*SYMBIOSIS* (2007) 44, 153–158  ISSN 0334-5114

# *Vibrio cholerae-Acanthamoeba castellanii* interaction showing endosymbiont-host relation

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*(Received October 20, 2006; Accepted April 10, 2007)*

**Abstract**

The causative agents of cholera *Vibrio cholerae* O1 and O139 species are extracellular and waterborne bacteria, which lose to predators in aquatic environment. Recently, it was shown that *V. cholerae* survived intracellularly during interaction with the free-living amoeba *Acanthamoeba castellanii*. The aim of this study is to examine symbiosis between these microorganisms. The interaction between *V. cholerae* O1 classical, *V. cholerae* O1 El Tor, and *V. cholerae* O139 and *A. castellanii*, respectively, was studied by viable counts of singly and co-cultivated microorganisms using gentamicin assay, microscopy and statistical analysis. Symbiosis between *A. castellanii* and *V. cholerae* was studied by recultivation of *A. castellanii* hosting intracellular *V. cholerae* as endosymbionts. The results showed an enhanced growth and survival of co-cultivated *V. cholerae* species in comparison to singly cultivated bacteria. *V. cholerae* grew in the cytoplasm of *A. castellanii* trophozoites and bacteria were also found in cysts. Intracellular growth and survival of the bacteria did not inhibit growth of *A. castellanii*. Growth and survival of both amoebae and bacteria in recultivation of *A. castellanii* harbouring intracellular *V. cholerae* indicate endosymbionts-host relation between these microorganisms.

**Keywords**: *V. cholerae*, *Acanthamoeba*, symbiosis

## 1. Introduction

*Vibrio* is a genus of gram-negative bacteria including human pathogenic species such as *Vibrio cholerae, V. parahaemolyticus*, *V. vulnificus* (Ruby et al., 2005) and non-pathogenic species as *V. fischeri* (Ruby, 1996). *Vibrio cholerae* species O1 and O139 produce cholera toxin and cause the diarrheal disease cholera in humans (Faruque et al., 2005).

Cholera outbreaks are associated with contaminated food and water and the disease affects many million persons annually (Tauxe et al., 1995). The ecological life cycle of *V. cholerae* is characterised by biocomplexity (Mervis et al., 1998) since *V. cholerae* can be found on abiotic surfaces in biofilms (Watnick et al., 2001). Brown and Barker (1999) have found a complex interaction between bacteria and eukaryotic cells in biofilms. In nature, the free-living amoebae feed on bacteria as a food source and serve as reservoirs or vectors for pathogenic bacteria, while other amoebae live in a symbiotic relationship with other microorganisms. Many free-living amoebae serve as predators and control bacterial populations (Cabral, 2004). Mitchell and Yankofsky (1969) reported that the decline in *Escherichia coli* in water was linked with an increase in abundance of the amoeba, *Vexillifera telmathalassa*. In this context, *E. coli* as well as *Klebsiella aerogenes* have been excellent food to *A. castellanii* and *A. polyphaga* (Weekers et al., 1993). In contrast, Qureshi et al. (1993) has reported that *P. aeruginosa* is able to secret inhibitors of unknown nature for growth of *Acanthamoeba* species. Moreover, *P. aeruginosa* killed *Dictyostelium discoideum* by affect of ExoU excreted by type III secretion system (TTSS) (Pukatzki et al., 2002). Recently, it has been shown that TTSS effector proteins ExoS, ExoT, ExoU and ExoY killed *A. castellnii* by inducing apoptosis and necrosis (manuscript in preparation).

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*Presented at the 5th International Symbiosis Society Congress, August 4–10, 2006, Vienna, Austria*

Reidl and Klose (2002) suggested a symbiotic relationship between *V. cholerae* and zooplankton, which may enhance its survival in aquatic habitats. Moreover, it has been shown that there is an association between *V. cholerae* and algae (Islam et al., 1988) and fresh water amoebae enhance initial growth of *V. cholerae* when co-cultivated (Thom et al., 1992). Recently, it has been shown that *V. cholerae* O139 grew in *A. castellanii* (Abd et al., 2005) and that growth of *V. cholerae* O1 inside *A. castellanii* was dependent on the growth of amoebae, since the amoebae could work as a biological incubator supporting intracellular growth and maintaining the overall viability of *V. cholerae* (Abd et al., 2007).

*V. cholerae* requires a high infection dose to cause cholera (Sack et al., 2004) and it may need a biological host able to enhance its growth in water. *V. cholerae* as well as *Acanthamoeba* species are found in the aquatic environments and in drinking water (Backer, 2002; Greub and Raoult, 2004).

