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***Protochlamydia* belonging to environmental chlamydiae can survive in
acanthamoebae following encystation**

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Running title: Survival of environmental chlamydiae in amoebal cysts

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Summary

Whether endosymbionts can survive amoebal encystations remains a significant challenge in cellular biology. The survival of the endosymbiotic bacteria *Protochlamydia* belonging to environmental chlamydiae in acanthamoebae following encystation was therefore assessed. The bacteria were observed in cysts and the bacterial transcripts (*16S rRNA* and/or *groEL*) were also detected in amoeba cultures following encystation. Furthermore, the bacterial replication and the growth was confirmed in trophozoites reverted from cysts. Thus, these results demonstrated that *Protochlamydia* could survive in acanthamoebae following encystation. Our findings suggest that amoeba cysts might be further studied in order to elucidate their role in the environmental survival of endosymbionts.

Introduction

The family *Parachlamydiaceae*, consisting of *Parachlamydia acanthamoebae*, *Neochlamydia hartmanellae* and *Protochlamydia amoebophila*, has recently been recognized as environmental chlamydiae that show a wide distribution range in the natural environment, such as in rivers and soil (Everett *et al.*, 1999; Horn *et al.*, 2000; Bush *et al.*, 2001; Corsaro *et al.*, 2006; Casson *et al.*, 2008). Meanwhile, members of the family *Chlamydiaceae*, which includes *Chlamydia trachomatis* and *Chlamydophila pneumoniae*, are well known as human pathogens and are the major cause of preventable blindness, sexually transmitted diseases, and respiratory diseases (Grayston 1989; Grayston 1996; Grayston *et al.*, 1989; Hahn *et al.*, 1991; Kauppinen and Saikku 1995; Norton *et al.*, 1995; Kalman *et al.*, 1999). There is accumulating evidence, however, supporting the pathogenic role of *Parachlamydiaceae* in humans and this bacterial group is thought to be an etiological agent of lower respiratory tract infections in humans (Birtles *et al.*, 1997; Lieberman *et al.*, 1997; Corsaro *et al.*, 2006; Casson *et al.*, 2008). *P. acanthamoebae*, which was originally isolated from an aborted bovine fetus, is also considered as a potential abortigenic agent (Birtles *et al.*, 1997; Lieberman *et al.*, 1997; Corsaro *et al.*, 2006; Casson *et al.*, 2008; Ruhl *et al.*, 2008; Greub 2009). Although ancient chlamydiae diverged into pathogenic and environmental chlamydiae 700 million years ago, environmental chlamydiae have evolved as endosymbionts of lower eukaryotes, and have acquired the ability to survive in and to persistently infect free-living amoebae, which are predators of environmental bacteria exclusively (Horn *et al.*, 2004). Consequently, 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 *Legionella pneumophila* (Hall and Voelz 1985; Horn *et al.*, 1999; Fritsche *et al.*, 2000; Horn and Wagner 2004; Jeon 2004; Heinz *et al.*, 2007), but whether endosymbionts can survive amoebal encystation, which occurs universally in natural environments, remains a significant challenge in cellular biology. Thus, we performed experiments using an *Acanthamoeba* strain and an endosymbiotic bacterium, *Protochlamydia*, belonging to the environmental chlamydiae to demonstrate the survival of endosymbiotic bacteria following encystation.

