Interaction between waterborne pathogenic bacteria and *Acanthamoeba castellanii*

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Stockholm 2006

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TO MY FAMILY

ABSTRACT

Waterborne bacteria cause global public health problems. *Francisella tularensis* causes tularemia, which is a fatal disease in humans. *Pseudomonas aeruginosa* is an opportunistic and nosocomial pathogen of humans. *Vibrio cholerae* O1 and *V. cholerae* O139 infect only humans and cause epidemic and pandemic cholera. The principal natural reservoirs of these pathogens are largely unknown. To find their aquatic reservoirs is an important factor in the epidemiology of the infections. *Acanthamoeba* is a genus of free-living amoebae, which are found in the aquatic system and include several species and seem to have an increased role as reservoirs to many pathogenic bacteria.

Acanthamoeba castellanii was co-cultured with each of the above-mentioned bacteria for more than two weeks in order to study the interaction. Growth of the microorganisms, localisation and survival of intracellular bacteria was estimated by cell count, viable count, flow cytometry, PCR, fluorescence as well as electron microscopy.

The results showed that *F. tularensis* localised in *A. castellanii*, multiplied within vacuoles and survived in intact trophozoites, excreted vesicles, and cysts. Co-cultivation enhanced growth of *F. tularensis*, which grew and survived intracellularly for more than 3 weeks. In contrast, growth of singly cultured bacteria decreased significantly to non-detectable level within 2 weeks confirming the intracellular behaviour of the bacterium. The co-cultivation decreased growth of the amoebae in comparision to growth of singly cultured amoebae.

Co-cultivation of *A. castellanii* with different strains of *P. aeruginosa* PA103 producing different effector proteins secreted by type III secretion system (TTSS) resulted in the death of the amoeba populations. Different analysis disclosed that the number of co-cultured amoebae decreased over time in comparision to the number of singly cultured cells. The TTSS effector proteins ExoU and ExoS induced necrotic cell death to the most of *A. castellanii*.

The interaction between *V. cholerae* and *A. castellanii* resulted in growth and survival of *V. cholerae* O1 as well as O139 in the cytoplasm of trophozoites and in the cysts of *A. castellanii*. Co-cultivation enhanced growth of *V. cholerae*, which grew and survived intracellularly for more than two weeks, whereas, singly cultured bacteria decreased significantly to non-detectable level within few days disclosing an intracellular behaviour of *V. cholerae*.

In conclusion, methods used in this project showed predation between *A. castellanii* and the extracellular *P. aeruginosa*, symbiosis between *A. castellanii* and each of the facultative intracellular bacterium *F. tularensis* as well as *V. cholerae*.

Keywords: *Francisella tularensis*; *Pseudomonas aeruginosa*; *Vibrio cholerae*; *Acanthamoeba castellanii*; predation; intracellular behaviour; symbiosis; environmental reservoir

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers:

I. Abd, H., Johansson, T., Golovliov, I., Sandström, G., and Forsman, M.
Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii.*
Applied and Environmental Microbiology 69:600-606, 2003.

II. Abd, H., Wretlind, B., Saeed, A., Idsund, E., Hultenby, K., and Sandström, G.
Pseudomonas aeruginosa utilizes its type III secretion system to kill the free-living amoeba *Acanthamoeba castellanii.*
Manuscript.

III. Abd, H., Weintraub, A., and Sandström, G.
Intracellular survival and replication of *Vibrio cholerae* O139 in aquatic free-living amoebae.
Environmental Microbiology 7:1003-1008, 2005.

IV. Abd, H., Saeed, A., Weintraub, A., Balakrish Nair, G., and Sandström, G.
Intracellular behaviour of *Vibrio cholerae* O1 strains during interaction with the environmental free-living amoeba *Acanthamoeba castellanii*.
Submitted.

TABLE OF CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine mono phosphate
ASV	a destabilized variant
ATCC	American Type Culture Collection
ATP	adenosine tri phosphate
cAMP	cyclic adenosine mono phosphate
CFU	colony-forming units
CLED	Cystine lactose electrolyte deficient
CT	cholera toxin
ctxA	gene encoding cholera toxin subunit A
DNA	deoxyribonucleic acid
EXO	exoenzyme
FACS	fluorescence activated cell sorter
FLA	free-living amoeba
GFP	green fluorescent protein
LB	Luria-Bertani
LVS	live vaccine strain
MIC	minimal inhibitory concentration
MSHA	mannose sensitive hemagglutinin
NAD	nicotine amide dinucleotide
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
rRNA	ribosomal RNA
TCBS	Thiosulfate-citrate-bile-sucrose
TCP	toxin co-regulated pilus
TTSS	type III secretion system
VBNC	viable but non-culturable

1 INTRODUCTION

1. 1 Acanthamoeba

1. 1. 1 Free-living amoeba

Free-living amoebae (FLA) are environmental eukaryotic cells distributed worldwide in nature (23, 83, 85). FLA includes several genera such as *Acanthamoeba*, *Balamuthia*, *Naegleria* (83), *Sappinia* (52) and *Dictyostelium* (121). As aquatic inhabitants they are found in fresh, brackish, seawater, swimming pools, water supply networks, and on biofilms. Moreover, they are found in contact lens equipment and disinfecting solutions, dental treatment units, dialysis machines, and air-conditioning systems. Furthermore, they are isolated from mammalian cell cultures, vegetables, human nasal mucosa as well as human and animal brain, skin, lung tissues and eyes (90, 97, 100).

Life cycle of FLA includes at least two stages, a feeding trophozoite and a dormant cyst. *Naegleria* species have an additional flagellate stage. The cyst is a resting stage and consists of two layers, the ectocyst and the endocyst (58). Some species such as *Balamuthia mandrillaris* has a third layer, the mesocyst (132).

Castellani (24) reported the presence of *Acanthamoeba* in yeast cultures and Hull et al. (65) isolated *Acanthamoeba* from monkey kidney tissue culture. *Acanthamoeba* species are widely distributed in the environment (81). The trophozoite is an active stage and multiplies by binary fission. It is a typical eukaryotic cell containing a nucleus with a large central nucleolus, smooth as well as rough endoplasmic reticula, free ribosomes, Golgi apparatus, mitochondria, microtubules, and different vacuoles. Plasma membrane surrounds the cytoplasmic contents of the trophozoite, which possesses spiny surface projections called acanthopodia (pseudopodia). The cytoplasmic vacuoles include contractile vacuoles, which are osmoregulatory to control the water content of the cell. Secretory vacuoles usually contain enzymes for specific functions such as excystation and phagocytic vacuoles are sites of food digestion (18). The trophozoite takes necessary oxygen from the water that passes into it through its cell membrane. Waste products such as carbon dioxide and water are eliminated through the cell membrane. The trophozoites of *Acanthamoeba* are 25 to 40 µm in length depending on the species. They have a growth temperature range of 12°C to 45°C and move sluggishly by the aid of pseudopodia (108).