*Acanthamoebae* have shown an increased role as host to *Legionella penumophila* (Zusman et al., 2004). Co-cultivation of *Acanthamoeba* enhances growth and survival of many pathogenic bacteria such as *Campylobacter jejuni* (Axelsson-Olsson et al., 2005), *Fransicella tularensis* (Abd et al., 2003), *Helicobacter pylori* (Winiecka-Krusnell et al., 2002) and *Salmonella typhimurium* (Gaze et al., 2003).

Interaction between prokaryotes and eukaryotes may provide a better understanding of their coevolution (Hilbi et al., 2007). It was found that the non-pathogenic species *V. fischeri* is a symbiont in certain squids and fishes (Ruby, 1996). A recent study shows that the genome sequence of *V. fischeri* ES114 revealed parallels with *V. cholerae* (Ruby et al., 2005).

The aim of this study is to examine if there is a symbiotic interaction between *V. cholerae* and *A. castellanii*, which may enhance growth and survival of the bacteria in aquatic environment during cholera epidemics.

## 2. Materials and Methods

*Acanthamoeba castellanii* (ATCC 30010) was obtained from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, USA. *V. cholerae* O1 classical- Ogawa strain 395, *V. cholerae* O1 El Tor-Inaba strain N16961 and *V. cholerae* O139 were from the collection of Department of Laboratory Medicine, Division of Clinical Microbiology F 82, Karolinska University Hospital, Huddinge, SE-141 86, Stockholm, Sweden.

The plasmid (pGFPuv) carrying GFPuv gene and confers resistance to ampicillin (100 μg $ml^{-1}$), was obtained from BD Biosciences Clontech, USA, and introduced by electroporation into *V. cholerae* O1 classical strain C-19385 to detect viability of intracellular bacteria by fluorescence microscopy as an additional method to the viable count by gentamicin assay.

Each bacterial strain was cultivated with *A. castellanii* and incubated in 75 $cm^2$ cell culture flasks filled with 50 ml ATCC medium 712 containing an initial concentration of $10^5$ cells $ml^{-1}$ of *A. castellanii* and $10^6$ CFU $ml^{-1}$ of each bacterial strain. Control flasks of bacteria and amoebae were prepared in the same way and with the same initial concentration of microorganisms. The flasks were incubated statically at 30°C for 2 weeks. Samples were withdrawn at 0, 1, 4 and 14 days for microscopy and viable counts of microorganisms in control, co-culture flasks and intracellular bacteria, as it was previously described (Abd et al., 2005).

Briefly, viable counts of bacteria in absence or presence of amoebae were prepared by ten-fold dilution and spread on blood agar plats, which incubated at 37°C for 24 h to count numbers of colonies. Gentamicin assay was used to kill extracellular bacteria in samples from co-cultivated flasks and viable counts of intracellular bacteria were performed as described above. Samples from *Acanthamoeba* in absence or presence of each bacterial species were diluted two-fold with 0.5% basic eosin solution and live cells, which did not take the stain, were counted in

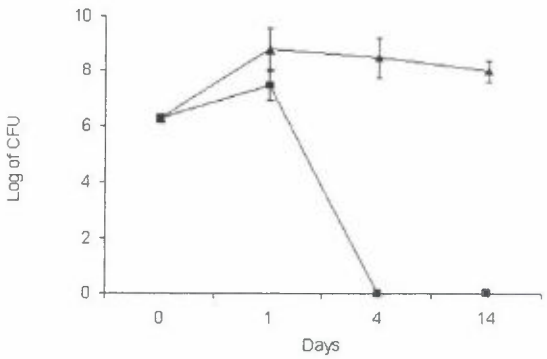


Figure 1. Counts of viable *V. cholerae*. Co-cultivated bacteria (▲) and singly cultivated bacteria (■). Data indicate mean values ± SD of *V. cholerae* O1 classical, El Tor, and O139.

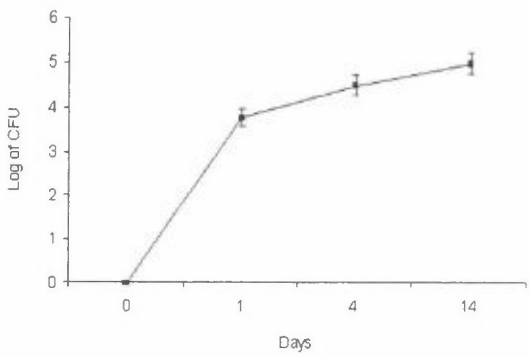


Figure 2. Counts of intracellular *V. cholerae*. Data indicate mean values ± SD of *V. cholerae* O1 classical, El Tor, and O139.

