity kit (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. Bacteria were then observed using fluorescence microscopy.

Infection procedure

A 24-well plate with *A. castellanii* C3 (5 x 10^5/well) suspended in PAS was infected with approximately 10^7 of bacteria (determined using a standard turbidimetrical assay with *E. coli*), and then incubated for 3 days at 30°C in a normal atmosphere. During the 3 days of culture, amoebae were regularly collected to calculate the rate of bacterial infection using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (Dojindo, Kumamoto, Japan) and fluorescence microscopy. The intracellular location of bacteria and morphological changes in amoeba were assessed using electron microscopy. The number of infective progeny was determined using the AIU assay.

DNA and RNA extraction and reverse transcription

Amoeba trophozoites (1×10^5) were harvested by centrifugation at 1,600 × g for 30 min, and the pellets were then used for DNA extraction. DNA extraction was performed using a DNA Mini Kit (QIAamp, Qiagen, Valencia, CA), according to the manufacturer's instructions. Also, amoeba isolates harboring bacterial endosymbionts were harvested in the same way, and the pellets were then used for RNA extraction. Total RNA extraction was performed using an RNeasy Mini Kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. The extracted total RNA was treated with DNase (Ambion, Austin, TX). The resulting RNA preparations were assayed for DNA contamination by

PCR without the reverse transcription step. Reverse transcription (RT) of 2 μg total RNA using avian myeloblastosis virus reverse transcriptase was performed with random primers in a commercial reaction mixture (Reverse Transcription System; Promega, Madison, WI).

PCR conditions and phylogenetic analysis

The amount of extracted DNA and cDNA synthesized by RT was determined using a spectrophotometer. template DNA (2 μl) was used for PCR. The detection of *Acanthamoeba* spp., bacteria minus the order *Chlamydiales*, and the order *Chlamydiales* was performed by conventional PCR that targeted *Acanthamoeba 18S rRNA* (Schroeder *et al*. 2001), bacterial *16S rRNA* excluding *Chlamydiales* (Heinz *et al*., 2007), and *Chlamydiales 16S rRNA* (Corsaro *et al*. 2002), respectively. The amplified products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. To prevent PCR amplicon contamination, the preparation of PCR reactions was performed in a separate room. For phylogenetic analysis of *Acanthamoeba* isolates and bacterial endosymbionts, the *Acanthamoeba* specific gene *ASA.S1* (Schroeader *et al*., 2001) and the nearly full-length bacterial *16S rRNA* (Horn *et al*., 1999) were also amplified and confirmed by direct sequencing (Macrogen, Seoul, Korea). Previously reported sequences were obtained from the DDBJ/EMBL/GeneBank databases. The sequences were aligned and the phylogenic tree was constructed with Neighbor-Jointing (Saitou and Nei, 1987) using MEGA4 software (Tamura *et al*., 2007). The primers used were as follows: *Acanthamoeba 18S rRNA* (*ASA.S1*): sense, 5’ –GGC CCA GAT CGT TTA CCG TGA

A–3’; antisense, 5’–TCT CAC AAG CTG CTA GGG AGT CA–3’ (Schroeder *et al*. 2001), bacterial *16S rRNA* excluding *Chlamydiales* (nearly full-length bacterial *16S rRNA*): sense, 5’–AGA GTT TGA TYM TGG CTC AG–3’; antisense, 5’–CAK AAA GGA GGT GAT CC–3’ (Horn *et al*., 2007), *Chlamydiales 16S rRNA*: sense, 5’–CGT GGA TGA GGC ATG CRA GTC G–3’; antisense, 5’–GTC ATC RGC CYY ACC TTV SRC RYY TCT–3’ (Corsaro *et al*., 2002).