The trophozoite feeds on bacteria, algae, and yeasts in the environment but can also take up nutrients in liquid through pinocytosis (17, 20). Uptake of food can occur by pseudopodia

to form food vacuoles in which phagocytosis and digestion occur within phagolysosomes (99) or by food cup formation and ingestion of particulate matter (105). In addition to uptake of debris and glasbeads *Acanthamoeba* can take up cells from cell cultures such as nerve cells (105). I observed that *Acanthamoeba* cells could also take up amoeba cells from the same amoeba cell cultures (unpublished data).

Under adverse conditions such as changes in pH, temperature, and food deprivation (19) or binding to a specific membrane protein antibodies (131) the trophozoite undergoes encystation to form a cyst, which is 15 to 28 μm and has a double wall, reduced metabolic activity and several functions such as protection against changes in the surrounding environment, sites for nuclear reorganization and cell division, as well as mode of transmission (139).

The structure of the cysts may explain their resistance to extremes of temperature (22), disinfection by some biocides (57), antibiotics (127) and to levels of chlorine, which could be present in adequately treated water supplies (117). However, it has been shown that treatment with Freon or methylene oxide or autoclaving destroys cysts (88). When favourable conditions occur, such as supply of suitable nutrition and temperature, the cysts hatch (excyst) and the trophozoites emerge to feed and replicate. Mazur et al. (86) demonstrated that cysts of *A. castellanii* emerged to trophozoites after 24 years storage in water at 4°C. Moreover, it has been mentioned that *Acanthamoeba* cysts can retain their viability from -20°C to 56°C (140).

1. 1. 2 Acanthamoeba species as human pathogens

There are nearly 25 identified species of *Acanthamoeba* (15). Several of them are human pathogens such as *A. astronyxis*, *A. castellanii*, *A. culbertsoni*, *A. hatchetti*, *A. polyphaga*, *A. rhysodes*. *A. griffini*, *A. quina*, and *A. lugdunensis* (81, 82). All pathogenic species have the ability to grow at 36°C to 37°C but the optimum is at 30°C, and the trophozoites enter human body through respiratory tract, injured skin, as well as invade the central nervous system and colonise the cornea (100).

Acanthamoebae have an increased role as human pathogens because the number of *Acanthamoeba* infections has increased worldwide (81, 82). Stehr-Green et al. mentioned that only 20 cases of keratitis were reported up to 1984 and the number of cases in the USA had increased to over 200 by 1989 (122). Several species of *Acanthamoeba* are opportunistic and non-opportunistic human pathogens, which can cause both granulomatous amoebic encephalitis and amoebic keratitis and have been associated with cutaneous lesions and sinusitis in acquired immune deficiency syndrome patients and other immunocompromised

individuals (34, 40, 81). As an evidence to the increasing importance of *Acanthamoeba* infections, we diagnosed the first Nordic case of fatal meningoencephalitis caused by *A. castellani* and two cases of amoebic keratitis, by direct microscopic examination of clinical specimens, cultivation of *Acanthamoeba* cells, as well as identification by fluorescence microscopy, and a modified PCR method amplifying eukaryotic18 rRNA gene (102) by using of specific primer for *Acanthamoeba* (unpublished data).

1. 1. 3. Acanthamoeba as model to study interaction between eukaryotes and prokaryotes

Undergoing encystation as well as excystation according to different environmental conditions helps *A. castellanii* to resist extreme changes in the temperatures, disinfection and to survive for a long time in culture and in the environment (Fig. 1).

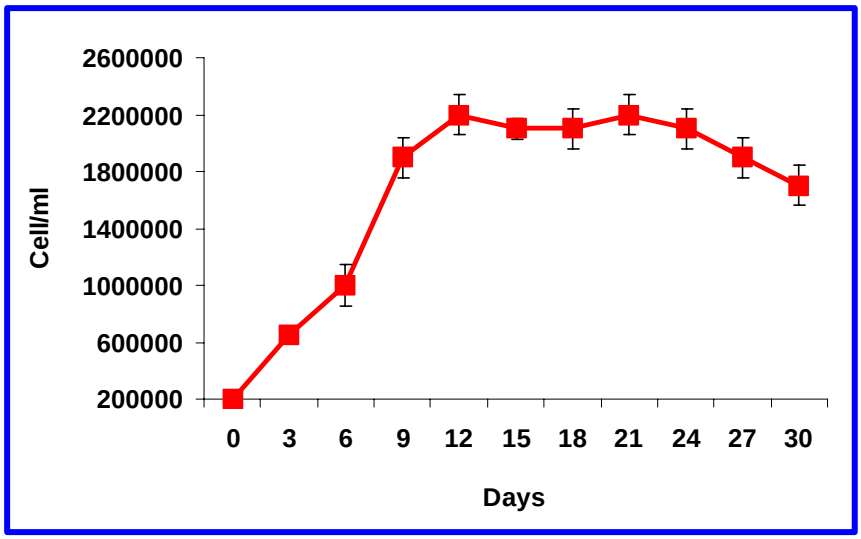

Fig. 1. Growth of *A. castellanii* in ATCC medium 712. Data indicate mean ± SD values of double measurements.

To study interaction between macrophages and some bacterial species in co-cultures takes no longer than three days but if *Acanthamoeba* is used in the experiment it will take several weeks. The trophozoite is able to phagocyte different cells and materials such as bacteria, yeasts, algae, amoeba, debris and glasbeads (Fig. 2).

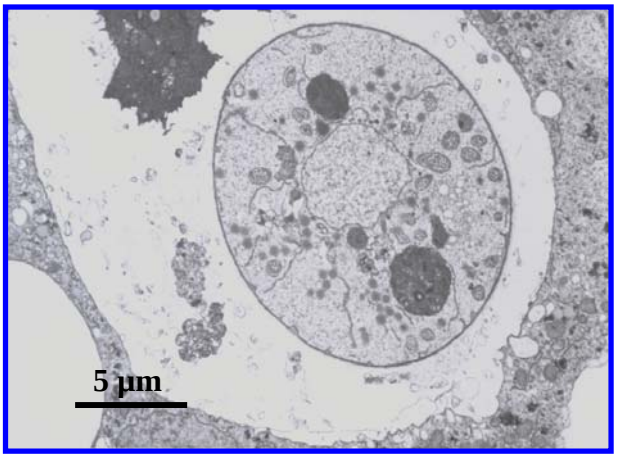

Fig. 2. *A. castellanii* trophozoite phagocytes a cyst in the same culture. Electron microscopy picture.

