

Molecular characterization of bacterial endosymbionts of *Acanthamoeba* isolates from infected corneas of Korean patients

Ying-Hua XUAN¹, Hak Sun YU³, Hae Jin JEONG³, Sung-Yong SEOL²,
Dong-Il CHUNG¹ and Hyun-Hee KONG¹*

¹Department of Parasitology and ²Department of Microbiology,
Kyungpook National University School of Medicine, Taegu 700-422,

³Department of Parasitology, Pusan National University School of Medicine, Busan 602-739, Korea

Abstract: The endosymbionts of 4 strains of *Acanthamoeba* (KA/E9, KA/E21, KA/E22, and KA/E23) isolated from the infected corneas of Korean patients were characterized via orcein stain, transmission electron microscopic examination, and 16S rDNA sequence analysis. Double membrane-bound, rod-shaped endosymbionts were distributed randomly throughout both the trophozoites and cysts of each of *Acanthamoeba* isolates. The endosymbionts of KA/E9, KA/E22, and KA/E23 were surrounded by electron-translucent areas. No lacunae-like structures were observed in the endosymbionts of KA/E21, the bacterial cell walls of which were studded with host ribosomes. Comparative analyses of the 16S rDNA sequences showed that the endosymbionts of KA/E9, KA/E22 and KA/E23 were closely related to *Caedibacter caryophilus*, whereas the KA/E21 endosymbiont was assigned to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. In the 4 strains of *Acanthamoeba*, the hosts of the endosymbionts were identified as belonging to the *Acanthamoeba castellanii* complex, which corresponds to the T4 genotype. *Acanthamoeba* KA/E21 evidenced characteristics almost identical to those of KA/E6, with the exception of the existence of endosymbionts. The discovery of these endosymbionts from *Acanthamoeba* may prove essential to future studies focusing on interactions between the endosymbionts and the amoebic hosts.

Key words: *Acanthamoeba*, bacterial endosymbiont, keratitis, 16S rDNA, 18S rDNA

INTRODUCTION

The *Acanthamoeba* genus is an opportunistic protozoan parasite which has adapted to live in a variety of

environments, including soil, air, fresh water, ocean sediment, wild animals, and humans. Some *Acanthamoeba* species have been determined to induce serious human infections, including amoebic keratitis, particularly in contact lens wearers, as well as granulomatous amoebic encephalitis (GAE) in immunocompromised individuals (Marciano-Cabral and Cabral, 2003). Amebic keratitis was considered to be a relatively rare type of corneal infection during the 10 years following the first case report by Jones et al. (1975). However, the incidence of amoebic keratitis

• Received 22 November 2006, accepted after revision 29 January 2007.

• This research was supported by Kyungpook National University Research Team Fund, 2002.

• This work was supported by the Brain Korea 21 Project in 2006.

*Corresponding author (e-mail: hhkong@knu.ac.kr)

infection has exponentially increased over the past 2 decades, as the consequence of the increasing use of contact lenses. Recently, the incidence of amoebic keratitis in Korea has also evidenced an increase; this may also be due to the high *Acanthamoeba* contamination rate of contact lenses cases in Korea, as has been reported in several papers (Lee et al., 1997; Yu et al., 2001; Kong et al., 2002).

Bacteria are a major food source for amoeba (Rodriguez-Zaragoza, 1994). However, some bacteria are capable of surviving within *Acanthamoeba*, and exploit them as hosts. Bacterial transient intracellular associations with *Acanthamoeba* have been previously reported, and have included species such as *Legionella pneumophila*, *Chlamydia pneumoniae*, *Mycobacterium avium*, and *Escherichia coli* O157 (Rowbotham, 1986; Essig et al., 1997; Steinert et al., 1998; Barker et al., 1999). Within the amoebal trophozoites, these bacteria may be protected against adverse environmental conditions, thereby suggesting that amoebae may play a crucial role in the survival and dispersal of bacteria within the environment (Winiecka-Krusnell and Linder, 2001). Such interactions may be of clinical relevance, particularly because the multiplication of *L. pneumophila* and *M. avium* within the amoebic host cell increases bacterial virulence (Cirillo et al., 1997).

In this study, we describe a new discovery regarding the presence of endosymbionts in 4 strains of *Acanthamoeba* (KA/E9, KA/E21, KA/E22, KA/E23) isolated from the infected corneas of Korean patients. The amoebic host and endosymbiotic bacteria were characterized via transmission electron microscopy and 18S and 16S rDNA sequence analyses.

