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Bacterial Endosymbionts of *Acanthamoeba* sp. Isolated from Cooling Tower Water

HYUN-HEE KONG AND DONG-IL CHUNG

Department of Parasitology, Kyungpook National University School of Medicine, Taegu 700-422, Korea.
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Abstract

Intracytoplasmic presence of bacterial endosymbionts within an isolate of *Acanthamoeba* from cooling tower water (KA/W4), was confirmed by transmission electron microscopy. The isolate was closely related with the L3a strain of *A. lugdunensis* based on ribosomal RNA gene (rDNA) PCR – restriction fragment length polymorphism (RFLP), mitochondrial (mt) DNA RFLP and multilocus enzyme electrophoresis (MLEE) analyses. The endosymbionts were randomly distributed in both trophozoites and cysts, and were gram-negative, rod-shaped bacteria measuring approximately 1.3 × 0.39 μm. They were neither included in lacunae-like structure nor surrounded by phagosomal or phagolysosomal membranes.

Key words: intracytoplasmic endosymbionts; Gram negative bacillus; *Acanthamoeba* sp.; cooling tower water.

Introduction

Acanthamoeba spp., which are widely distributed among human environment (De Jonckheere, 1991), are the protozoa of medical significance. Several species of the genus *Acanthamoeba* are known to cause life-threatening granulomatous amebic encephalitis and vision-threatening keratitis (Martinez, 1987). Furthermore, some protozoa including *Acanthamoeba* have been suspected as possible carriers or vectors in the dispersion and dissemination of pathogenic microbes, *Mycobacteria* spp. (Jadin, 1975), *Legionella* spp. (Anand *et al.*, 1983; Barker *et al.*, 1992, 1993; Kwaik *et al.*, 1994), *Vibrio* spp. (Thom *et al.*, 1992), and *Listeria* spp. (Ly and Muller, 1990a, b; Harf, 1994). In addition to these well known microbes, unculturable bacterial endosymbionts have been known to dwell within cytoplasm of *Acanthamoeba* spp. (Proca-Ciobanu *et al.*, 1975; Hall and Voelz, 1985; Drozanski, 1991; Fritsche *et al.*, 1993; Gautom and Fritsche, 1995; Yagita *et al.*, 1995).

The authors have carried out ecological studies of *Acanthamoeba* and *Legionella* in hospital cooling tower water. An isolate of *Acanthamoeba* from cooling tower water showed peculiar patterns when mitochondrial (mt) DNA RFLP analysis was performed. The sum of the fragments sizes of the isolate was far larger than those of the others. The mt DNA RFLP of the isolate showed extra fragments as well as common fragments which the other strain showed. The RFLP pattern remained unchanged after repeated clonal isolations and overnight digestion with restriction endonucleases. That might indicate the possibility of presence of intracellular endosymbionts which have circular DNAs.

The present study confirmed intracellular presence of prokaryotic endosymbionts within an isolate of *Acanthamoeba* from cooling tower water by transmission electron microscopy and characterized the amoeba isolate by molecular and biochemical methods.

Correspondence: Dong-Il Chung, dichung@bh.kyungpook.ac.kr

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Materials and Methods

Isolation of Acanthamoeba *KA/W4 and Axenic culture*

Fifty fold concentrated cooling tower water was inoculated on an 1.5% agar plate which has been covered with heat-inactivated *Escherichia coli* (American Type Culture Collection # 25922, free of

plasmid) at 60°C for 1 hour. Culture on the plate, clonal isolation, and axenic culture were carried out by the same techniques as described previously (Yagita and Endo, 1990). An isolate KA/W4 which was suspecious to host endosymbionts was used in the present study.

Reference strains of Acanthamoeba

Acanthamoeba KA/W2 was isolated from the same sample of cooling tower water where KA/W4 was isolated but harboured no bacterial endosymbionts. Thirty strains of *Acanthamoeba* spp. including *A. lugdunensis* L3a (ATCC # 50240), *A. quina* Vil3 (# 50241) and *A. castellanii* Castellani (# 30011) were purchased from ATCC and used as reference strains.