Results and discussion

Experimental design for bacterial detection from amoeba trophozoites and cysts

Environmental *Acanthamoeba* strain (genotype T4), persistently harboring *Protochlamydia* sp. and isolated from a river of Sapporo city, Japan, was used for this study. The genotype of the amoeba and the species of the endosymbiotic bacteria were determined by phylogenetic analysis of amoeba 18S rRNA sequence (Gene accession number: AB508964) and bacterial 16S rRNA sequence [Gene accession number: AB506679 (99.2% identities against *P. amoebophila* UWE25 16S rRNA sequence)]. Amoebae harboring bacteria were maintained in broth including 0.75% peptone, 0.75% yeast extract and 1.5% glucose (PYG) at 30°C according to methods described previously (Matsuo *et al.*, 2008). The infected amoebae grew normally and compared with *A.*

castellanii (standard strain purchased from ATCC) no difference in growth rates was seen (data not shown). The prevalence of infected amoeba, defined by DAPI staining, was always approximately 100%. Lytic amoeba and free bacteria in cultures were few during the maintenance or experimental culture periods, indicating that a stable interaction between bacteria and amoebae occurred (data not shown). Encystation was simply performed by putting amoebae on a non-nutrient agar plate for 3 days. The remaining trophozoites were removed by the treatment with 0.5% SDS, according to the conditions described previously (Lorenzo-Morales *et al.*, 2008). Amoebal cysts were then induced back to trophozoites by culturing in PYG broth for 5 days. The numbers of trophozoites and cysts in cultures were assessed using the trypan blue exclusion method and morphological analysis under a phase-contrast microscope. The percentage of amoebae changing to cysts was approximately 100% and neither trophozoite nor immature precysts, which lacked the outer layer and were defined as a not spore-like form (Lorenzo-Morales *et al.*, 2008), in suspension were found after the SDS treatment showing that the contamination of trophozoites in the cyst preparation was negligible (Fig. 1). Amoebae cultures were collected at several time points for the assessment of bacterial ultrastructures within amoebae by transmission electron microscopy (TEM) and for the detection of bacterial transcripts by RT-PCR. The increase of infectious progeny in amoebae reverted from cysts was also assessed according to the method described previously (Matsuo *et al.*, 2008).

Morphology of bacteria in cysts and in trophozoites reverted from cysts

After encystation, most trophozoites changed to cysts with double-thick cell wall structures composed of outer (See single star in Fig. 2A) and inner layers (See double stars in Fig. 2A), which are a typical feature of cyst formation (Bowers and Korn 1969). The cyst retained the spore-like infectious elementary body (EB) (however, most EBs in the cysts changed to crescent bodies which are a morphological trait in of environmental chlamydiae) (Greub *et al.*, 2002; Greub *et al.*, 2003; Greub *et al.*, 2005), but no dividing intracellular reticulate body (RB) was observed in the cysts. Interestingly, the EBs in the cysts appeared to lack being surrounded by inclusion membranes (Fig. 2B). Although the exact reason for this lacking remains unknown, the finding suggest that the interactions between bacteria and amoebae are stably maintained, possibly for the acquirement of energy.

The morphological images showing trophozoites reverted from cysts are also of interest. The morphological traits of bacteria, including EBs (but not crescent bodies), RBs and dividing bacteria (See white arrows in Fig. 2D) were observed in the cytoplasm of trophozoites reverted from cysts (Fig. 2C and D) as well as in trophozoites before encystation (data not shown). The reason why few crescent bodies were seen in the cytoplasm of trophozoites reverted from cysts remains unknown; however the morphological change to crescent bodies may be specific for *Protochlamydia* in amoeba cysts. It has been reported that crescent bodies of *Planctomycetes* (but not environmental chlamydiae) can be an effect of the fixation procedure for electron microscopy (Lindsay *et al.*, 1995). However, because no crescent bodies was observed in the trophozoites fixed with the same method as the cysts, the effect on fixation may differ among bacterial