The emitting of autofluorescence by trophozoite as well as cyst helps in the diagnosis of *Acanthamoeba* and its viability (Fig. 3).

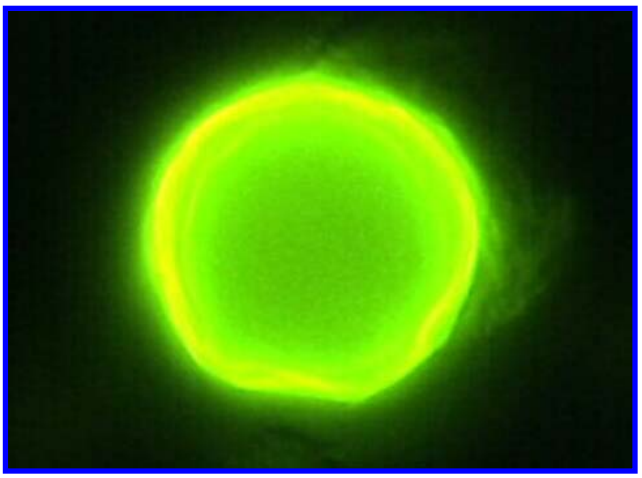

Fig. 3. Fluorescence microscopy micrograph of *A. castellanii* showing autofluorescence, 630 x.

The importance of *Acanthamoeba* species is their ability to be predators controlling microbial communities by utilising several bacteria as a food source and as hosts to pathogenic bacteria. More than 30 strains of pathogenic bacteria could be internalised by

Acanthamoeba species (50, 134). Fritsche et al. (50) has found that 24% of *Acanthamoeba* isolates contained bacterial endosymbionts.

These characteristics, long life because of encystation as well as excystation, phagocytosis, autofluorescence**,** resistance to many antibiotics, predator and host to different bacteria, make *Acanthamoeba* an ideal cell organism for the study of the interaction between eukaryotes and prokaryotes. Moreover, it may be used as a powerful tool for the culture of some intracellular bacteria (58).

1. 2 Extracellular and intracellular bacteria

According to fate following phagocytosis, pathogenic bacteria can be divided into intracellular and extracellular bacteria. Obligate intracellular bacteria including *Chlamydia, Coxiella, Mycoplasma*, and *Rickettsia* multiply strictly inside the host cells, while facultative intracellular bacteria including *Brucella*, *Francisella*, *Legionella*, *Listeria*, *Mycobacterium*, *Salmonella*, *Shigella*, and *Yersinia* can multiply inside- and outside the host cells (69). The intracellular bacteria are able to survive and grow within professional phagocytes giving chronic and/or recurrent disease.

Extracellular bacteria including a large number of bacteria such as *Bacillus* species, *Pseudomonas* species, *Staphylococcus* species, *Streptococcus* species, *Escherichia coli,* and *Vibrio* species can multiply in the body fluid, and damage tissues only as long as they remain outside the cells (Fig. 4).

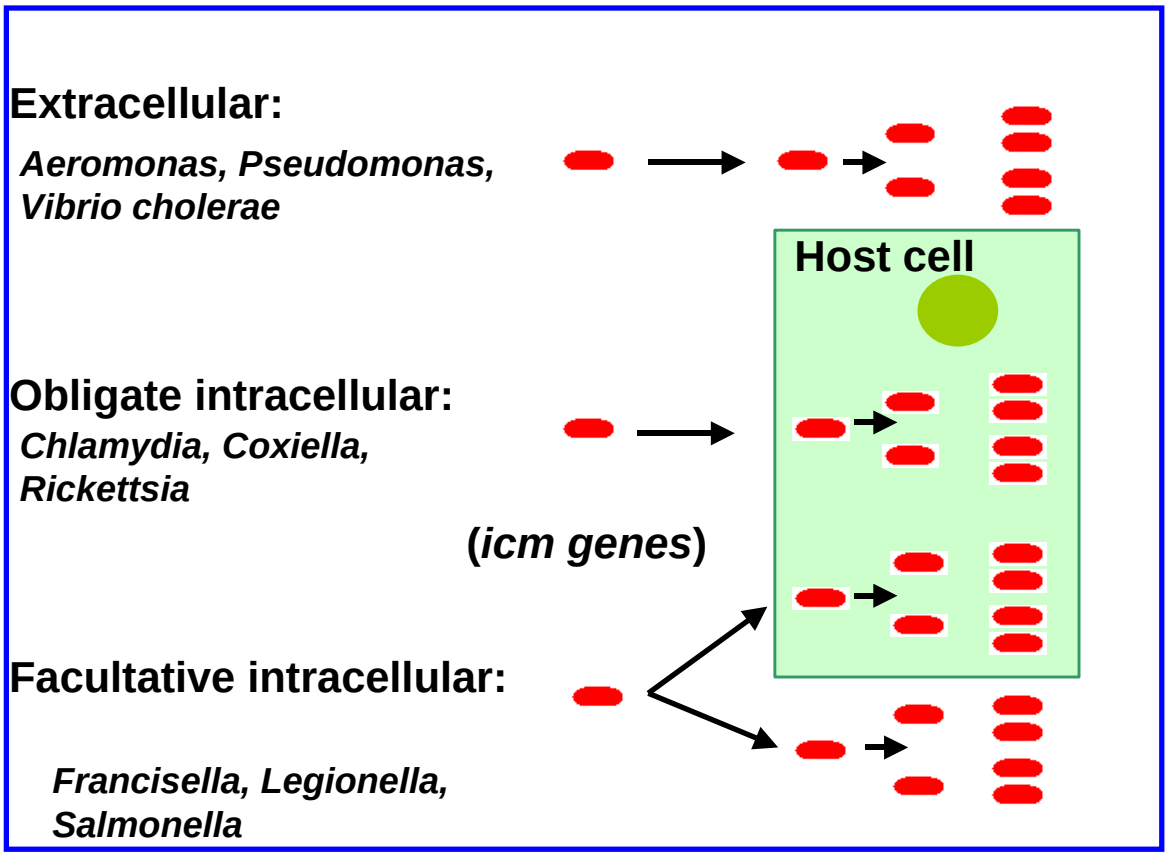

Fig. 4. Localisation and growth of extra- and intracellular bacteria in their host. The figure is modified from Fig. 7-4 and Table 7-3 (141).

Recent studies have shown that many of previously considered extracellular bacteria like *Staphylococcus*, *Enterococcus*, *Campylobacter*, pathogenic *E. coli*, and *Helicobacter* may be considered as facultative intracellular bacteria (10, 11, 36, 53, 79). Generally, extracellular bacteria can be eliminated by the humoral immune response, whereas, intracellular bacteria can only be eliminated by a cellular immune response.