Genetic characterization of Acanthamoeba *by rDNA PCR-RFLP, Mitochondrial (mt) DNA RFLP and Multilocus enzyme electrophosis (MLEE) of the* Acanthamoeba *lysates*

The sequences of primers for PCR were originated from that of SSU rRNA of *A. castellanii* Neff strain reported by Gunderson and Sogin (1986). The sequences were 5'-TTT GAA TTC GCT CCA ATA GCG TAT ATT AA-3' and 5'-TTT GAA TTC AGA AAG AGC TAT CAA TCT GT-3'. Expected size of the PCR product was estimated to be approximately 920 bp. Extraction of the genomic DNA, conditions of PCR, and digestion of the product with restriction enzymes were followed as described by Kong and Chung (1996). The restriction patterns of the isolate were compared with those of the reference strains of *Acanthamoeba* spp. Mt DNA RFLP was followed as described previously by Yagita and Endo (1990), and MLEE was done as described previously by Kong *et al.* (1995).

Transmission electron microscopy

The trophozoites suspension was centrifuged for 10 minutes at 2,000 rpm and the sediment was washed 3 times in cold phosphate buffered saline (PBS). The sediment was prefixed with 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4 for 2 hours. After rinse with 0.1 M cacodylate buffer, the sediment was postfixed with 1% osmium tetraoxide for 3 hours, rinsed twice with 0.1 M maleate buffer, pH 5.2, dehydrated with ethyl alcohol and treated with propylene oxide for 30 minutes. The pellet was immersed propylene oxide-resin (1:1) overnight with continuous shaking. The pellet was embedded in resin and incubated overnight at 60°C. Ultrathin sections cut on a Reichert-Jung ultramicrotome were stained with uranyl acetate and lead citrate. The sections were observed under a Hitachi H-7000 electron microscope.

Isolation of endosymbionts

Isolation of bacterial endosymbionts was followed as described as Gautom and Fritsche (1995). Briefly, endosymbiont-bearing amoeba isolate was grown in axenic medium in 150 cm^2 culture flask and harvested at the end of log phase growth. The amoebae suspension was washed with cold sterile PBS by low speed centrifugation (5 min at 150g). The resulting pellet was freeze-thawed. The disrupted cell slurry was resuspended in 5 ml of cold sterile PBS and filtered through a 5 μm syringe filter. The filtrate was washed with cold sterile PBS and the pellet was used for EM study or stored at –80°C for further study.

Results

The *Acanthamoeba* isolate KA/W4 belonged to morphological group II with unreticulated ectocyst and polyhedral endocyst (Fig. 1). rDNA PCR-RFLP of the isolate showed the identical pattern with *A. lugdunensis* L3a (the type strain) and KA/W2 strains but slightly different from *A. quina* Vil3 and *A. castellanii* Castellani strains (Fig. 2). The restriction fragment pattern of extranuclear circular DNA from the isolate revealed extra fragments as well as common fragments which were observed from *A. lugdunensis* L3a and KA/W2 strains (Fig. 3). MLEE performed on polyacrylamide gels showed almost identical pattern with *A. lugdunensis* L3a and KA/W2 strains (Fig. 4).

The endosymbionts were randomly distributed within the cytoplasms of the amebic trophozoites and cysts (Fig. 5A and B). Compared with mitochondria, the endosymbionts were approximately four times frequently prevalent within the trophozoites, and 1.2 times within the cysts. They were straight rod with rounded ends, and measured approximately 1.3×0.39 μm (Fig. 5C). They were