phylum and it is possible that the effect on environmental chlamydiae is minimal. Curiously, typical inclusion membranes were not seen around the bacteria observed in the cytoplasm of trophozoites reverted from cysts; however it has been reported that environmental chlamydia could replicate and survive in the inclusion membrane of amoeba trophozoites (Horn *et al.*, 2000; Greub and Raoult 2002; Greub *et al.*, 2003; Greub *et al.*, 2005; Casson *et al.*, 2008). The reason of this difference remains unknown, but it is possible that the natural infectious mode of endosymbiont bacteria during symbiosis with amoebae may differ from that of bacteria coercively internalized in amoebae by experimental infection. Moreover, it is noteworthy that miniature bodies (small dots with high electron density, sized approximately 10 to 50 nm), which are considered characteristic of pathogenic chlamydial (*Chlamydophila pneumoniae*) EBs (Wolf *et al.*, 2000), were observed in bacteria within the trophozoites reverted from cysts (Fig. 2D, white arrowhead). Since miniature bodies in dividing bacteria were rare, it appears that the presence of miniature bodies in bacteria may be a trait of mature *Protochlamydia*.

Assessment of bacterial transcripts and bacterial numbers in trophozoites, cysts, or trophozoites reverted from cysts

The transcripts of *Protochlamydia* 16S rRNA were detected following encystation, while the transcripts of bacterial *groEL*, which is a major gene in the stress response of common bacteria (Langer *et al.*, 1992), were weakly detected only in SDS-treated amoebae after encystations (Fig. 3A). These results imply that *Protochlamydia* in amoebae may not be

stressed and therefore not synthesizing GroEL, in contrast to that of other intracellular bacteria representative to *L. pneumophila* (Moffat and Tompkins 1992; Cirillo *et al.*, 1994; Gao and Kwaik 2000), possibly suggesting that the endosymbiont *Protochlamydia* sp. intimately adapts to acanthamoebae. As expected, the increase of infectious progeny in amoebae reverted from cysts was also confirmed (Fig. 3B). Several studies have shown that, in contrast with trophozoites, cysts possess a high-powered bacterial degradation pathway that depends on hydrolysis or free radicals (Rosenthal *et al.*, 1969; Aksozek *et al.*, 2002; Hughes *et al.*, 2003); therefore, it is believed that intracellular bacteria, except for *L. pneumophila* which can replicate in the cytoplasm of trophozoites or cysts (Kilvington and Price 1990), could not grow nor survive in the cytoplasm of amoeba cysts. However, our results clearly indicate that *Protochlamydia* could survive in amoeba cysts. Although we examined whether *P. acanthamoebae*, which is also representative of environmental chlamydia, could survive in the cysts as well as *Protochlamydia*, we could not confirm the survival of *P. acanthamoebae* following encystation because of unsuccessful encystation of amoebae infected with *P. acanthamoebae* (data not shown). Thus, it is possible that the survival of *Protochlamydia* in cysts may be a special event, even in environmental chlamydiae.

In conclusion, although the mechanism remains unknown, we demonstrated that *Protochlamydia* could survive in acanthamoebae following encystation. Amoeba harboring environmental chlamydiae may be a very valuable model for understanding unknown endosymbiotic interactions and for exploring the novel concept of intracellular bacteria that can escape bacterial elimination systems in cells. Furthermore, our findings

suggest that amoeba cysts might be further studied in order to elucidate the their role in the environmental survival of endosymbionts.

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Legends to figures

Fig. 1. Number of amoeba trophozoites and cysts in culture before and after encystation, with or without SDS treatment. See the experimental design in the text. In brief, encystation was performed by putting amoebae on a non-nutrient agar plate. The remaining trophozoites were removed by SDS treatment. Amoebal cysts were induced back to trophozoites by culturing in PYG broth. The number of amoebae was assessed by the trypan blue exclusion method and morphological analysis using phase-contrast microscopy. Statistical analysis was performed with the unpaired Student *t*-test. A *p* value of less than 0.05 was considered significant. * and **, $p < 0.05$ vs. normal cultures.