1. 3 Waterborne pathogenic bacteria

Since the waterborne outbreaks of cholera in England 1831 (142), tularemia in Italy 1982 (56) and *P. aeruginosa* infections had been reported (13) the waterborne pathogens still have caused global problems to the public health. Therefore, our project focuses on the interaction between *A. castellanii* and *F. tularensis*, *P. aeruginosa* and *V. cholerae*

1. 3. 1 Francisella tularensis

McCoy and Chapin isolated a small gram-negative bacterium from ground squirrels with a plague-like illness in Tulare County, California in 1911 and the bacterium was called *Bacterium tularensis* (87). Edward Francis recognized the illness as a fatal disease in humans, which was called tularemia (49). Thereafter, the bacterium was renamed to *Francisella tularensis* in recognition of E. Francis contributions (44). *F. tularensis* is a small gram-negative, capsulated, non-motile, aerobic bacterium (64).

The genus *Francisella* comprises two species: *F. tularensis* and *F. philomiragia*. *F. tularensis* includes four subspecies. *F. tularensis* subspecies were identified on the basis of virulence, citrulline ureidase activity (conversion of L-citrulline to ornithine), and acid production from glycerol as described by Ellis et al. (42). Two subspecies of *F. tularensis* are most virulent to humans and animals. *F. tularensis* subsps. *tularensis* fermenting glycerol is highly virulent, and is found in North America. *F. tularensis* subsps. *holarctica* does not ferment glycerol, is moderately virulent, and is found in Europe, Asia and North America. *F. tularensis* subsps. *mediaasiatica* is not known to cause tularemia in humans and is found in Central Asia. *F. tularensis* subsps. *novicida* is low virulent and causes a tularemia-like illness in humans and has been isolated in the United States and Canada (63).

Tularemia is a zoonotic disease, which is transmitted from animals to humans and results in fever, rash and swollen lymph nodes. The transmission of the bacterium occurs by several modes such as bites by infected ticks, flies or mosquitoes (33), or intake of contaminated water, food or soil as well as inhalation of aerosol containing the bacteria and other direct contact means such as handling of tissues or fluids from infected animals (44). According to the mode of transmission, the bacterium can infect humans through the skin, mucous membranes, and gastrointestinal tract. The major target organs are the lymph nodes, spleen, liver, lungs, and kidney. Therefore, it can cause different forms of tularemia including ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoid like and septic forms (38).

F. tularensis is a facultative intracellular organism, which can multiply and survive within macrophages and hepatocytes (29, 47, 48). It has been shown that the live vaccine strain (LVS) is very well adapted to the intracellular environment of macrophages, survives in phagosomes (5, 55) and exerts a cytopathogenic effect on murine macrophages (6, 14, 55). Furthermore, it has been found that LVS localizes within an acidic vesicle, which facilitates its iron uptake (48) and that LVS releases an acid phosphatase that inhibits the respiratory burst in neutrophils (112). Moreover, nitric oxide has been shown not to be involved in the killing of *F. tularensis* by alveolar macrophages (107) and LVS induces apoptosis in murine macrophages (73).

F. tularensis is a facultative intracellular bacterium, which induces both humoral and cellular immune response in man (118). It possesses a capsule, which protects the bacteria from phagocytosis. It has been shown that intradermal injection with live *F. tularensis* but not with killed bacteria in mice can induce production of interleukin-12, tumor necrosis factor alpha and interferon gamma, which are involved in the activation of T cells (128). Recently, it has been shown that *F. tularensis* possesses pili, which may contribute to its virulence (54).

Outbreaks of disease in humans are often parallel with outbreaks of tularemia in wild animals. However, it is not clear whether these animal species are the true reservoir of the bacterium in the environment. A wide range of arthropod vectors have also been implicated in the transmission of tularemia between mammalian hosts such as ticks, biting flies and mosquitoes (96). These vectors play a role both in the transmission of the disease within wild animal populations and in the transmission of disease to humans (38).

There is evidence that the bacterium can persist in watercourses, possibly in association with amoebae (12).

The bacterium has been isolated from more than 250 animal species such as hares, rabbits and rodents (96) and it can be recovered from contaminated water and soil (101) but its principal natural reservoir is unknown.

1. 3. 2 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a common environmental bacterium, which has the ability to colonize multiple environmental niches (31). In 1862 Luke observed the presence of rod-shaped particles in blue-green pus of some infections (80).

P. aeruginosa is a Gram-negative, aerobic bacterium, which is an opportunistic and nosocomial pathogen in humans. It utilises different virulence factors such as cell

components, extracellular products by quorum sensing as well as different type secretion systems including the type III secretion system (TTSS).

Cell components include capsule, flagellum, pilus and adhesive factors, which are fimbrial and non-fimbrial such as capsular glycocalyx, lipopolysaccharide and alginate.

Pseudomonas produces the alginate slime that forms the matrix of biofilm to anchor bacterial cells to their environment as well as to protect those cells from the host defences.

Several virulence factors of *P. aeruginosa* such as extracellular enzymes, rhamnolipids, pyocyanin, and exotoxin A are produced under control of two quorum-sensing systems Las and Rhl (75). Quorum sensing is cell-to-cell communication or cross talk between the cells in a bacterial population unit to find more suitable environment, nutrient supply, survival strategies, protection and biofilm formation. The bacteria generate signal molecules (autoinducers) to communicate with each other. Production and accumulation of autoinducers (compounds of homoserine lactone) occur when cell density reaches a certain threshold level. The autoinducer enters other bacterial cell and binds N terminus of the transcriptional activator protein whose C terminus binds DNA to induce gene expression and translation of virulence factors as well as more autoinducers (30, 37, 104).

To date TTSS effector proteins include four exoenzymes Exo S, Exo T, Exo U and Exo Y (119). ExoS and ExoT are ADP-ribosyltransferases (67, 136), which catalyze the transfer of an ADP-ribose from NAD to target protein. The ADP-ribose is toxic to the target protein and inhibits DNA synthesis as well as phagocytosis in the host cell (91). ExoU is a phospholipase and ExoY is an adenylate cyclase. The phospholipase has cytotoxic effects and induce necrosis, whereas, the adenylate cyclase has a lysis effect (45, 130).

P. aeruginosa is resistant to most antibiotics by different mechanisms. It has a large genome containing 6.26 Mbp encoding 5567 genes, which may increase the probability of mutation in chromosomal genes regulating resistance genes in addition to the presence of numerous efflux pumps. The bacterium is able to acquire resistance genes from other organisms via plasmids, transposons, and bacteriophages. Its cell wall is characterised by low permeability to antimicrobial agents (74).

P. aeruginosa can be found in soil, water, plankton, biofilm, and hospital environment. It may infect any part of the human body in immunosuppressed and hospitalised patients with cancer, cystic fibrosis, and burns causing serious health problems. Accordingly, it is important to study its ecological niche, which is not well defined (46, 93) (Paper II).