MATERIALS AND METHODS

Isolation and maintenance of *Acanthamoeba*

Four *Acanthamoeba* isolates; KA/E9, KA/E21, KA/E22, and KA/E23, all of which harbored bacterial endosymbionts, were isolated from the infected corneas of Korean patients. Corneal samples collected by scraping from keratitis patients were loaded onto 1.5% agar plates covered with heat-inactivated *Escherichia coli* (ATCC 25922, plasmid-free,

Washington D.C., USA). Culturing on the plates, clonal isolation, and axenic culturing were conducted via the same techniques as previously described (Yu et al. 2001).

Orcein staining

Orcein staining was employed in order to visualize the presence of endosymbionts within the *Acanthamoeba*, as described by Beier et al. (2002). Thin films of *Acanthamoeba* were prepared on slides and allowed to air-dry. The slides were then placed in 1% KMnO₄ for 5 min, washed in water, and rinsed for 30 sec in 3% oxalic acid, and finally rinsed off in distilled water. The films were covered with a few drops of orcein-carmin staining solution. The slides were examined 1 hr after staining under a light microscope (Olympus IX70, Tokyo, Japan), using the oil immersion objective.

Mitochondrial (Mt) DNA restriction fragment length polymorphism (RFLP)

The *Acanthamoeba* MtDNA was extracted via the method described by Yagita and Endo (1990). *Acanthamoebae* trophozoites harvested at the end of the logarithmic growth phase were washed in cold PBS. The amoebas were lysed with fresh 1% sodium dodecyl sulfate (SDS) solution in 0.2 N NaOH and potassium acetate buffer, and incubated for 30 min on ice. The MtDNA was then extracted with phenol and phenol-chloroform (1:1) and recovered via precipitation with cold absolute ethanol in the presence of sodium acetate. The extracted MtDNA was then digested with the *Bgl* II restriction enzyme (Promega, Madison, Wisconsin, USA) at 37°C. The digested DNA was electrophoresed in 0.7% agarose gel stained with ethidium bromide. The MtDNA RFLP patterns were observed and photographed under a UV transilluminator.

Transmission electron microscopy (TEM)

The trophozoites were washed 3 times with cold PBS and prefixed for 2 hr with 4% glutaraldehyde in 0.1 M cacodylate buffer. After rinsing with 0.1 M cacodylate buffer, the sediment was post-fixed for 3 hr with 1% osmium tetroxide, rinsed twice with 0.1 M

maleate buffer, pH 5.2, dehydrated with ethyl alcohol, and treated for 30 min with propylene oxide. The pellets were immersed overnight in propylene oxide-resin (1:1) with continuous shaking, then embedded in resin and incubated overnight at 60°C. Ultrathin sections were cut on a Reichert-Jung ultramicrotome, and then stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (Hitachi H-7000, Tokyo, Japan).

Sequence analysis of 16S rDNA of endo-symbionts and 18S rDNA of *Acanthamoeba*

In order to amplify the 16S rDNA of the bacterial endosymbionts, the MtDNA of each *Acanthamoeba* was employed as a template. The nucleotide sequences of the primers were as follows: forward; 5'-TCGACAACAGAGTTTGATCCTGGCTCAG-3' reverse; 5'-ATCCAAGCTTAAGGAGGTGATCCAGCC-3'. 16S rDNA amplification reactions were separately conducted in a thermal capillary cyler using a premix of 50 µl scale (iNtRON, Seongnam, Korea), which included 1.25 U of *Taq* polymerase (iNtRON). The PCR reactions were conducted at an initial 3 min at 94°C, followed by 30 cycles of 94°C for 1.5 min, 50°C for 1 min, 72°C for 4 min, and a final 7-min elongation step at 72°C.

The chromosomal DNA of *Acanthamoeba* isolates acquired via the method described by Kong and Chung (1996) was utilized as a template for 18S rDNA PCR. The amplification of nearly full-length amoebic 18S rDNA gene fragments was conducted using primers originating from the ssu rRNA sequence of the *A. castellanii* Neff strain, as reported by Gunderson and Sogin (1986). The sequences of the primers were as follows: forward; 5'-CCGAATTCGTCGA-CAACCTGGTTGATCCTGCCAGT-3' reverse; 5'-GGATCCAAGCTTGATCCTTCTGCAGGTTACCTAC-3'. The 18S rDNA amplification reactions were separately conducted in a thermal capillary cyler using premix of 50 µl scale (iNtRON). The PCR reactions were conducted for an initial 3 min at 94°C, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2.5 min, and a final elongation step of 10 min at 72°C. The presence and size of all of the amplified

products were evaluated via agarose gel electrophoresis and ethidium bromide staining.

The amplified PCR products were ligated into the T/A cloning vector pGEM-T easy System I (Promega), with subsequent transformation into *E. coli* by each vector. DNA sequencing was conducted by an outside company (Macrogen, Seoul, Korea).