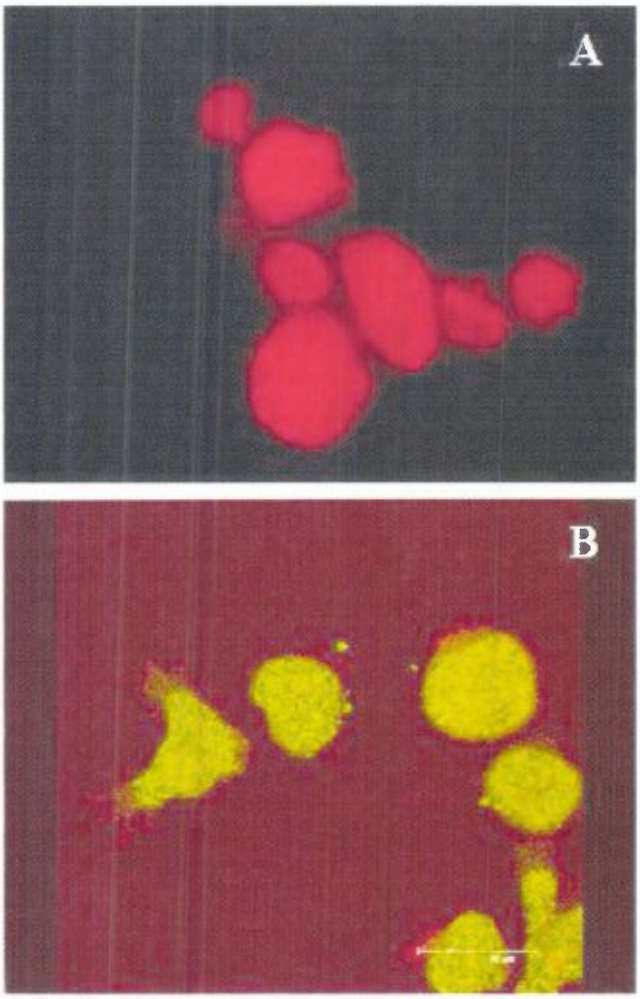


Figure 3. Fluorescence microscopy analysis. A. Amoebae cells emitting red autofluorescence as negative control. B. Showing intracellular localisation of *V. cholerae* O1 Classical GFP emitting green fluorescence inside *A. castellanii* trophozoites and cysts at day 3 of co-cultivation.

Bürker chamber. Moreover, 5 ml samples of *Acanthamoeba* in presence of each bacterial species were prepared for electron microscopy as previously described (Abd et al., 2005).

*V. cholerae* O1 GFP classical strain C-19385 was cultivated with *A. castellanii* in the ATCC medium 712 containing 100 μg $ml^{-1}$ ampicillin to visualise intracellularly located bacteria by fluorescence microscopy.

Student's *t*-test was used to examine for significant differences in growth of alone- and co-cultivated microorganisms.

## 3. Results

Interaction between different *V. cholerae* species and *A. castellanii* was studied by different methods. The analysis showed that co-cultivated *V. cholerae* strains grew hundred folds after one day and survived two weeks in comparision to singly cultivated bacteria (Fig. 1). Student's *t*-test showed that presence of the amoebae enhanced growth and survival of the bacteria ($p<0.001$).

Gentamicin assay confirmed intracellular growth of *V. cholerae* since the number of intracellularly grown bacteria was $10^3$ and $10^5$ CFU $ml^{-1}$ on days one and 14, respectively (Fig. 2).

Intracellular localisation of *V. cholerae* was studied by microscopy, samples from co-cultures containing *A. castellanii* and *V. cholerae* O1 GFP were washed, mounted and examined under fluorescence and electron microscopy.

Fluorescence microscopic analysis showed an intracellular localisation of *V. cholerae* O1 inside *Acanthamoeba* cells. Viability of intracellular bacteria was confirmed by emission of green fluorescence, which was detected by fluorescence microscopy (Fig. 3). The electron microscopic pictures confirmed that *V. cholerae* was localised in vacuoles of trophozoites and multiplication occurred in the cytoplasm of trophozoites (Fig. 4). The bacteria were also found in cysts of *A. castellanii* (Fig. 4).