1. 3. 3 Vibrio cholerae

Vibrio cholerae is a straight or curved gram-negative, facultatively anaerobic bacterium, which possesses a polar flagellum as well as many pili. It is a free-living cell in aquatic environment (28) and it is held to be an extracellular bacterium (58). The bacterium was described and called *Vibrio cholerae* by the Italian Pacini in 1854 (106).

The *V. cholerae* species comprise nearly 200 serogroups based on the O-antigenic structures (137). *V. cholerae* O1 and *V. cholerae* O139 infect only humans and cause epidemic and pandemic cholera. The major difference between the O1 and the O139 strains is that the latter possesses a capsular polysaccharide (70). The serogroup *V. cholerae* O1 is subdivided into two biotypes, classical and El Tor depending on haemolysis of sheep erythrocytes, biochemical properties and phage sensitivity. Each biotype has three O-antigens (A, B, C) divided in three serotypes: Ogawa (A, B), Inaba (A, C) and Hikojima (A, B, C) (71).

V. cholerae O1 El Tor as well as O139 possesses mannose-sensitive hemagglutinin, which is required for colonization of zooplankton (26). Moreover, the common property of *V. cholerae* serogroups is their ability to possess toxin co-regulated pilus, which is a colonisation factor to the human intestine (71).

V. cholerae colonises the mucosa of the human small intestine, produces neuraminidase that removes sialic acid from gangliosides exposing ganglioside GM1, which is the specific receptor for cholera toxin (CT) (95). The bacterium secretes CT, which is composed of A subunit and B subunits (98). When B subunits bind to GM1 on mucosal epithelial cell, change of the toxin structure occurs to release A subunit to enter inside the epithelial cell. The intracellular glutathione reduces the disulfide bond of the A subunit that dissociates into A1 and A2. A1 hydrolyses enzymatically NAD to ADP-ribose and nicotinamide. The ADP-ribose activates adenylate cyclase to hydrolyse ATP to cyclic AMP (cAMP) (143).

The increased concentration of PO_4^{-3} by high production of cAMP inside the cell increases the electrical gradient, which stimulates mucosal cells to pump large amounts of Cl^- into the intestinal contents. H_2O, Na^+, K^+ and HCO_3^- follow the osmotic and electrical gradients caused by the loss of Cl^-. The lost H_2O and electrolytes in mucosal cells are replaced from the blood and causes diarrhoea and dehydration that are characteristic of cholera (143).

Cholera is a severe diarrhoeal disease in Asia, Africa, and America and it affects many million persons annually (71, 124, 125). *V. cholerae* is widely distributed in aquatic environments and cholera outbreaks are associated with contaminated food and water supplies. The seasonality of cholera has been associated with physico-chemical and biological

factors (77). However, many factors affect the survival of *V. cholerae* in aquatic environments such as attachment to plankton, entering into and resuscitation from viable but non-culturable (VBNC) state, and loss to predators (32). Factors regulating the level of viable cells of *V. cholerae* O1 and O139 in aquatic environment are still being investigated (43). The infective dose of *V. cholerae* to cause cholera is approximately 10^8 cells (116). Therefore, the bacteria need a biological reservoir in order to grow and survive in high concentrations to infect humans. Finding of aquatic reservoirs of *V. cholerae* is an important factor in the epidemiology of cholera.

1. 4 Interaction between waterborne microorganisms

Interaction between organisms includes predation when one of them is eaten and symbiosis when they live together. In symbiosis, there is a benefit for at least one of the partners.

If the second partner is injured, symbiosis is called parasitism, commensalism if it is relatively unaffected, and mutualism if it benefits. The benefits might be a protective environment, nutrition provided by the host or that the symbiont protects the host by making it more difficult to be colonized by pathogenic bacteria (62).

Studies about the interaction between pathogenic bacteria and eukaryotic cells (amoebae as well as macrophages) have previously shown that facultative intracellular bacteria have different mechanisms for their intracellular growth. *L. pneumophila* can survive in the eukaryotic phagosomes (1) and *S. dysenteriae* can survive in the cytoplasm of such cells (114). Furthermore, Abu Kwaik (1) has found that besides being an environmental host, *Acanthamoeba*e mimic the interaction of macrophages with bacteria. The known genetic factors required by *Legionella* to infect protozoa are also required for the infection process in the mammalian cells (60, 61).

Free-living amoebae such as *Acanthamoeba* species are commonly found in natural water systems (83), in soil, as well as in biofilms (21) and in drinking water (9). The human pathogenic bacteria *F. tularensis* (64, 101), *P. aeruginosa* (89) and *V. cholerae* (58) are connected with water systems.

Acanthamoeba species have an increased role as reservoirs, vectors and hosts to many pathogenic bacteria such as *Campylobacter jejuni* (8), *Chlamydia* species (4) *Helicobacter pylori* (135), *Legionella penumophila* (138) and *Salmonella typhimurium* (51).

Previous studies of the interaction between waterborne pathogenic bacteria and *A. castellanii* have shown that growth of *F. tularensis* is enhanced in media preconditioned by amoebae (59). *Acanthamoeba* and *Pseudomonas* were isolated from eyewash stations (103) and from a contaminated drinking water system in hospitals (89). Although it has been shown that there is an association of *V. cholerae* with algae (68) as well as with fresh water amoebae (126) and that *V. cholerae* strains are able to attach to zooplankton (111). Much less is known about *V. cholerae's* behaviour and symbiosis with eukaryotic cells in the environment.

In this project, we have tested the hypothesis that *Acanthamoebae* may comprise a significant environmental reservoir for the facultative intracellular bacterium *F. tularensis* (paper I).

Since it has been reported that *P. aeruginosa* is able to secret inhibitors of unknown nature for growth of *Acanthamoeba* species (110), our project aimed to examine the effect of different components of TTSS such as ExoS, ExoT, ExoU and ExoY as well as exotoxin A on the growth of *A. castellanii* as an example to interaction between phagocytic eukaryotic cells and extracellular prokaryotic cells in the aquatic environment (paper II).

Kaper (71) has mentioned that the environmental reservoirs for *V. cholerae* in endemic areas are not well defined, therefore, we have investigated the interaction between *A. castellanii* and *V. cholerae* O139 (study III) as well as *V. cholerae* O1 (study IV).

2 AIMS OF THE PROJECT

The aims of the project are:

i) Establish methods to study amoebae-bacteria interactions over a long period of time;

ii) Find out if the human pathogenic bacteria *F. tularensis*, *P. aeruginosa* and *V. cholerae* can survive and multiply intra-amoebically;

iii) Compare the interaction of different *V. cholerae* strains with amoebae;

iv) Evaluate if there is a symbiotic relation between *Acanthamoeba* and its interacted bacteria in order to disclose a role of free-living amoebae as environmental hosts for pathogenic bacteria.