Phylogenetic analyses

Nucleotide sequences were compared to the sequences of published strains via BLAST search (Altschul et al., 1990). CLUSTAL X (Thompson et al., 1997) was used for pairwise alignment and calculations of the percentages of sequence dissimilarity. The percentages of similarity correspond to the number of sites that are identical between the 2 isolates, aligned with one another, as in the master alignment. Phylogeny was evaluated using the CLUSTAL X 1.82 program with a low gap penalty, and the phylogenetic trees were drawn using TreeView computer program.

RESULTS

Orcein staining

Bacterial endosymbionts were readily visualized within the orcein-stained *Acanthamoeba* under phase-contrast light microscopy (Fig. 1). Darkly stained rod-shaped bacterial endosymbionts were found to be distributed randomly within the cytoplasm of the trophozoites of *Acanthamoeba* KA/E9, KA/E21, KA/E22, and KA/E23. In comparison, no bacteria were detected in *A. castellanii* Castellani, which was employed as a control.

Mitochondrial DNA (MtDNA) restriction phenotypes

Fig. 2 shows the agarose gel electrophoretic patterns of *Bgl* II-digested MtDNA from 4 clinical *Acanthamoeba* isolates and the reference strains. The restriction fragment patterns of the extranuclear circular DNA from *Acanthamoeba* strains KA/E9, KA/E21, KA/E22, and KA/E23 evidenced extra fragments, as well as common fragments which were also observed

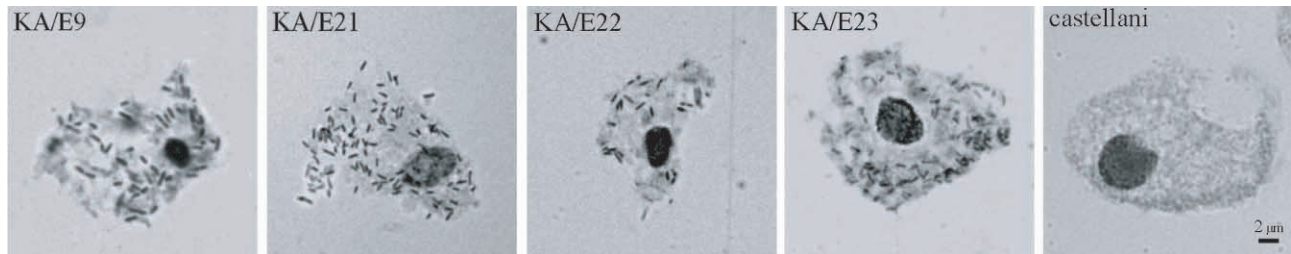


Fig. 1. Phase-contrast micrographs of endosymbionts of *Acanthamoeba* stained with orcein-carmin. Numerous bacterial symbionts in the cytoplasm of trophozoites of *Acanthamoeba* KA/E9, KA/E21, KA/E22, and KA/E23 are visible. *A. castellani* Castellani is used as a control. Bar = 2 μ m.

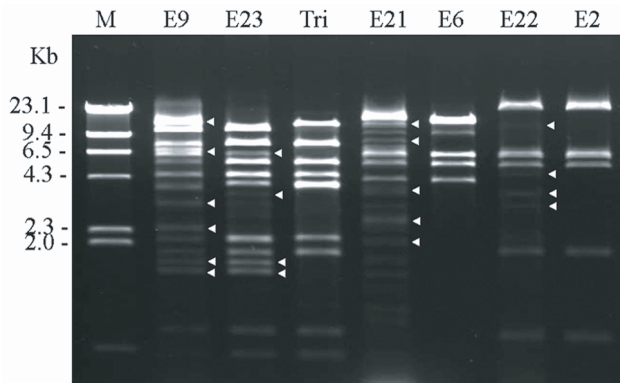


Fig. 2. Agarose gel electrophoretic restriction fragment patterns of *Bgl* II-digested mitochondrial DNA of host *Acanthamoeba* spp. and the reference strains. Lane 1; KA/E9, 2; KA/E23, 3; *A. triangularis* SH621, 4; KA/E21, 5; KA/E6, 6; KA/E22, 7; KA/E2. M; Hind III digested lamda phage DNA as DNA molecular size standard. *Acanthamoeba* KA/E9, KA/E21, KA/E22, KA/E23 show extra bands (arrow heads) as well as common bands with the other reference strains.

in the reference strains. *Acanthamoeba* KA/E9 and KA/E23 evidenced extremely similar MtDNA RFLP patterns with *A. triangularis* SH621, whereas *Acanthamoeba* KA/E21 and KA/E22 were identical to the MtDNA RFLP patterns of KA/E6 and KA/E2, respectively, obtained from other corneal isolates of Korean patients.