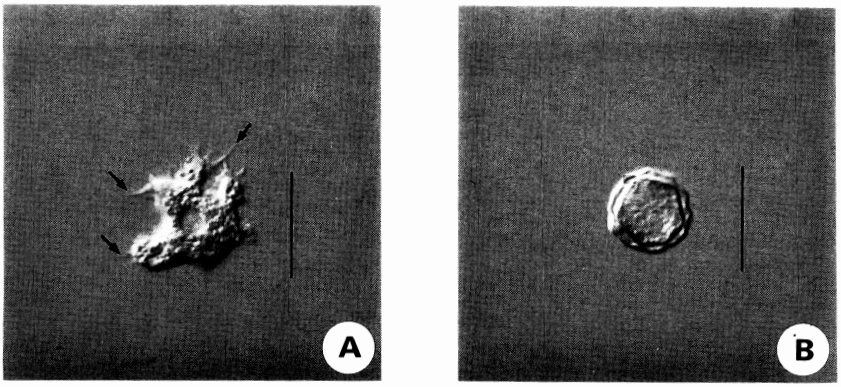

Fig. 1 Photomicrographs of *Acanthamoeba* isolate KA/W4. (A) The trophozoite showing numerous acanthopodia (arrows). (B) The cyst not reticulated. Endocyst polyhedral and ectocyst closely applied to endocyst. Arms not prominent. Bars = 20 μm.

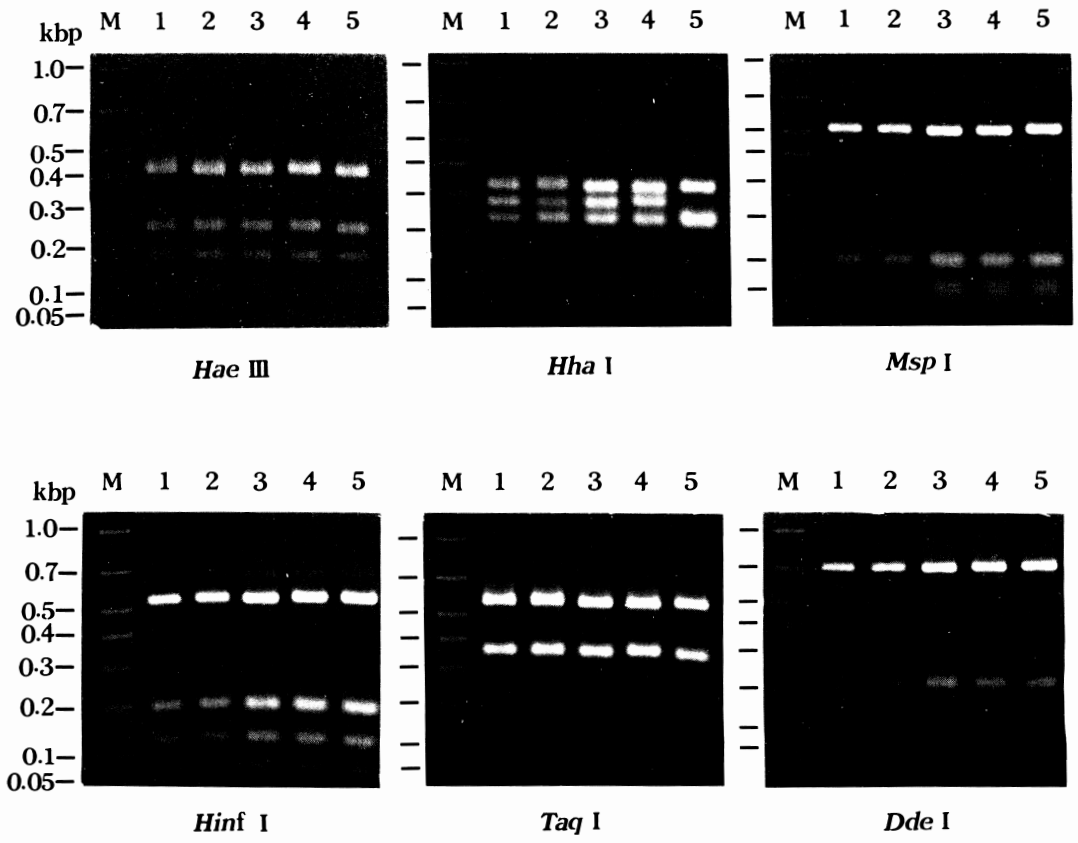

Fig. 2 Agarose gel electrophoretic restriction fragment patterns of PCR amplified ssu rDNA conserved region. Lane: 1, *Acanthamoeba* sp. KA/W4; 2, *A. lugdunensis* KA/W2; 3, *A. lugdunensis* L3a; 4, *A. quina* Vil3; 5, *A. castellanii* Castellani strain; M, DNA size standard.