3 MATERIALS AND METHODS

3. 1 Microorganisms

Acanthamoeba castellanii (ATCC 30234) was obtained from the American Type Culture Collection (Manassas, VA) and used in all experiments.

Francisella tularensis LVS (Live Vaccine Strain, type B) was from the US Army Medical Research Institute of Infectious Diseases, Fort Detrik, MD, USA. *F. tularensis* LVS carrying pKK214 plasmid containing a gene for a destabilized variant (ASV) form of green fluorescent protein (GFP) and a gene for tetracycline resistance (72). *F. tularensis* LVS/GFP/ASV was used in paper I and III.

P. aeruginosa PA103 and isogenic mutants described in Table 1 were kindly supplied by Dara Frank, Medical College of Wisconsin, USA and used in paper II.

Table 1. *P. aeruginosa* strains and their effector proteins (129).

Strain	Effectors
PA103	ExoT, ExoU, exotoxin A
PA103 toxA::Ω	ExoT, ExoU
PA103ΔexoUexoT::Tc pUCPexoS	ExoS
PA103ΔexoUexoT::Tc pUCP exoY	ExoY
PA103ΔexoUexoT::Tc	None

Non-pathogenic *Escherichia coli* DH5α was obtained from Brendan P. Cormark, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305-5402, USA.

Vibrio cholerae O139, AI1838 is a clinical isolate that was obtained from the culture collection of Laboratory Science Division, International Centre for Diarrhoeal Disease Research, Bangladesh. *Escherichia coli* and *Vibrio cholerae* O139 were used in paper III.

Twelve strains of *V cholerae* O1 classical and El Tor listed in Table 2 were from the culture collection of Laboratory Science Division, International Centre for Diarrhoeal Disease Research, Bangladesh. The plasmid (pGFPuv) carrying GFPuv gene and confers resistance to ampicillin (100 μg/ml), was obtained from BD Biosciences Clontech, USA and introduced by electroporation into *V. cholerae* O1 classical strain C-19385 and *V. cholerae* O1 El Tor strain AK-38670. All mentioned *V. cholerae* O1 strains were used in paper IV.

Table 2. *V. cholerae* strains used in Paper IV.

Strain No.	Biotype	Laboratory ID	Year of Isolation
1	Classical	C-19385	1965
2	El Tor	Q-5970	1977
3	Classical	F-2427	1968
4	El Tor	AE-8182	1989
5	El Tor	AK-38670	1995
6	Classical	H-18	1970
7	El Tor	AR-32732	2002
8	Classical	X-19850	1982
9	Classical	Y-8661	1983
10	El Tor	AS-6522	2003
11	El Tor	MQ-1194	2001
12	Classical	AA-5117	1985

3. 2 Determination of the half-life of GFP/ASV in F. tularensis LVS

Samples containing 2.0 x 10^9 cfu/ml were inactivated by treatment with 250 µg/ml gentamicin (Sigma, St. Louis, MO) for 1 h at room temperature in darkness. After confirming that the cells were unable to grow, as determined by viable counts, following this treatment, viable count and flow-cytometry analysis were carried out in parallel for seven days. No growth on Modified Thayer-Martin agar was observed and the fluorescence gradually decreased.

3. 3 Culture media and growth conditions

3. 3. 1 Growth of amoeba

A. castellanii was grown without shaking at 30^{o}C to a final concentration of 10^6 /ml in ATCC medium no.712 (ATCC, Manassas, VA).

3. 3. 2 Growth of bacteria

F. tularensis LVS/GFP/ASV was grown on Modified Thayer-Martin agar plates containing 36 g/l GC base medium (Difco Laboratories, Detroit, MI), 10 g/l haemoglobin (Difco), 10

mg/l IsoVitaleX (BBL Microbiology Systems, Cockeysville, MD) and 10 mg/l tetracycline (Paper I and III). *P. aeruginosa* strains were grown on cystine lactose electrolyte deficient (CLED) agar plates (Merck, Darmstadt, Germany), for 24 h at 37^{o}C (Paper II). *E. coli* was grown on Luria-Bertani (LB) agar plates (Merck, Germany). *V. cholerae* O139 was grown on Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar plates (Oxoid, England) for 24 h at 37^{o}C (Paper II). *V. cholerae* O1 strains were grown on blood agar plates for 24 h at 37^{o}C (Paper IV). *E. coli*, *P. aeruginosa* and *V. cholerae* strains were grown in Luria-Bertani (LB) broth (Merck) to an absorbance of 0.6 at 600 nm and a 1.0 absorbance at 600 nm suspension of *F. tularensis* colonies in PBS was performed.

3. 3. 3 Co-cultures

Co-cultures of each bacterial strain and *A. castellanii* were incubated in 75 cm^2 cell culture flasks (Corning Incorporated Costar, USA) filled with 50 ml ATCC medium 712 containing an initial concentration of 10^5 cell/ml *A. castellanii* and 10^3 cell/ml of each *P. aeruginosa* PA103 strains, as well as 10^6 cell/ml of each *E. coli*, *F. tularensis* and *V. cholerae* strains, respectively. For fluorescence microscopy analysis of *V. cholerae* O1 GFP strains, co-cultures of each *V. cholerae* O1 GFP classical strain C-19385 and *V. cholerae* O1 GFP El Tor strain AK-38670 and *A. castellanii* in the ATCC medium 712 containing 100 μg/ml ampicillin were performed.

3. 3. 4 Cultures of control microorganisms

Control flasks for each microorganism were cultivated separately and prepared in the same way and with the same initial concentration as for co-cultivated microorganisms. The flasks were incubated at 30^{o}C without shaking.

3. 4 Analysis

At different time intervals samples were withdrawn for analysis.

3. 4. 1 Flow-cytometry analysis

The flow cytometer (FACSort, Becton Dickinson Immuno Systems, San Jose, CA), equipped with an argon laser giving a 488-nm primary emission line, was calibrated using unlabeled and labeled beads (Becton Dickinson) and FACSComp software (Becton Dickinson). Unlabeled cells were adjusted for forward scatter (relative size), side scatter (relative

granularity), FL1 (green colour), FL2 (red colour) and FL3 (deep red colour). The measuring time per sample was 15-50 sec with a medium flow rate of 60 µl/min. From each sample 10,000 events were registered and data were analysed using Cell Quest software (Becton Dickinson). Samples (3 ml) of cell suspension from the co-culture flasks were centrifuged for 10 min at 300 g in Beckman Model TJ6 centrifuge (Beckman Instruments, Palo Alto, CA) and washed six times with FacsWash solution (Becton Dickinson) prior to the analyses.