Fig. 2. TEM images of *Protochlamydia* in amoeba cysts after SDS treatment (A and B) and in amoeba trophozoites reverted from cysts (5 days after reversion) (C and D). The squares in images (A) and (C) are enlarged in images (B) and (D), respectively. TEM was performed according to previously described methods (Matsuo *et al.*, 2008). In brief, amoebae trophozoites or cysts harboring bacteria were immersed in fixative containing 3% glutaraldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, at 4°C for 24 h, and then post-fixed in 1 % osmium tetroxide in 0.1 M PBS for 2 h. After a brief wash with PBS, they were processed for alcohol dehydration and embedding in Epon 813. After staining with lead citrate and uranium acetate, ultrathin sections of amoebae were prepared and viewed by TEM. Black arrows show representative crescent bodies. White arrows show dividing bacteria. Scale bars show scale of enlargement in each image.

White arrowhead in the image shows representative miniature bodies. E, EB. R, RB. *, outer layer of cyst. **, inner layer of cyst. mt, mitochondria.

Fig. 3. Detection of *Protochlamydia* transcripts in trophozoites, cysts, trophozoites reverted from cysts (A) and number of bacterial progenies in trophozoites reverted from cysts (B). (A) RT-PCR was performed as below. Amoebae (1×10^5) were harvested by centrifugation at $1,600 \times g$ for 30 min, and the pellets were then used for RNA extraction. Total RNA extraction was performed using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The extracted total RNA was treated with DNase (Ambion, Austin, TX, USA). Absence of DNA contamination in the resulting RNA preparations was confirmed 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, USA). The PCR conditions for amplification of the genes specific to *Acanthamoeba 18S rRNA*, *Chlamydiales 16S rRNA* and bacterial *groEL* were as described previously (Everett *et al.*, 1999; Schroeder *et al.*, 2001; Teng *et al.*, 2001). The primers used were as follows: *Acanthamoeba 18S rRNA*: 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), *Chlamydiales 16S rRNA*: sense, 5'–CGG CGT GGA TGA GGC AT–3'; antisense, 5'–TCA GTC CCA GTG TTG GC–3' (Everett *et al.*, 1999), bacterial *groEL*: sense, 5'-GGN GAC GGN ACN ACN ACN GCA

ACN GT-3'; antisense, 5'-TCN CCR AAN CCN GGY GCN TTN ACN GC-3' (Teng *et al.*, 2001). The transcripts were detected by RT-PCR as described in the text. *, 5-day culture after reversion. N, negative control. P, positive control. (B) Number of infectious progenies in amoebae reverted from cysts. The number of infectious progenies of amoebae infected with *Protochlamydia* was determined by the AIU assay described previously (Matsuo *et al.*, 2008). *, $p < 0.05$ vs. immediately after reversion from cysts (0 day).