The symbiotic relationship between intracellularly growing *V. cholerae* and the host cell *A. castellanii* was examined by recultivation of $4\times10^5$ cell $ml^{-1}$ *A. castellanii* hosting $2\times10^5$ cell $ml^{-1}$ *V. cholerae* in 50 ml ATCC medium 712 for 2 weeks. The result showed an enhanced growth of both microorganisms. Viable *A. castellanii* number increased from $4\times10^5$ cell $ml^{-1}$ on day 0 to $2.3\times10^6$ cell $ml^{-1}$ on day 14 and the viable count of *V. cholerae* increased from $2\times10^5$ cell $ml^{-1}$ on day 0 to $2.5\times10^9$ and to $3\times10^{10}$ cell $ml^{-1}$ on days 1 and 4 and bacteria survived during 14 days at a concentration of $3\times10^8$ cell $ml^{-1}$ (Fig. 5).

## 4. Discussion

Microorganisms are found in aquatic environments and in biofilms, where a complex interaction between bacteria and eukaryotic cells occur. *V. cholerae* interacts symbiotically with zooplankton (Reidl and Klose, 2002) and associates with algae (Islam et al., 1988). Ability of *V. cholerae* O139 to grow inside *A. castellanii* has been proven (Abd et al., 2005) and growth of *V. cholerae* O1 inside *A. castellanii* has been found to be dependent on the growth of amoebae, since the amoeba acts as a biological incubator supporting intracellular growth and maintaining overall viability of *V. cholerae* (Abd et al., 2007).

The present study examined if the interaction between *V. cholerae* and *A. castellanii* showed endosymbiont-host relation. The results showed enhanced growth and survival of co-cultivated *V. cholerae* species, which did not inhibit growth of the amoebae in co-cultivation. Growth of *V. cholerae* inside *A. castellanii* trophozoites did not affect

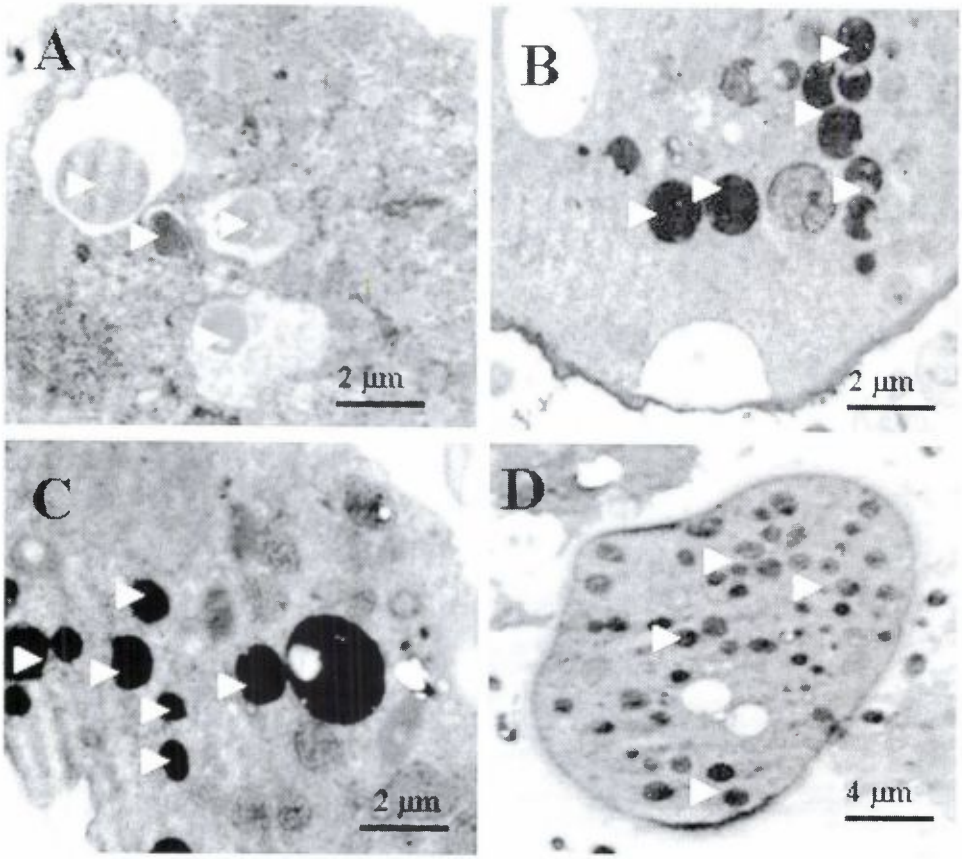


Figure 4. Electron microscopy analysis. Arrowheads point out bacteria. A. Intracellular localisation of *V. cholerae* O1 in the vacuoles of *A. castellanii* trophozoite 1 day after co-cultivation. B. Existence of *V. cholerae* O1 in *A. castellanii* cyst 7 days after co-cultivation. C. Multiplication of *V. cholerae* O139 in the cytoplasm of *A. castellanii* trophozoite 7 day after co-cultivation. D. Existence of *V. cholerae* O139 in *A. castellanii* cyst 7 days after co-cultivation.