3. 4. 2 Microscopy analysis

3. 4. 2. 1 Light, fluorescence and confocal microscopy

A. castellanii cells in the absence and presence of bacteria were counted in a Bürker chamber (Merck Eurolab) under a light microscope (Carl Zeiss) while the abundance and distribution of *F. tularensis* LVS/GFP/ASV were analysed by fluorescence microscopy (Leica Microscopy Systems). Prior to analysis, 2-ml samples of cell suspension from the co-culture flask were centrifuged for 10 min at 300 g in Beckman Model TJ6 centrifuge and the resulting pellets were washed six times with FacsWash solution.

Antibody labeling of extracellular *F. tularensis* LVS/GFP/ASV was performed by adding 10 µl biotin-labelled antibodies, specific for *F. tularensis* LVS (German Armed Forces Medical Academy, Munich, Germany), to 1-ml samples of cell suspension from co-culture flasks. The samples were incubated for 20 min at room temperature, washed and reincubated for 20 min with 10 µl phycoerythrin (PE) conjugated Streptoavidin (DAKO, Glostrup, Denmark), washed again and examined under the fluorescence microscope.

Intracellular localisation of *V. cholerae* O1 GFP inside *Acanthamoeba* cells were analysed by confocal microscopy. Two ml samples of cell suspension from the co-culture flasks containing *A. castellanii* and each of *V. cholerae* O1 GFP classical strain C-19385 and *V. cholerae* O1 GFP El Tor strain AK-38670 were centrifuged for 10 min at 300 x g and the resulting pellets were washed six times with PBS, mounted and examined by confocal microscope (Leica TCS SP2 AOBS).

3. 4. 2. 2 Electron microscopy

The intracellular localisation of *F. tularensis*, *P. aeruginosa* wild type strain, *V. cholerae* O1 classical C-19385 and El Tor AK-38670 were analysed by electron microscopy. Five ml samples of cell suspension from co-culture flasks were centrifuged for 10 min at 300 x g in Labofuge GL centrifuge (VWR International). The resulting pellets were washed with PBS. Each pellet of infected amoebae was fixed in 2.5% glutaraldehyde in 0.1 M sodium

cacodylate buffer pH 7.3 with 0.1 M sucrose and 3 mM $CaCl_2$ for 30 min at room temperature. Samples were then washed in sodium cacodylate buffer and postfixed in 2% osmium tetroxide in the same buffer for 1 h. The samples were centrifuged and the pellets were dehydrated and embedded in Epoxy resin LX-112. The embedded samples were cut into ultra-thin sections, placed on grids, stained with uranyl acetate and lead citrate. Sections of *P. aeruginosa*, *V. cholerae*, and *F. tularensis* were examined with a transmission electron microscope (Philips 420) and (Carl Zeiss 900), respectively.

3. 4. 3 Growth and survival of singly and co-cultured bacteria

To estimate growth and survival of singly and co-cultured bacteria with *A. castellanii* by viable counts, 1 ml from each bacterial control flask and from co-cultured flasks containing both bacteria and amoebae was withdrawn. Samples were prepared by tenfold dilution from 10^1 to 10^{10} and spread on agar plates and incubated according to culture media and growth conditions of each strain. Thereafter the numbers of colonies were counted.

3. 4. 4 Growth and survival of intracellular bacteria

To examine intracellular growth and survival of the bacteria in *A. castellanii* cells by viable count assay, 2 ml of cell suspension from co-culture flasks were diluted in 8 ml PBS, centrifuged for 10 min at 300 x g and washed 6 times in PBS to minimise extracellular bacteria contamination. The pellets were resuspended in 1 ml PBS and incubated with 250 µg/ml of gentamicin for 1 h at room temperature. The samples were then diluted in 9 ml PBS and centrifuged for 10 min at 300 x g. The pellets were resuspended in 1 ml PBS solution and centrifuged for 10 min at 300 x g. One hundred µl of the supernatants were spread on blood agar plates and each pellet was diluted two-fold with 0.1% sodium deoxycholate (0.5% sodium deoxycholate) for *F. tularensis*. Series of tenfold dilution from 10^1 to 10^4 were prepared of the sample and spread on agar plates and incubated according to culture media and growth conditions of each strain and viable counts were performed.

3. 4. 5 DNA isolation and PCR amplification

Two ml samples of cell suspensions from co-culture flask containing *A. castellanii* and each of *V. cholerae* O139, *V. cholerae* O1 classical strain C-19385 and *V. cholerae* O1 El Tor strain AK-38670, respectively, were diluted in 8 ml PBS, centrifuged for 10 min at 300 x g and washed six times in PBS to minimise extracellular *V. cholerae* contamination. The pellets were resuspended in 1 ml PBS and incubated with 250 µg/ml of gentamicin for 1 h at room

temperature to kill extracellular bacteria. The samples were then diluted in 4 ml PBS and centrifuged for 10 min at 300 x g. The pellet was resuspended in 2 ml PBS solution and DNA was extracted according to Qiagen DNA mini kit (Qiagen, Hilden, Germany). The PCR method and detection of cholera toxin gene (*ctxA*) and 18S rRNA gene was performed as previously described by Lipp et al. (78) and Pasricha et al. (102).

3. 5 Statistical analysis

Chi-square test and Student's t-test were used to examine for significant differences in growth between alone and co-cultivated amoebae as well as bacteria.

4 RESULTS

4. 1 Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii* - Paper I

4. 1. 1 GFP-fluorescence from intracellular F. tularensis LVS/GFP/ASV in A. castellanii analysed by flow cytometry

Flow cytometer analysis of samples taken from singly cultured and co-cultured *A. castellanii* with *F. tularensis* LVS/GFP/ASV after washing out of the extracellular bacteria from co-cultured samples, showed that the fluorescence intensity increased with time in *A. castellanii* populations, from 0% at day 0 to 4, 8 % in singly cultured (upper panel) and to 94 % in co-cultured amoebae (lower panel) at day 15 (Fig. 5).