Fluorescent in situ hybridization (FISH)

The following oligonucleotide probes were used to confirm the location of bacteria in host cells. Proca 438, specific for *Procabacter*, 5’-CGA TTT CCT CCC RGA CAA-3’ (Schmitz-Esser *et al*., 2008); CC23a, specific for α-*Proteobacterium*, 5’-TTC CAC TTT CCT CTC TCG-3’ (Schmitz-Esser *et al*., 2008); C_{22}-658, specific for *Neochlamydia*-like bacteria, 5’-TCC ATT TTC TCC GTC TAC-3’ (Fritsche *et al*., 2000) and EUK516, targeted to eukaryote 18S rRNA, 5’-ACC AGA CTT GCC CTC C-3’ (Amann *et al*., 1997). The prokaryotic probes and the eukaryotic probe were labeled with Alexa Fluor 532 and Alexa Fluor 488, respectively (Invitrogen Corporation, Carlsbad, CA). Hybridizations were performed for 90 min at 46°C, according to methods described previously (Matsuo *et al*., 2008).

Transmission electron microscopy (TEM)

For TEM analysis, cells were immersed in a fixative containing 3% glutaraldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, for 24 h at 4°C. After a brief wash with PBS,

they were processed for alcohol dehydration and embedding in Epon 813, as described previously (Matsuo *et al*., 2008). Ultrathin sections of cells were stained with lead citrate and uranium acetate before viewing by electron microscopy.

Statistical analysis

Comparisons between the infectious rates of bacteria were assessed by an unpaired *t* test. A *p* value of less than 0.05 was considered significant.

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Figure Legends

Fig. 1 Detection and phylogenetic analysis of bacterial endosymbiont DNA in environmental amoeba isolates. (A) Representative results of PCR products amplified from amoebae, separated by agarose gel electrophoresis. M, molecular weight marker; N, negative control; P, positive control. (B and C) Phylogenic trees showing the relationships between bacterial PCR amplicons and previously identified sequences. Trees were constructed using the Neighbor-Joining method in MEGA (version 4). The accession numbers used were as follows: *Caedibacter caryophila* (AJ238683, X71837), *Caedibacter macronucleorum* (AM236090), endosymbiont of *Acanthamoeba polyphaga* HN-3 (AF132138), endosymbiont of *Acanthamoeba* sp. AC305 (AY549548), endosymbiont of *Acanthamoeba* sp. EI3 (AM408790), endosymbiont of *Acanthamoeba* sp. UWC9 (AF132137), endosymbiont of *Acanthamoeba* sp. UWET39 (AF132139), *Holospora obtuse* (X58198), *Trojanella thessalonices* (AF069496), '*Candidatus* Procabacter acanthamoebae' (AF177427), '*Candidatus* Procabacter sp.' OEW1 (AM412761), '*Candidatus* Procabacter sp.' Page 23 (AF177425), '*Candidatus* Procabacter sp.' TUMSJ-226 (AF352385), '*Candidatus* Procabacter sp.' TUMSJ-341 (AF352386), '*Candidatus* Procabacter sp.' UWC6 (AF177426), '*Candidatus* Procabacter sp.' UWE2 (AF177424). *Chlamydophila pneumoniae* TW-183 (NC_005043), *Chlamydia trachomatis* 434/Bu (NC_010287), endosymbiont of *Acanthamoeba* sp. UWE1 (AF083614), endosymbiont of *Acanthamoeba* sp. UWC22 (AF083616), endosymbiont of *Acanthamoeba* sp. TUME1 (AF098330), *Neochlamydia hartmannellae* (AF177275),

Parachlamydia acanthamoebae Bn9 (Y07556), *Parachlamydia acanthamoebae* Hall's coccus (AF366365), *Parachlamydia acanthamoebae* Seine (DQ309029), *Parachlamydia acanthamoebae* UV-7 (AJ715410), *Protochlamydia amoebophila* UWE25 (AF083615), *Protochlamydia naegleriophila* KNic (DQ632609), and *Simkania negevensis* Z (U68460).