TEM

The morphological and ultrastructural characteristics of the endosymbionts were elucidated via transmission electron microscopy. The rod-shaped endosymbiont of *Acanthamoebae* KA/E9 was estimated to be approximately $1.32 \times 0.4 \mu$ m in size (Fig. 3A-3C). In addition, the endosymbionts were determined

to be randomly distributed within the host cytoplasm, and were frequently surrounded by an electron-translucent area (Fig. 3C). The endosymbionts of *Acanthamoebae* strains KA/E22 and KA/E23 were morphologically identical to those of KA/E9.

However, the endosymbionts of *Acanthamoeba* KA/E21 were also determined to be randomly distributed within the cytoplasm of both the trophozoites and cysts (Fig. 3D-3F). They appeared as straight rods with rounded ends, and measured approximately $1.08 \times 0.35 \mu$ m. The endosymbionts were bound by double membranes, and the bacterial cell surfaces were studied with a number of host cell ribosomes (Fig. 3F). They were not enclosed within vacuoles. No specific features could be observed within the bacteria in either case.

16S rRNA sequence analysis of endosymbionts

Comparative analysis with the 16S rRNA gene sequences showed that the endosymbionts of KA/E9 (84%), KA/E22 (83%) and KA/E23 (83%) evidenced a high degree of sequence similarity with *Caedibacter caryophilus*, a member of the α -Proteobacteria. Phylogenetic treeing methods showed that the endosymbionts of KA/E9 KA/E22 and KA/E23, *Caedibacter caryophilus*, *Holospira obtusa* and 'NHP bacterium' constitute a monophyletic cluster within the α -Proteobacteria, and are closely related to the *Rickettsia*s (Fig. 4).

The results of comparative sequence analysis supported the assignment of the endosymbiont of *Acanthamoeba* KA/E21 to the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum. The KA/E21 endosymbionts evidenced the highest degree of similarity, with