viability of the trophozoites to develop to cysts hosting viable *V. cholerae*. Recultivation of *A. castellanii* hosting intracellular *V. cholerae* as endosymbionts enhanced growth and survival of the amoebae as well as the bacteria, which is in contrast to other extracellular bacteria such as *Aeromonas hydrophila* and *Pseudomonas aeruginosa* both of which kill *A. castellanii* in co-cultures (unpublished data). Behaviour of *V. cholerae* also differs from the extracellular and non-pathogenic *Escherichia coli* and *Klebsiella aeroginosa* that are utilised as food to *A. castellanii* and *A. polyphaga* (Abd et al., 2005; Weekers et al., 1993). However, *V. cholerae* resembles many facultative intracellular bacteria such as *Salmonella typhimurium* (Gaze et al., 2003) and *Shigella* species (Manuscript in preparation), which do not kill their host cells since they are needed for intracellular survival.

It has been shown that toxigenic *V. cholerae* (Marsh and Taylor, 1998) resembles facultative intracellular bacteria such as *L. pneumophila* (Allen, 2003; Liles, 1999) and *F. tularensis* (Gil et al., 2004) since they can survive in *A. castellanii* (Abd et al., 2003; Abd et al., 2005; Zusman et al., 2004). The *icmF* and *icmH* genes are required for intracellular multiplication of *L. pneumophila* in *A. castellanii*. The proteins of these genes, IcmF and IcmH are found in many bacteria such as *Yersinia pestis*, *Salmonella enterica*, and *V. cholerae*, which associate with eukaryotic cells (Zusman et al., 2004), and thus *V. cholerae* possesses the *icmF* gene (Das et al., 2002).

All these evidences show that *V. cholerae* differs from strictly extracellular bacteria and instead resembles facultative intracellular bacteria since it can grow and survive in the presence of *A. castellanii*. Furthermore, the intracellular survival of the bacteria does not inhibit growth of the amoebae indicating endosymbionts-host relation between *V. cholerae* and *A. castellanii*.

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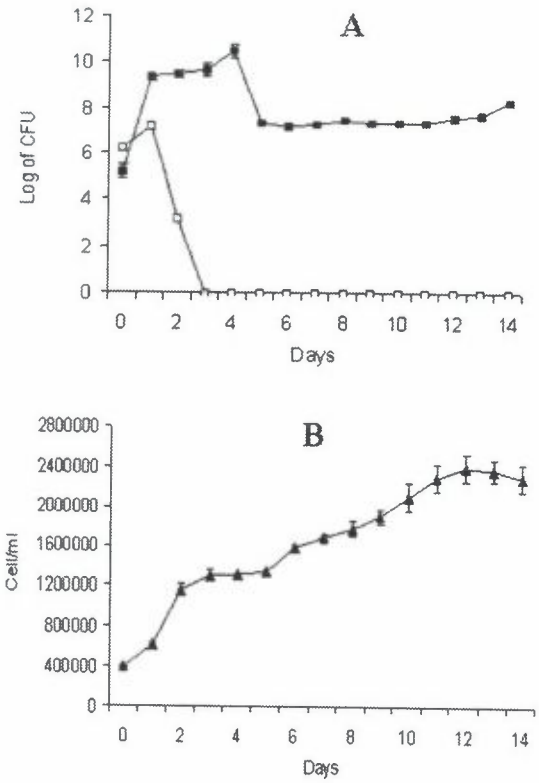


Figure 5. Symbiotical growth of amoebae and bacteria. A. Growth of *V. cholerae* O1 from recultivation of amoebae hosting intracellular bacteria (black squares) and growth of singly cultivated bacteria (white squares). Data indicate mean values ± SD of double measurements. B. Growth of recultivated *A. castellanii* hosting intracellular *V. cholerae* O1. Data indicate mean values ± SD of double measurements.

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