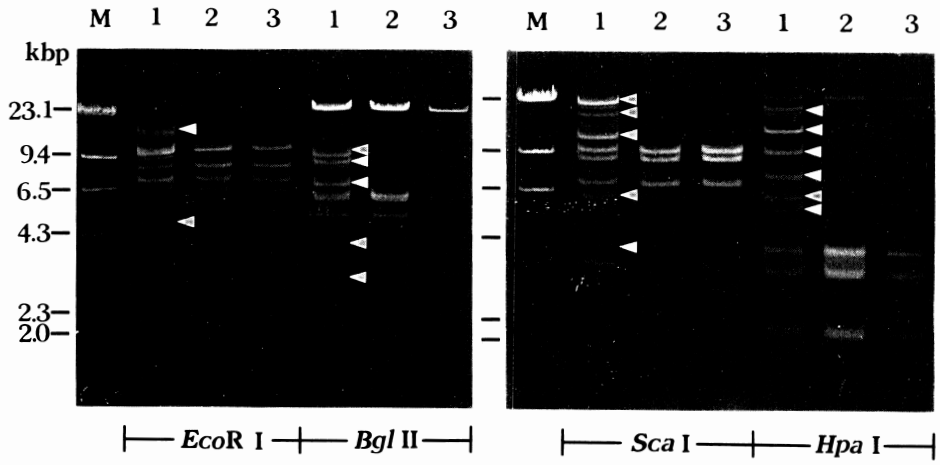

Fig. 3 Agarose gel electrophoretic restriction fragment patterns of extranuclear circular DNA from *Acanthamoeba* isolates. *Acanthamoeba* sp. KA/W4 (Lane 1) revealed extra DNA fragments (arrow heads) as well as shared ones. Lane: 1, *Acanthamoeba* sp. KA/W4; 2, *A. lugdunensis* KA/W2; 3, *A. lugdunensis* L3a; M, *Hind* III digested λ phage DNA.