Fig. 1

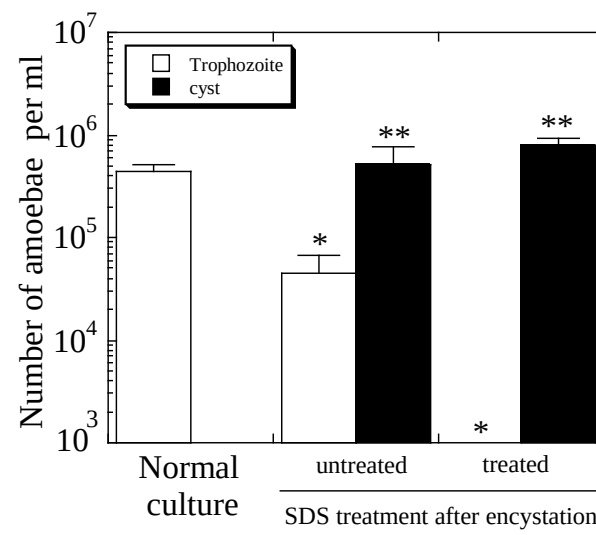


Fig. 2

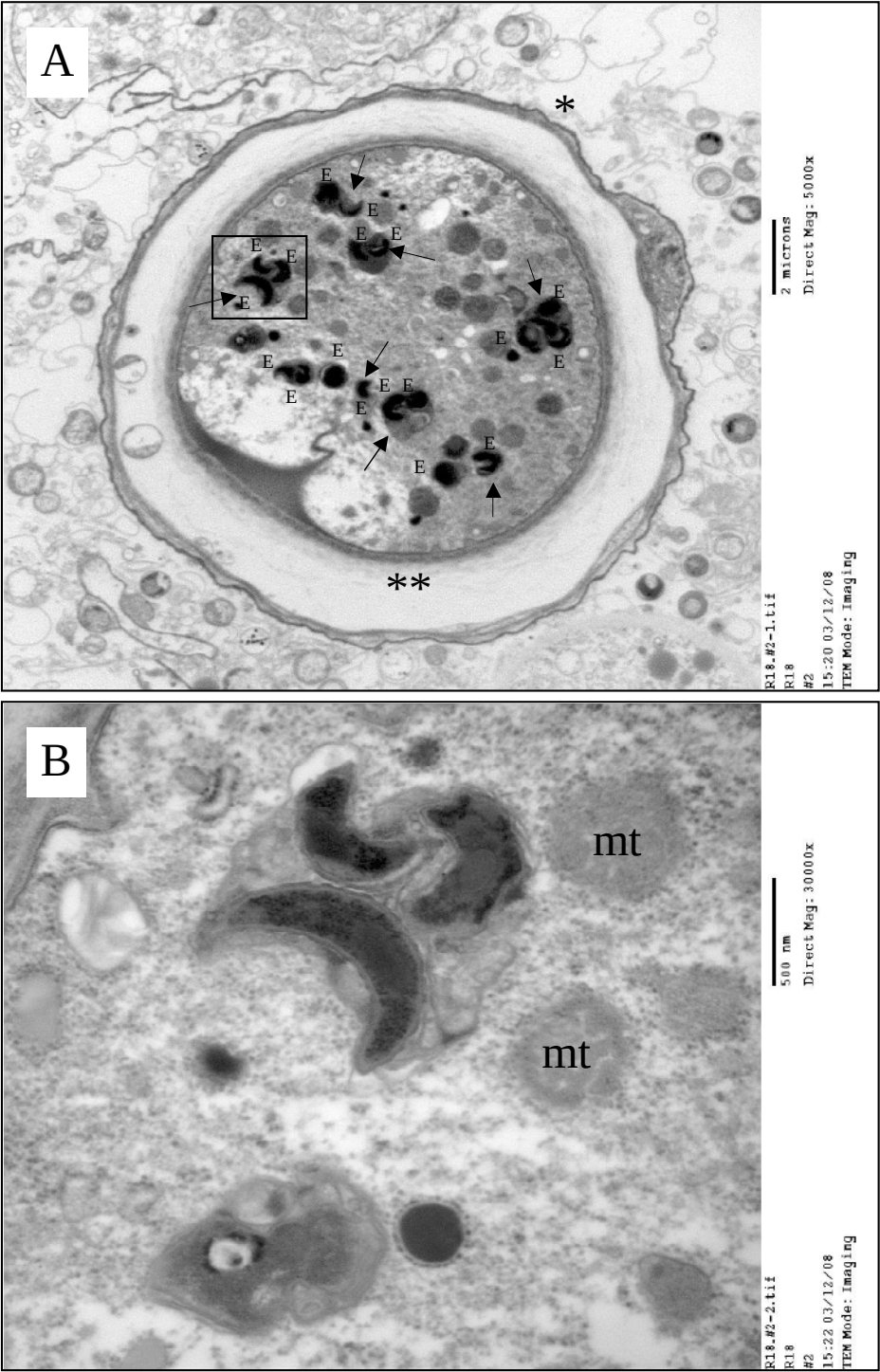