Fig. 2 Representative FISH (A) and TEM (B) images of amoebae with bacterial endosymbionts. (A) Amoebae harboring bacterial endosymbionts (a, S13; b, R18; c, S23; d, S31; e and e', S40) were fixed and then hybridized with Alexa Flour 488-labeled EUK 516, which targets eukaryotic 18S rRNA (a-e', Green color), Alexa Fluor 532-labeled C_{22}-658, which is specific for *Neochlamydia*-like bacteria (Fritsche *et al*., 2000) (a, b, e', Red color), CC23a, which is specific for α-*Proteobacterium* (Schmitz-Esser *et al*., 2008) (c, d, Red color), or Proca 438, which is specific for *Procabacter* (Schmitz-Esser *et al*., 2008) (e, Red color). Magnification, x 200. (B) TEM images showing the ultrastructure of amoebae with bacterial endosymbionts (a, S13; b, R18; c, S23; d, S31; e, S40) and of *P. acanthamoebae*-infected amoebae 1 day after infection (f). Arrows indicate representative bacterial morphologies (a-f). Arrowheads show other bacteria present in S40 (e). Bar = 500 nm.

Fig. 3 Representative images of LIVE/DEAD stained bacterial endosymbionts drawn

from amoebae (A) and of RT-PCR of amoebae with endosymbionts (B). (A) Bacterial endosymbionts drawn from amoebae were stained with LIVE/DEAD staining kit (See the experimental procedures). The heat-killed bacteria were prepared by heating to 85 °C for 30 min. Magnification, x 200. (B) Representative results of RT-PCR products amplified from amoebae and separated by agarose gel electrophoresis. M, molecular weight marker; N, negative control; P, positive control.

Fig. 4 Ability of bacterial endosymbionts to transfer to amoebae. *A. castellanii* C3 suspended in PAS was infected with bacteria (MOI of 100), and then incubated for 3 days at 30°C in a normal atmosphere (See the experimental procedures). (A) Representative images of DAPI stained amoebae infected with bacterial endosymbionts (eR18, eS13). (B) The infectious rate of amoebae by bacteria was assessed by DAPI staining and fluorescence microscopy. (C) The number of infective progeny of amoebae infected with eR13 endosymbiont, as determined by the AIU assay. *, $p < 0.05$. (D) Representative TEM images of bacterial attachment and invasion of amoeba. Arrows indicate crescent bodies, which are a typical morphological feature indicating the elementary bodies of environmental chlamydia in the developmental stage. Arrowheads indicate digested bacteria. Bar = 500 nm.