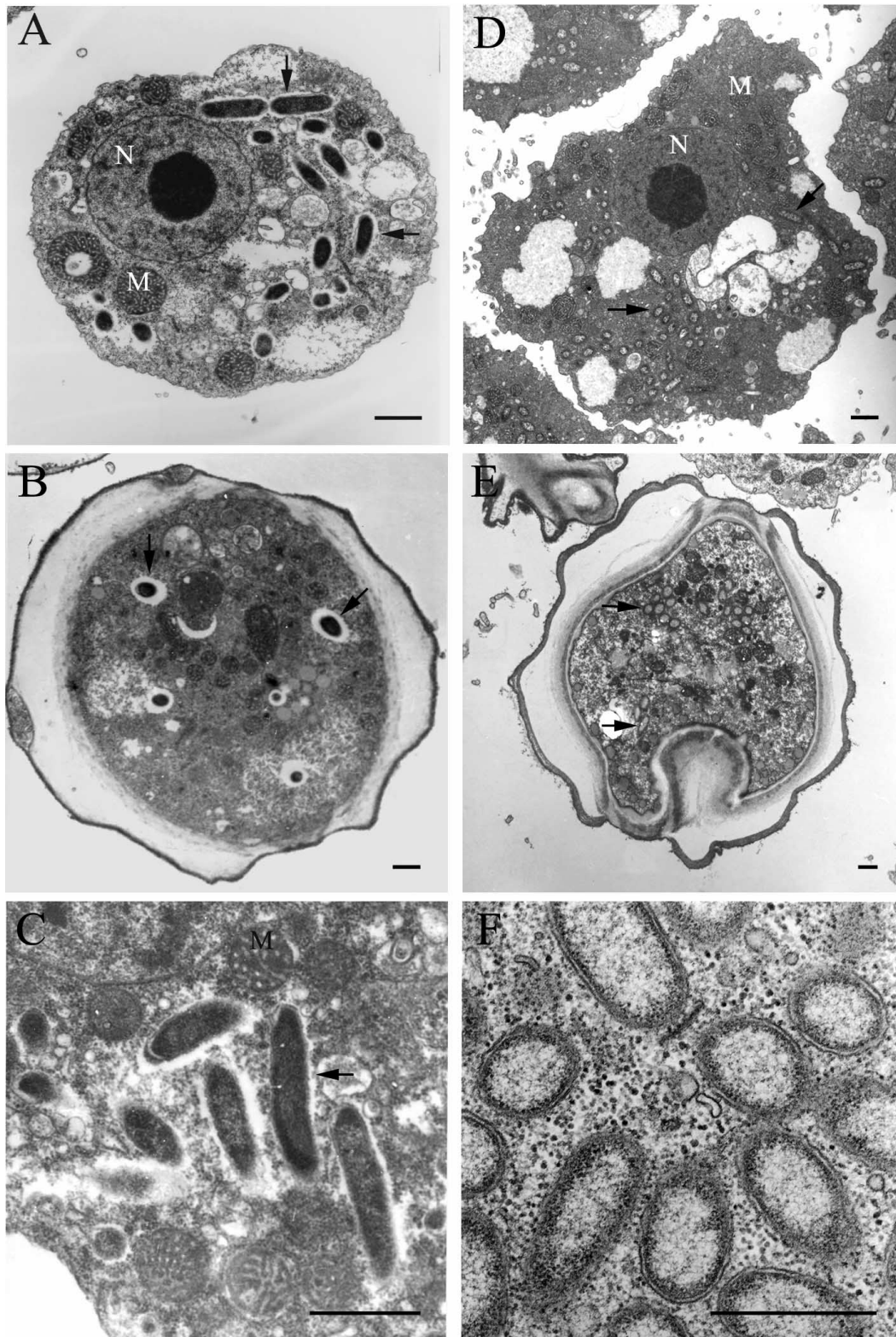


Fig. 3. Transmission electron micrographs of *Acanthamoeba* KA/E9 (A-C) and KA/E21 (D-F) with bacterial endosymbionts. **A, D:** Trophozoites with numerous rod-shaped endosymbionts (arrows) in the cytoplasm. **B, E:** Cysts with endosymbionts. **C:** Ultrastructural feature of the bacteria surrounded by an electron-translucent area. **F:** Bacterial endosymbionts closely associated with host ribosomes. N, nucleus; M, mitochondria. Bar = 1 μ m.

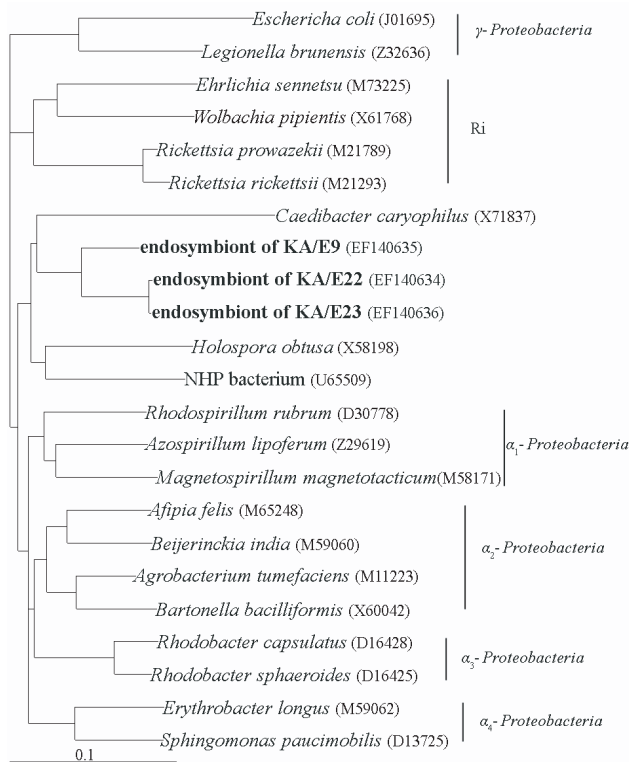


Fig. 4. Phylogenetic tree, based on comparative 16S rDNA analysis, showing relationships of endosymbiont of *Acanthamoeba* KA/E9, KA/E22 and KA/E23 to representative members of the α -subclass of *Proteobacteria*.

97% 16S rDNA sequence similarity to a recently published bacterium, *Candidatus Amoebophilus asiaticus* (Horn et al., 2001). This symbiont also evidenced a high degree of similarity with the endosymbionts of the tick, *Ixodes scapularis* (90%) and with those of the parasitic white fly, *Encarsia pergandiella* (89%). However, the endosymbiont of KA/E21 evidenced some similarity (less than 80%) to other species within the CFB phylum. The results of our phylogenetic analyses consistently demonstrated that the endosymbiont of KA/E21, *Candidatus Amoebophilus asiaticus*, the endosymbiont of *I. scapularis*, and the endosymbiont of *E. pergandiella* constitute a monophyletic lineage within the CFB phylum (Fig. 5).