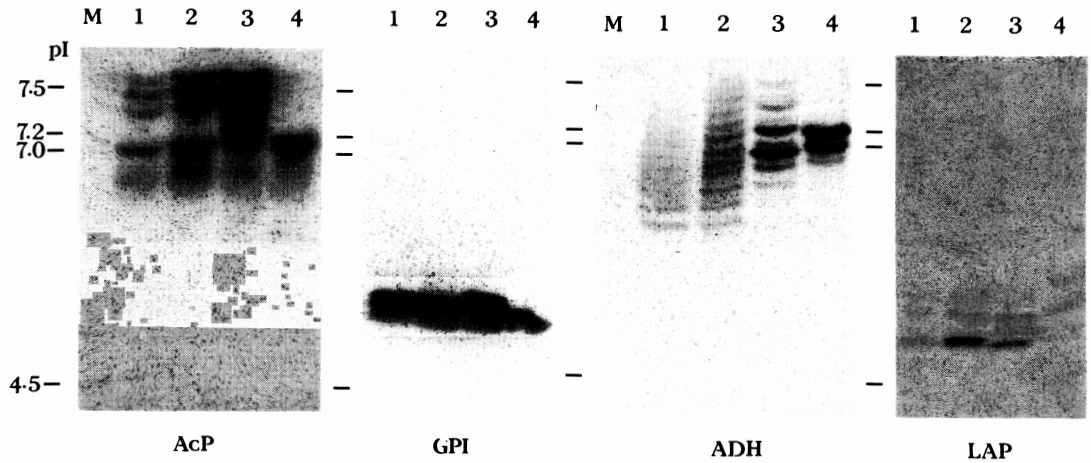

Fig. 4 Polyacrylamide gel isoelectric focusing patterns for 4 isoenzymes of *Acanthamoeba* isolates. Lane: 1, *Acanthamoeba* sp. KA/W4; 2, *A. lugdunensis* KA/W2; 3, *A. lugdunensis* L3a; 4, *A. quina* Vil3; M, IEF standard. AcP, acid phosphatase; GPI, glucose phosphate isomerase; ADH, alcohol dehydrogenase; LAP, leucine aminopeptidase.

bounded by double membranes, inner plasma membrane and outer cell wall. The cell wall of intracytoplasmic endosymbiont was seen to be studded with numerous host cell ribosomes, whereas that of freed one was studded few, if any, ribosomes (Fig. 5D). Freed endosymbionts revealed no flagella (Fig. 5D).

Discussion

This is the first record to demonstrate endosymbiotic bacteria from the Korean isolate of *Acanthamoeba*. Based on rDNA PCR-RFLP, mt DNA RFLP and MLEE analyses, the isolate studied was closely related to the L3a strain of A. *lugdunensis*