Fig. 1

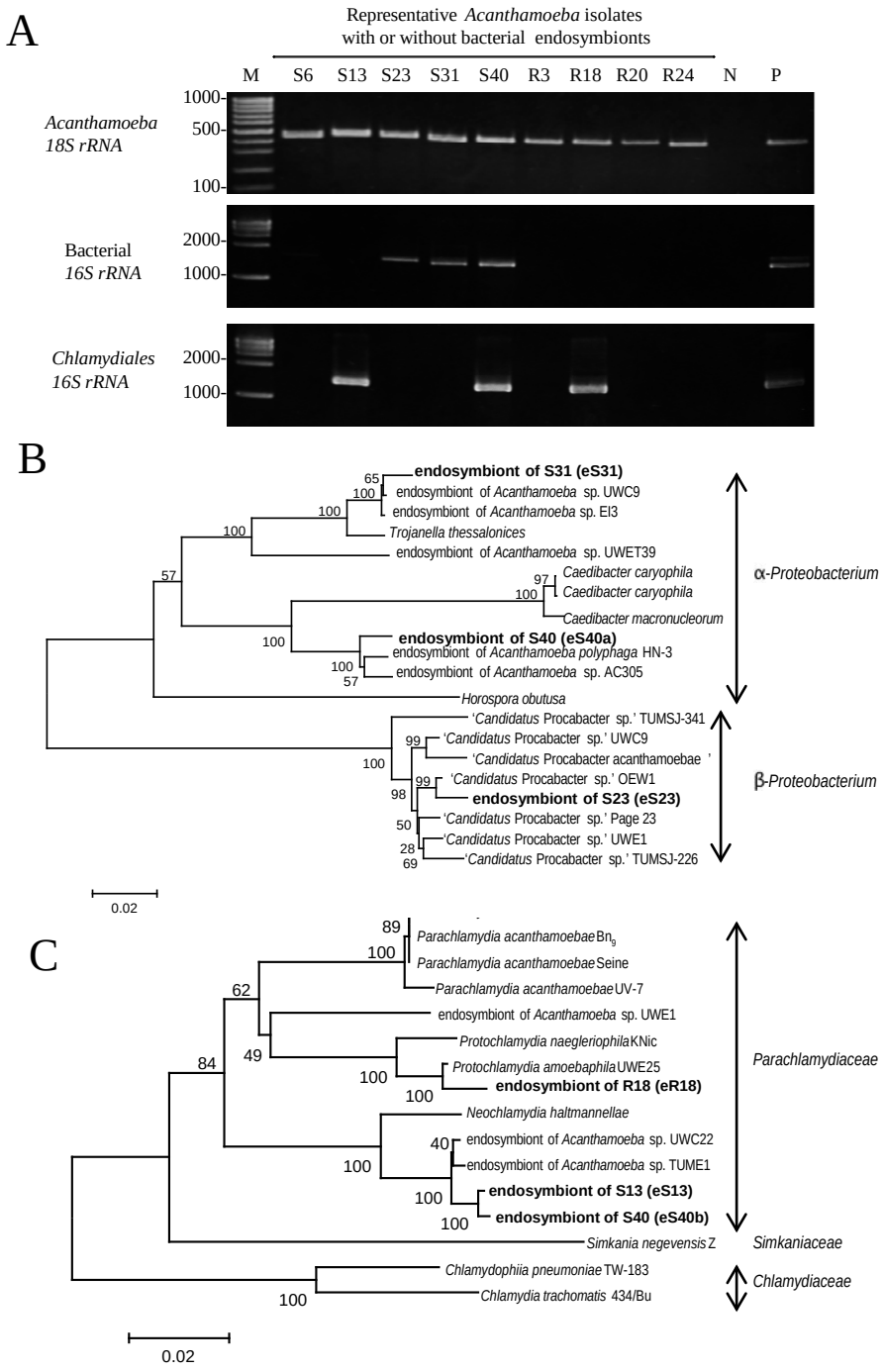

A
Representative *Acanthamoeba* isolates
with or without bacterial endosymbionts
M S6 S13 S23 S31 S40 R3 R18 R20 R24 N P
Acanthamoeba 18S rRNA
1000
500
100
Bacterial *16S rRNA*
2000
1000
Chlamydiales 16S rRNA
2000
1000
B
endosymbiont of S31 (eS31)
endosymbiont of *Acanthamoeba* sp. UWC9
endosymbiont of *Acanthamoeba* sp. EI3
Trojanella thessalonices
endosymbiont of *Acanthamoeba* sp. UWET39
Caedibacter caryophila
Caedibacter caryophila
Caedibacter macronucleorum
endosymbiont of S40 (eS40a)
endosymbiont of *Acanthamoeba polyphaga* HN-3
endosymbiont of *Acanthamoeba* sp. AC305
Horospora obutusa
'*Candidatus* Procabacter sp.' TUMSJ-341
'*Candidatus* Procabacter sp.' UWC9
'*Candidatus* Procabacter acanthamoebae '
'*Candidatus* Procabacter sp.' OEW1
endosymbiont of S23 (eS23)
'*Candidatus* Procabacter sp.' Page 23
'*Candidatus* Procabacter sp.' UWE1
'*Candidatus* Procabacter sp.' TUMSJ-226
α-*Proteobacterium*
β-*Proteobacterium*
0.02
C
Parachlamydia acanthamoebae Bn9
Parachlamydia acanthamoebae Seine
Parachlamydia acanthamoebae UV-7
endosymbiont of *Acanthamoeba* sp. UWE1
Protochlamydia naegleriophila KNic
Protochlamydia amoebaphila UWE25
endosymbiont of R18 (eR18)
Neochlamydia haltmannellae
endosymbiont of *Acanthamoeba* sp. UWC22
endosymbiont of *Acanthamoeba* sp. TUME1
endosymbiont of S13 (eS13)
endosymbiont of S40 (eS40b)
Simkania negevensis Z
Chlamydophiia pneumoniae TW-183
Chlamydia trachomatis 434/Bu
Parachlamydiaceae
Simkaniaceae
Chlamydiaceae
0.02