18S rRNA sequence analysis of 3 strains of *Acanthamoeba*

Comparative sequence analysis verified the morphology-based identification of this isolate as

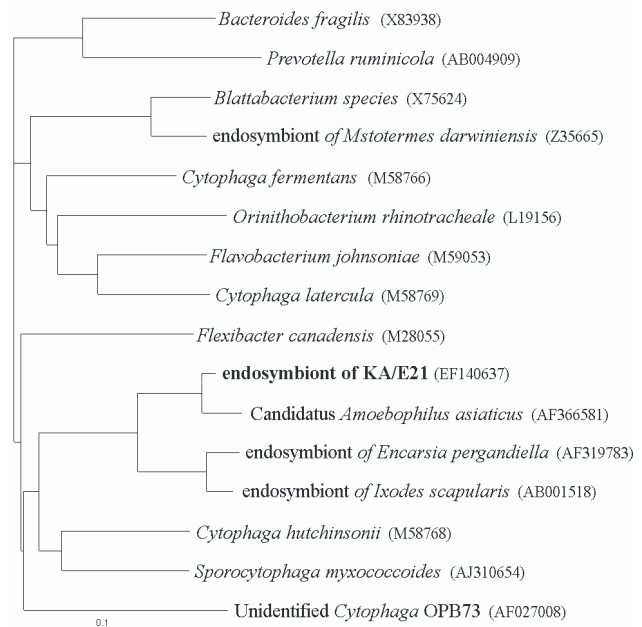


Fig. 5. Phylogenetic tree, based on comparative 16S rDNA analysis, showing relationships of endosymbiont of *Acanthamoeba* KA/E21 to representative members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum.

Acanthamoeba sp. *Acanthamoebae* KA/E9, and KA/E23 evidenced the most pronounced sequence similarity (98%) with *A. triangularis* SH621. In addition, *Acanthamoeba* KA/E21 and KA/E22 evidenced very high 18S rDNA sequence similarity (99%) with the clinical isolates KA/E6 and KA/E2 (isolated from Korean keratitis patients), respectively. The dendrogram constructed on the basis of the 18S rDNA sequence analyses is shown in Fig. 6. Using the 95% similarity value for the definition of the *Acanthamoeba* 18S rDNA sequence types (Stothard et al., 1998), the *Acanthamoeba* host should be considered a constituent of the *A. castellanii* complex, which corresponds to genotype T4, the genotype encompassing the majority of clinical and environmental isolates of *Acanthamoeba*.

DISCUSSION

Bacterial endosymbionts are commonly observed in protozoans (Winiecka-Krusnell and Linder, 2001). Approximately 24% of *Acanthamoeba*, isolates from both environmental and medical samples were determined to harbor endosymbionts (Fritsche et al., 1993).

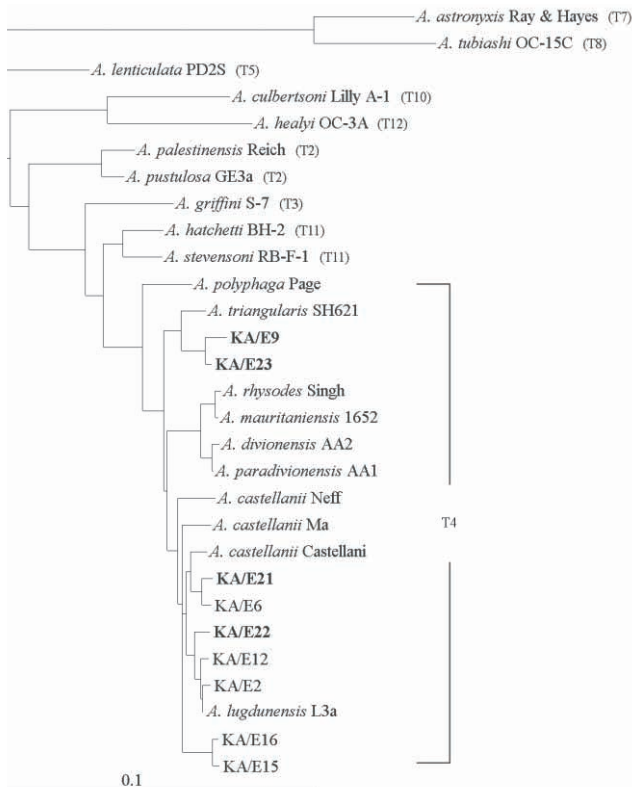


Fig. 6. 18S rRNA-based Phylogenetic tree reflecting the affiliation of host *Acanthamoeba* sp. with selected members of genus *Acanthamoeba*. The reference with their corresponding accession numbers are: *A. astronyxis* Ray & Hayes, AF019064; *A. tubiash* OC-15C, AF019065; *A. lenticulata* PD2S, U94741; *A. culbertsoni* Lilly A-1, AF019067; *A. healyi* OC-3A, AF019070; *A. palestinensis* Reich, U07411; *A. pustulosa* GE3a, AF019050; *A. griffini* S-7, U07412; *A. hatchetti* BH-2, AF019068; *A. stvensoni* RB-F-1, AF019069; *A. polyphaga* Page, AF019061; *A. triangularis* SH621, AF316547; *A. rhysodes* Singh, AF351644; *A. mauritaniensis* 1652, AY351647; *A. divionensis* AA2, AY351646; *A. paradiionensis* AA1, AY351645; *A. castellanii* Castellani, U07413; *A. lugdunensis* L3a, AF005995; *A. castellanii* Neff, AF690457; *A. castellanii* Ma, U07414; strain KA/E2, AF005998; KA/E6, AF349044; KA/E9, AF316546; KA/E12, AF316545; KA/E15, AY148961; KA/E16, AY148962; KA/E21, EF140633; KA/E22, EF140638; KA/E23, EF140625.