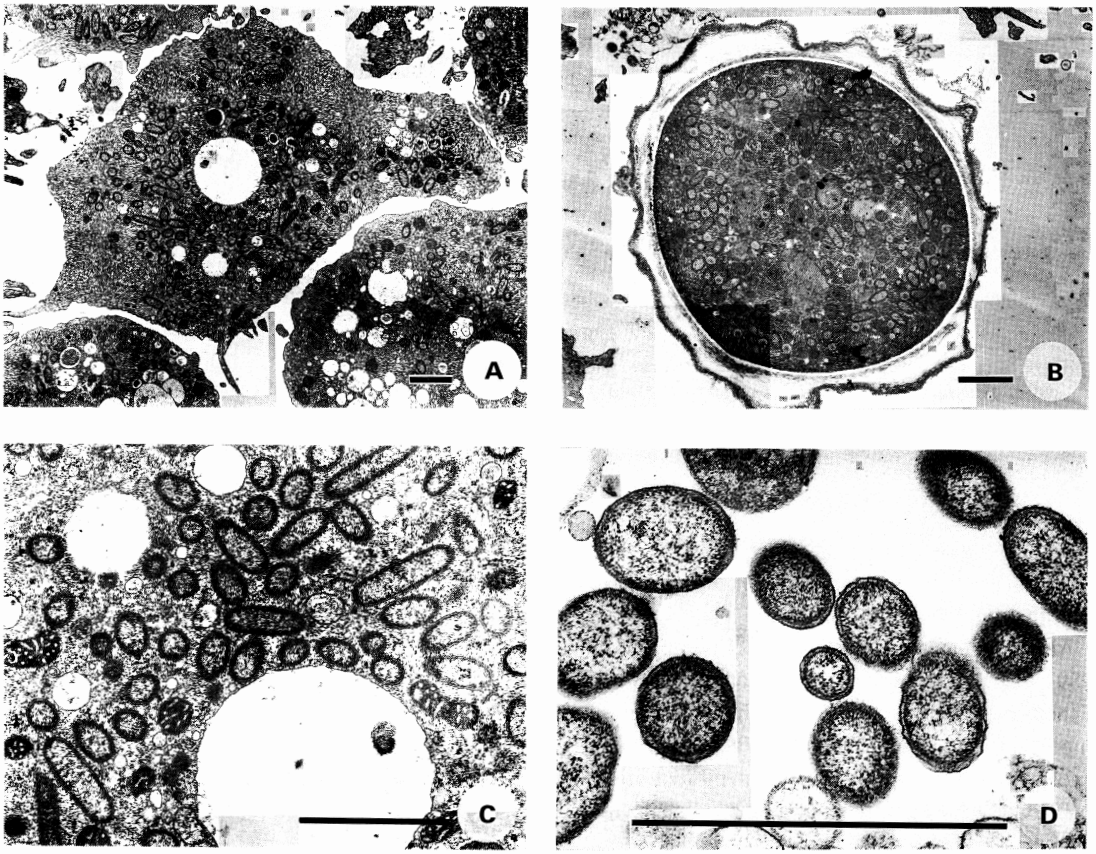

Fig. 5 Electron micrographs of *Acanthamoeba* sp. KA/W4 strain. (A) Trophozoite harbours numerous rod-shaped endosymbionts in its cytoplasm. (B) Cyst. (C) Bacterial endosymbionts closely associated with host ribosomes. (D) Freed endosymbionts. Bars = 2 μm.

isolated from a swimming pool of France. The L3a strain is known to be virulent, killing more than 50% of mice when inoculated intracranially (De Jonckheere, 1980). Pathogenicity and *in vitro* cytopathic effects of the isolate have yet to be assessed. Mt DNA RFLP type of this isolate was the most frequently occurring one (KA/L1 type) of the *Acanthamoeba* from contact lens paraphernalia of Korean lens wearers (unpublished data). One (KA/E2) of four isolates from Korean keratitis patients diagnosed in 1996 showed the identical rDNA PCR-RFLP, mt DNA RFLP and MLEE patterns with the isolates analyzed in the present study.

In comparison with the endosymbionts previously reported (Table 1), the endosymbionts are morphologically similar to those reported by Yagita *et al.* (1995). However, abundance of the present endosymbiont (33–70 per section) was far greater than that (8–10 per section) reported by Yagita *et al.* (1995). Except for *Sarcobium lyticum* (Drozanski, 1991) which clustered in lacunae, all the endosymbionts parasitizing *Acanthamoeba* sp., including the present one, are known to be randomly distributed at extravacuolar location. *S. lyticum* is also unique for its lytic effect on host cell.

Yagita *et al.* (1995) reported Southern hybridization using cytochrome b gene as the probe can differentiate mt DNA from the other circular DNA of endosymbiontal origin. In the present study, a simpler method, restriction enzyme digestion of circular DNAs extracted from the amoeba isolate, could also inform the presence of the other circular

Table 1 Comparison of unculturable endosymbionts from *Acanthamoeba* spp

		Present study (1996)	Yagita *et al.* (1995)	Drozanski (1991)	Hall & Voelz (1985)	Proca-Ciobanu *et al.* (1975)
Host	species	*Acanthamoeba* sp.	*Acanthamoeba* sp.	*A. castellanii*	*Acanthamoeba* sp.	*A. castellanii*
	strain	KA/W4	PAC/C13	nd	HN-3	Snagov
	origin	CTW†, Korea	pond, the Philippines	nd	human nasal swab	lake, Romania
	CPE*	nd‡	nd	nd	–	+
Endosymbionts	species	unknown	unknown	*Sarcobium lyticum*	unknown	unknown
	location	extravacuolar	extravacuolar	vacuole/lacuna	extravacuolar	extravacuolar
	distribution	random	random	clustered	random	random
	host ribosomal stud	+/–	+	–	–	–
	shape	Gram (–) rod	Gram (–) rod	Gram (–) rod	Gram (–) rod	rod
	size	1.3 × 0.39 μm	1.3 × 0.43 μm	1.9 × 0.6 μm	0.7~3.3 × 0.22~0.33 μm	0.7~1.0 × 0.4~0.5 μm
	lysis of host cell	–	–	+	–	–

*CPE: cytopathic effect
†CTW: cooling tower water
‡nd: not defined

DNA than mt DNA.

According to the previous report (Jung *et al.*, 1989), *Legionella* spp. had been isolated from water of the same cooling tower, from which the present amoebic isolate was obtained. We tried several times to confirm whether the endosymbiont might be *Legionella* with PCR using *Legionella* specific primers, but failed (data not shown).

Multiple attempts to culture the endosymbionts in amoeba-free media failed. Genetic characterization of the endosymbiont is a subject of future study.

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