Fig. 2

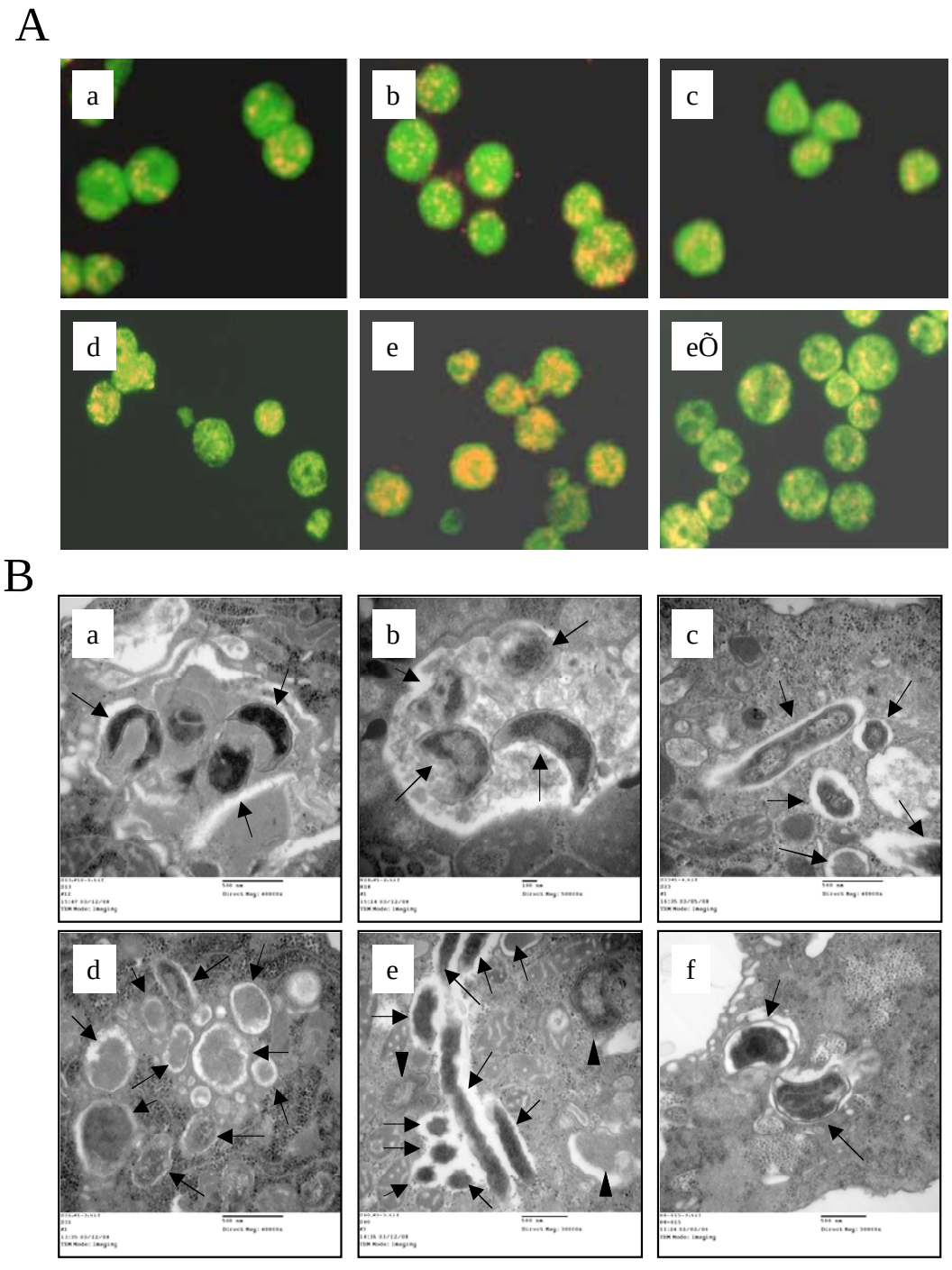


Fig. 3

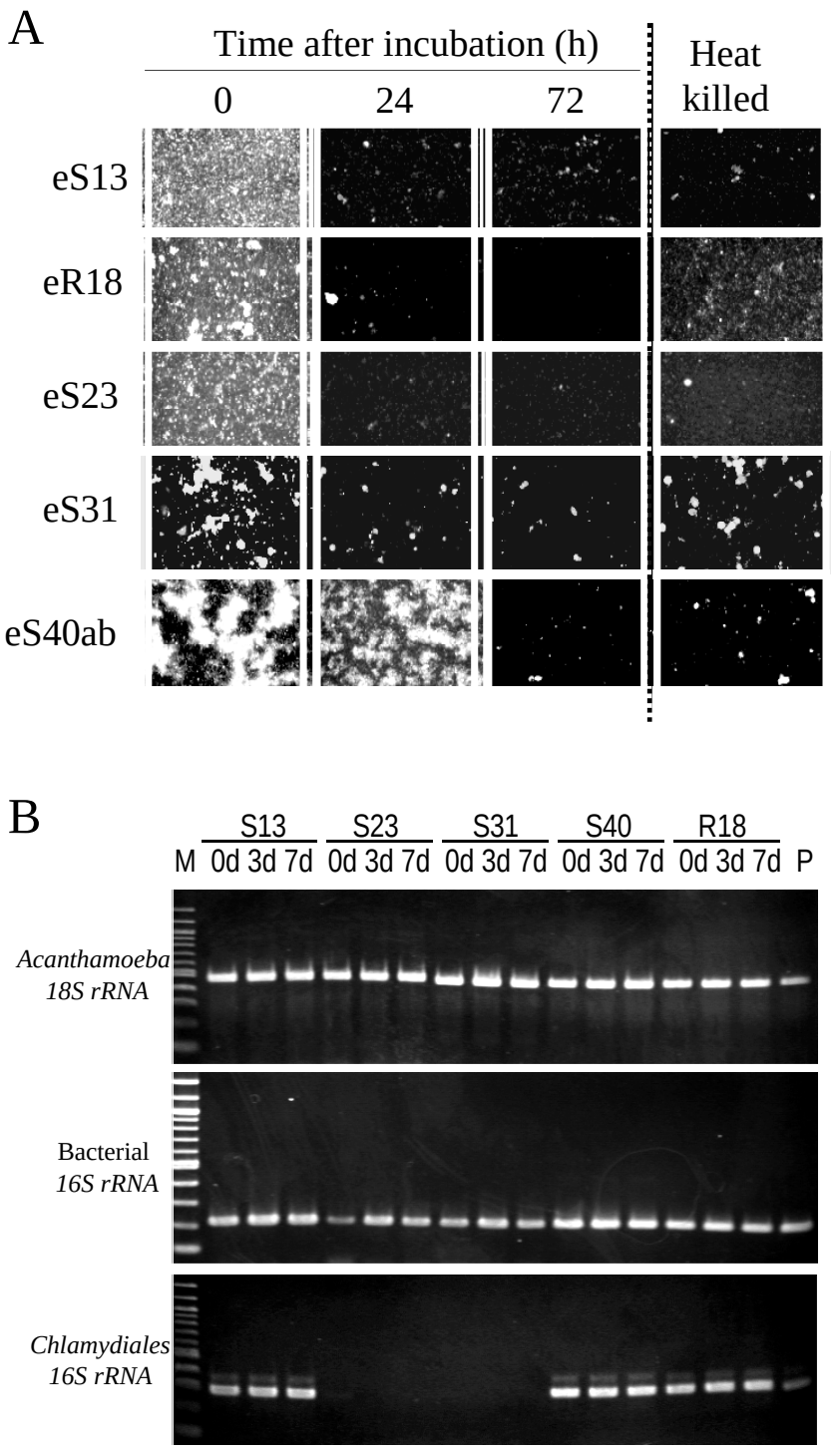
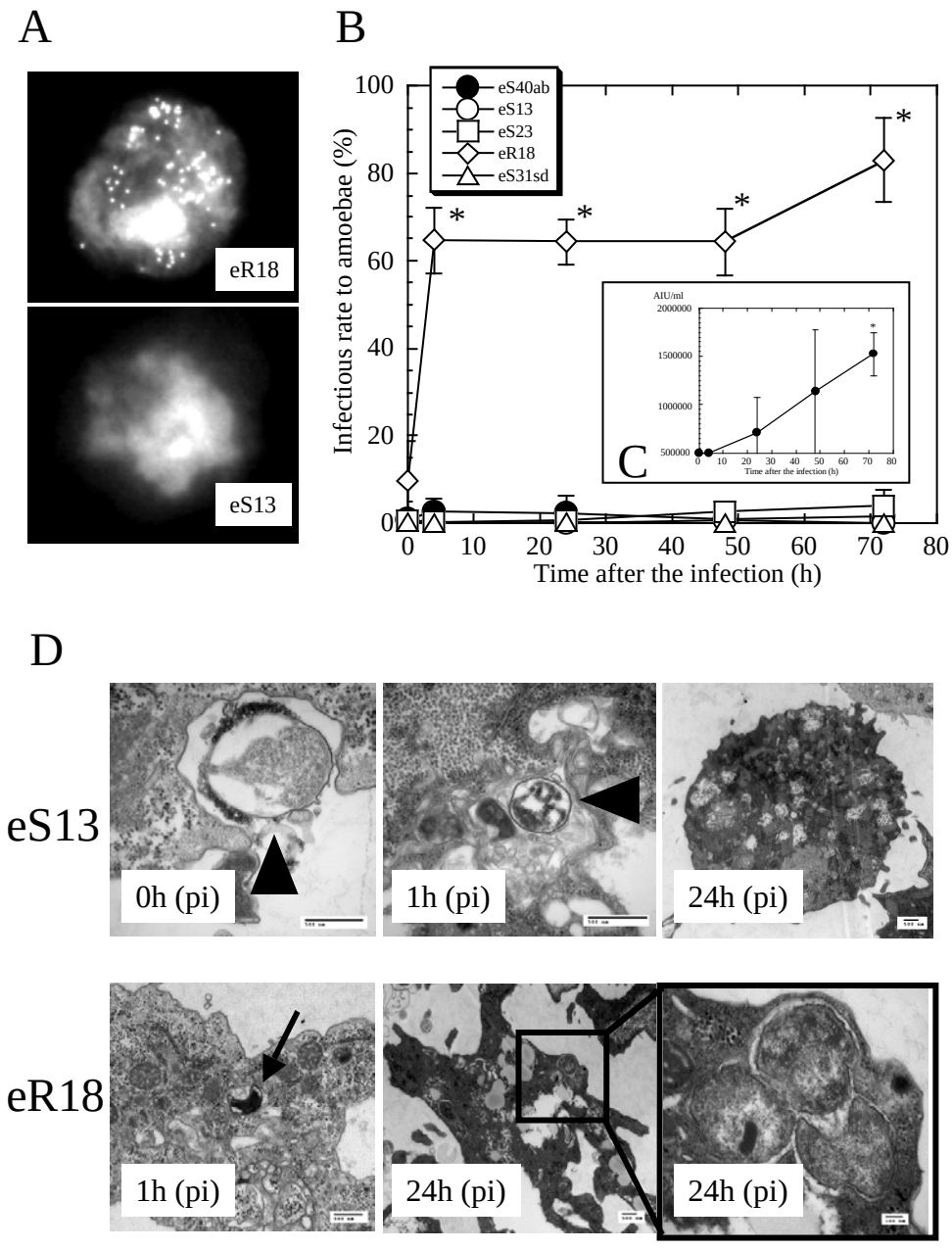

A
Time after incubation (h)
0
24
72
Heat killed
eS13
eR18
eS23
eS31
eS40ab
B
S13
S23
S31
S40
R18
M 0d 3d 7d 0d 3d 7d 0d 3d 7d 0d 3d 7d 0d 3d 7d P
Acanthamoeba 18S rRNA
Bacterial 16S rRNA
Chlamydiales 16S rRNA

Fig. 4


A
B
eR18
eS13
eS40ab
eS13
eS23
eR18
eS31sd
Infectious rate to amoebae (%)
Time after the infection (h)
C
AIU/ml
Time after the infection (h)
D
eS13
0h (pi)
1h (pi)
24h (pi)
eR18
1h (pi)
24h (pi)
24h (pi)