In this study, we have investigated, for the first time, the presence of endosymbiont bacteria in clinical *Acanthamoeba* isolates from infected corneas of Korean patients.

Four *Acanthamoeba* isolates suspected to harbor endosymbionts evidenced very peculiar MtDNA RFLP patterns. The estimated size of the MtDNA of

these isolates, based on the numbers and sizes of the DNA fragments generated by RFLP, was much larger than the general size of *Acanthamoeba* MtDNA reported by Burger et al. (1995). These results might indicate that the 4 isolates of *Acanthamoeba* may possibly harbor intracellular endosymbiotic bacteria containing circular DNAs in *Acanthamoeba*.

The KA/E9, KA/E22, and KA/E23 endosymbionts were determined to be surrounded by electron-translucent areas in the TEM examination, a result similar to that reported by Horn et al. (1999). Hall and Voelz (1985) previously demonstrated that this translucent area was the consequence of the presence of bacterial capsular material. With regard to the endosymbionts of KA/E21, our results indicated that they were surrounded by a double membrane, which was decorated with numerous ribosomes of the *Acanthamoeba* host. These morphological characteristics are very consistent with what has been observed with the endosymbionts of *Acanthamoeba* isolated previously from contact lens storage cases in Korea (Chung et al., 1997).

On the basis of the results of comparative 16S rDNA sequence analyses, the endosymbionts of KA/E9, KA/E22 and KA/E23 are members of the α -subclass of *Proteobacteria*, and are clearly affiliated with a monophyletic cluster of intracellular bacteria that are distinct from, but related to, the *Rickettsia*s. The α -*Proteobacteria* cluster comprises a number of as-yet uncultured microorganisms that appear to be capable of infecting distantly-related eukaryotes, including *Paramecium caudatum*. Within this cluster, the KA/E9, KA/E22, and KA/E23 endosymbionts evidence sequence similarities of 84%, 83%, and 83%, respectively, to *C. caryophilus*. Because Stackebrandt and Goebel (1994) mentioned that $\geq 97\%$ 16S rDNA sequence similarity refers to one species, the endosymbionts of KA/E9, KA/E22, and KA/E23, which evidence less than 97% sequence similarity, might be representative of a species other than *C. caryophilus*.

The 18s rDNA sequence analyses of the 2 hosts, KA/E9 and KA/E23, evidenced a 99% similarity with each other, and both were quite closely related to *A.*

triangularis SH621. The 16S rDNA sequence analysis of the endosymbionts of KA/E9 and KA/E23 also resulted in a finding of 92% similarity, and were identical to the endosymbionts of *C. cariophilus*. On the basis of this finding, it is possible to suggest that a particular endosymbiont may exhibit a preference to certain specific host characteristics.

The effects of bacteria on amoebic hosts are less well known. The external bacteria enhance the growth and binding of *Acanthamoeba* trophozoites to contact lenses, and also enhance the survival of amoeba in biocidal lens care solution (Cengiz et al., 2000). However, the mechanisms by which bacteria affect the virulence and pathogenicity of *Acanthamoeba* infections have yet to be thoroughly elucidated. With the exception of the presence of endosymbionts, *Acanthamoeba* KA/E9 and KA/E23 with *A. triangularis* SH621, *Acanthamoeba* KA/E21 with KA/E6, and *Acanthamoeba* KA/E22 with KA/E2 were found to be closely phylogenetically related, on the basis of MtDNA RFLP and 18S rDNA sequence analyses. This isogenetic pair can be employed in studies of the relationship between the bacteria with their host, *Acanthamoeba*. Further studies will focus on the roles of these endosymbionts that augment amoebic cytopathogenicity.