Fig. 2 continued

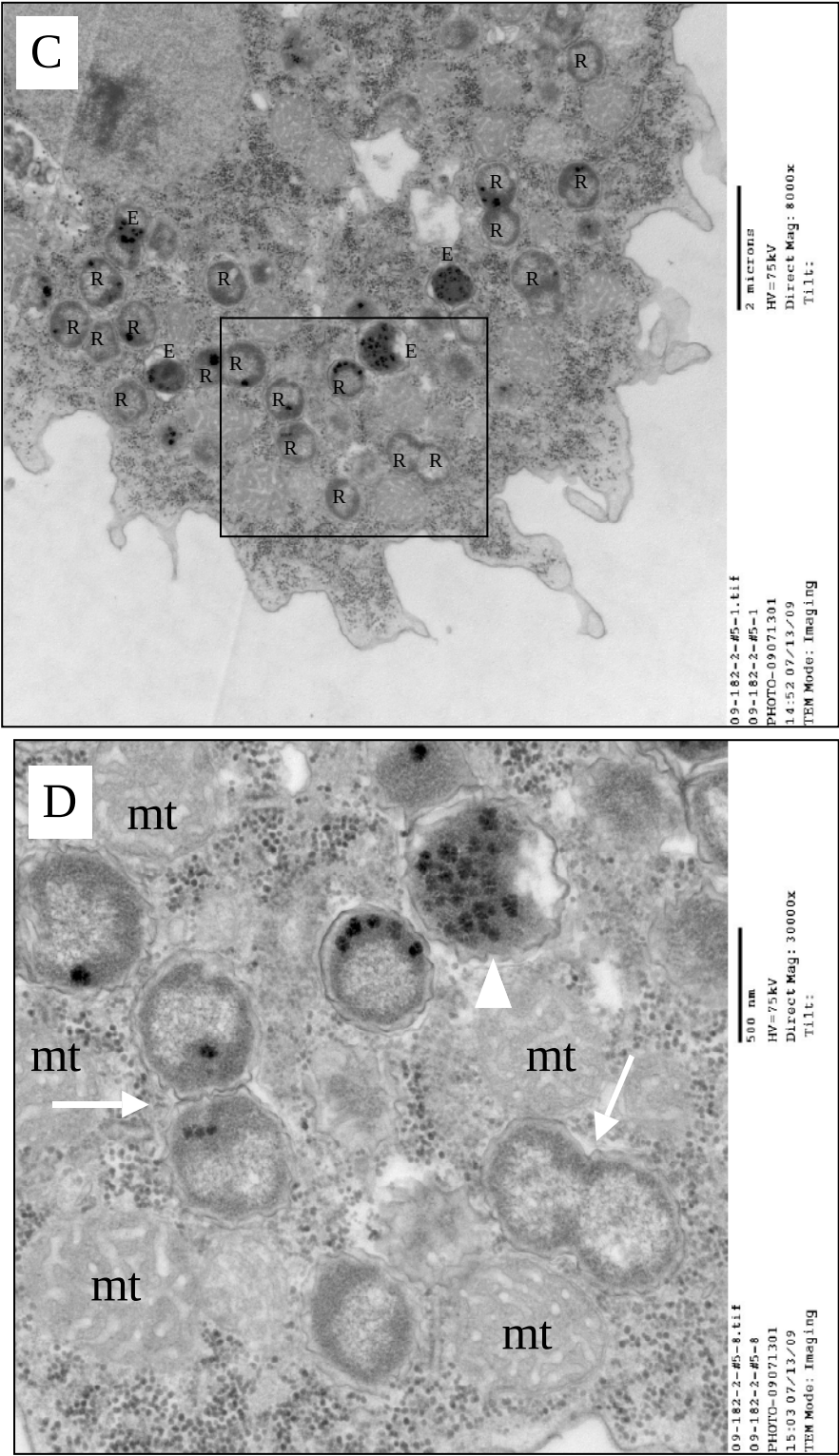


Fig. 3