REFERENCES

- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* **215**: 403-410.
- Barker J, Humphrey TJ, Brown MW (1999) Survival of *Escherichia coli* 0157 in a soil protozoan: implications for disease. *FEMS Microbiol Lett* **173**: 291-295.
- Beier CL, Horn M, Michel R, Schweikert M, Gortz HD, Wagner M (2002) The genus *Caedibacter* comprises endosymbionts of *Paramecium* spp. related to the *Rickettsiales* (Alphaproteobacteria) and to *Francisella tularensis* (Gammaproteobacteria). *Appl Environ Microbiol* **68**: 6043-6050.
- Burger G, Plante I, Lonergan KM, Gray MW (1995) The mitochondrial DNA of amoeboid protozoan, *Acanthamoeba castellanii*: complete sequence, gene content and genome organization. *J Mol Biol* **245**: 522-537.
- Cengiz AM, Harmis N, Stapleton F (2000) Co-incubation of *Acanthamoeba castellanii* with strains of *Pseudomonas aeruginosa* alters the survival of amoeba. *Clin Experiment Ophthalmol* **28**: 191-193.
- Chung DI, Kong HH, Kim TH, Hwang MY, Yu HS, Yun HC, Seol SY (1997) Bacterial endosymbiosis within the cytoplasm of *Acanthamoeba lugdunensis* isolated from a contact lens storage case. *Korean J Parasitol* **35**: 127-133.
- Cirillo JD, Falkow S, Tompkins LS, Bermudez LE (1997) Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infect Immun* **65**: 3759-3767.
- Essig A, Heinemann M, Simnacher U, Marre R (1997) Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Appl Environ Microbiol* **63**: 1396-1399.
- Fritsche TR, Gautam RK, Seyedirashdi S, Bergeron DL, Lindquist TD (1993) Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from corneal and environmental specimens and contact lenses. *J Clin Microbiol* **31**: 1122-1126.
- Gunderson JH, Sogin ML (1986) Length variation in eukaryotic rRNAs: small subunit rRNAs from the protists *Acanthamoeba castellanii* and *Euglena gracilis*. *Gene* **44**: 63-70.
- Hall J, Voelz H (1985) Bacterial endosymbionts of *Acanthamoeba* sp. *J Parasitol* **71**: 89-95.
- Horn M, Fritsche TR, Gautam RK, Schleifer KH, Wagner M (1999) Novel bacterial endosymbionts of *Acanthamoeba* spp. related to the *Paramecium caudatum* symbiont *Caedibacter caryophilus*. *Environ Microbiol* **1**: 357-367.
- Horn M, Harzenetter MD, Linner T, Schmid EN, Muller KD, Michel R, Wagner M (2001) Members of the *Cytophaga-Flavobacterium-Bacteroides* phylum as intracellular bacteria of *Acanthamoebae*: proposal of 'Candidatus Amoebophilus asiaticus'. *Environ Microbiol* **3**: 440-449.
- Jones BR, McGill JL, Steele AD (1975) Recurrent suppurative kerato-uveitis with loss of eye due to infection by *Acanthamoeba castellanii*. *Trans Ophthalmol Soc U K* **95**: 210-213.
- Kong HH, Chung DI (1996) PCR and RFLP variation of conserved region of small subunit ribosomal DNA among *Acanthamoeba* isolates assigned to either *A. castellanii* or *A. polyphaga*. *Korean J Parasitol* **34**: 127-134.
- Kong HH, Shin JY, Yu HS, Kim J, Hahn TW, Hahn YH, Chung DI (2002) Mitochondrial DNA restriction fragment length polymorphism (RFLP) and 18S small-subunit ribosomal DNA PCR-RFLP analyses of *Acanthamoeba* isolated from contact lens storage cases of residents in southwestern Korea. *J Clin Microbiol* **40**: 1199-1206.
- Lee SM, Choi YJ, Ryu HW, Kong HH, Chung DI (1997)

- Species identification and molecular characterization of *Acanthamoeba* isolated from contact lens paraphernalia. *Korean J Ophthalmol* **11**: 39-50.
- Marciano-Cabral F, Cabral G (2003) *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev* **16**: 273-307.
- Rowbotham TJ (1986) Current views on the relationship between amoebae, legionellae and man. *Isr J Med Sci* **22**: 678-689.
- Rodriguez-Zaragoza S (1994) Ecology of free living amoebae. *Crit Rev Microbiol* **20**: 225-241.
- Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**: 846-849.
- Steinert M, Birkness K, White E, Fields B, Quinn F (1998) *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Appl Environ Microbiol* **64**: 2256-2261.
- Stothard DR, Schroeder-Diedrich JM, Awwad MH, Gast RJ, Ledee DR, Rodriguez-Zaragoza S, Dean CL, Fuerst PA, Byers TJ (1998) The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *J Eukaryot Microbiol* **45**: 45-54.
- Thomson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL-X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**: 4876-4882.
- Winiecka-Krusnell J, Linder E (2001) Bacterial infections of free-living amoebae. *Res Microbiol* **152**: 613-619.
- Yagita K, Endo T (1990) Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains in Japan. *J Protozool* **37**: 570-575.
- Yu HS, Choi KH, Kim HK, Kong HH, Chung DI (2001) Genetic analyses of *Acanthamoeba* isolates from contact lens storage cases of students in Seoul, Korea. *Korean J Parasitol* **39**: 161-170.