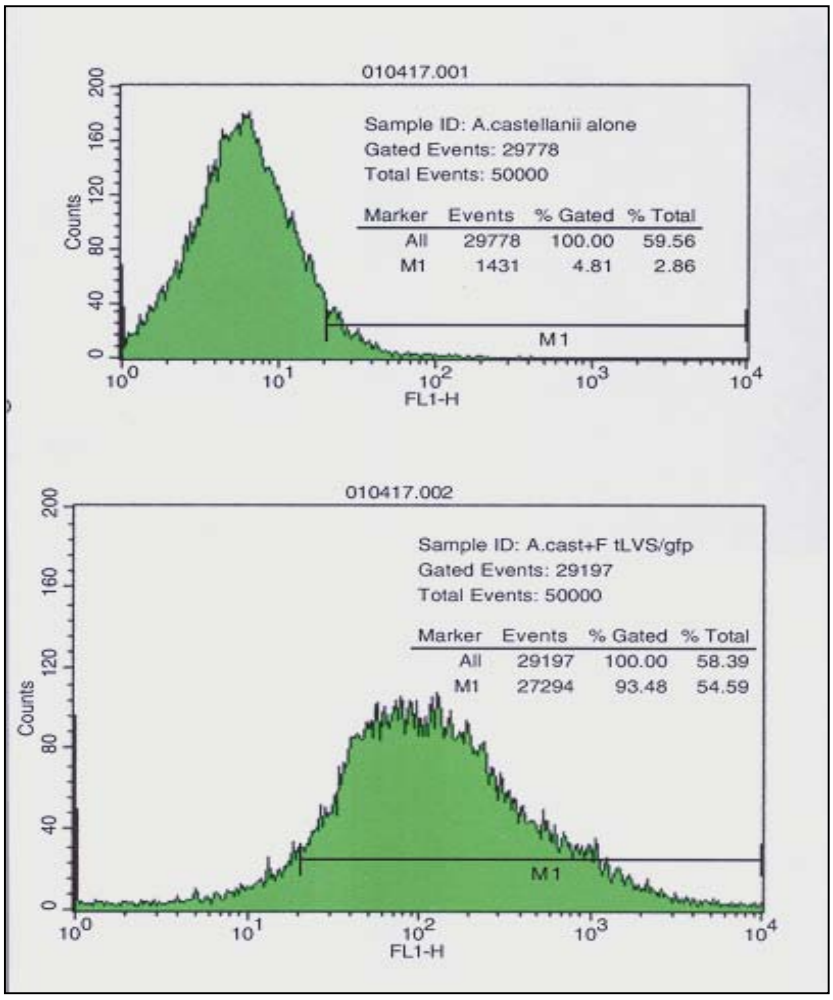

Fig. 5. FACS analysis showing GFP fluorescence from *F. tularensis* LVS/GFP/ASV inside the gated *A. castellanii* cells population at day 15.

4. 1. 2 Intracellular localisation of F. tularensis in A. castellanii - Fluorescence microscopy analysis

Samples taken from co-cultures of *F. tularensis* LVS/GFP/ASV and *A. castellanii* were washed and examined by the fluorescent microscopy. The results showed the localisation of green fluorescent bacterial cells inside *A. castellanii* cells. The numbers of intracellularly grown bacteria increased with time. Different stages of infection were observed, including growth in intracellular membrane limited vacuoles, release of vesicles and cysts containing bacteria as well as disintegrating amoeba cells filled with green fluorescent *F. tularensis* (Fig. 6).

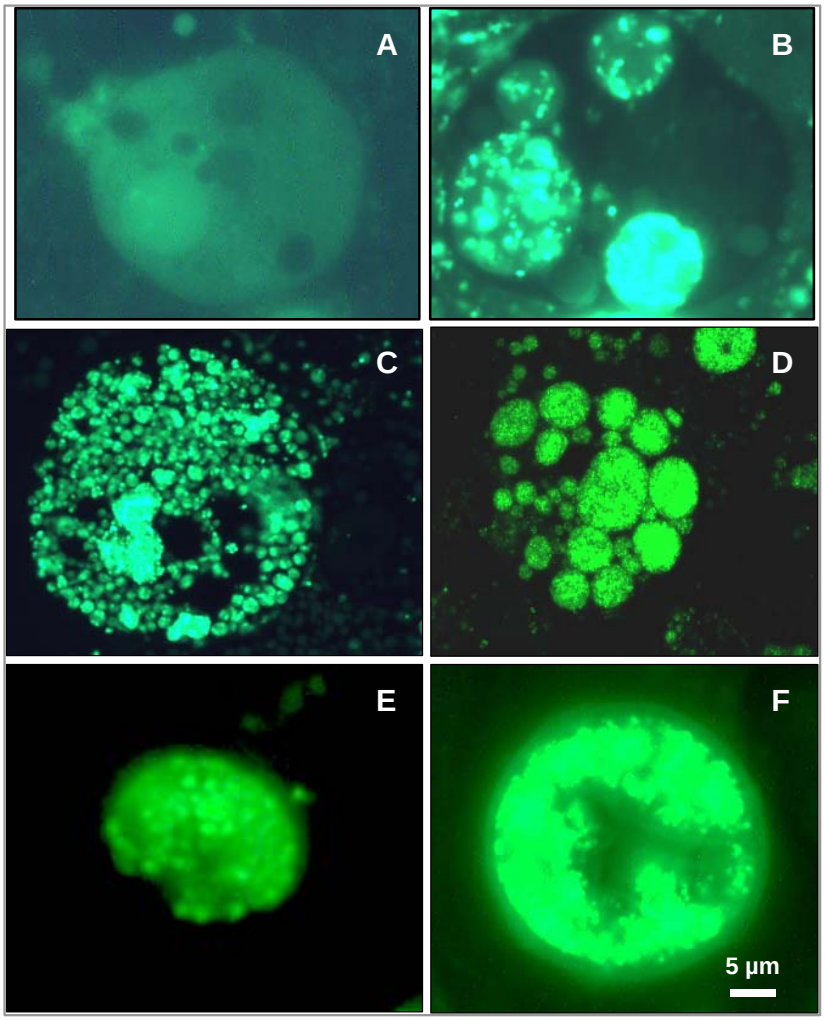

Fig. 6. Fluorescence microscopy analysis. A. *A. castellanii* trophozoite without intracellular *F. tularensis* (day 0). B. Intact *A. castellanii* with *Francisella*-filled vacuoles (day 10). C. Disintegrating *A. castellanii* filled with *F. tularensis* LVS/GFP/ASV (day 15). D. *Francisella*-filled vesicles, enclosed within the cell membrane of a dead *A. castellanii* trophozoite (day 18). E. *Francisella*-filled vesicle (day 18). F. *A. castelanii* cyst containing *F. tularensis* LVS/GFP/ASV inside the double-wall (day 40).

4. 1. 3 Differentiation between extracellular and intracellular F. tularensis

Samples taken at day 18 from the co-culture were mixed with PE-labelled antibodies specific for *F. tularensis* and the antibody-directed staining was visualised by fluorescence microscopy. The results showed that GFP-labelled bacteria within the *A. castellanii* were not accessible to the antibodies and hence exhibited green fluorescence. In contrast, extracellular bacteria were recognised by the antibodies and thus exhibited red fluorescence. The intracellular localisation of *F. tularensis* was found in amoeba cells disintegrated as vesicles filled with bacteria in excreted vesicles and in cysts (Fig. 7).

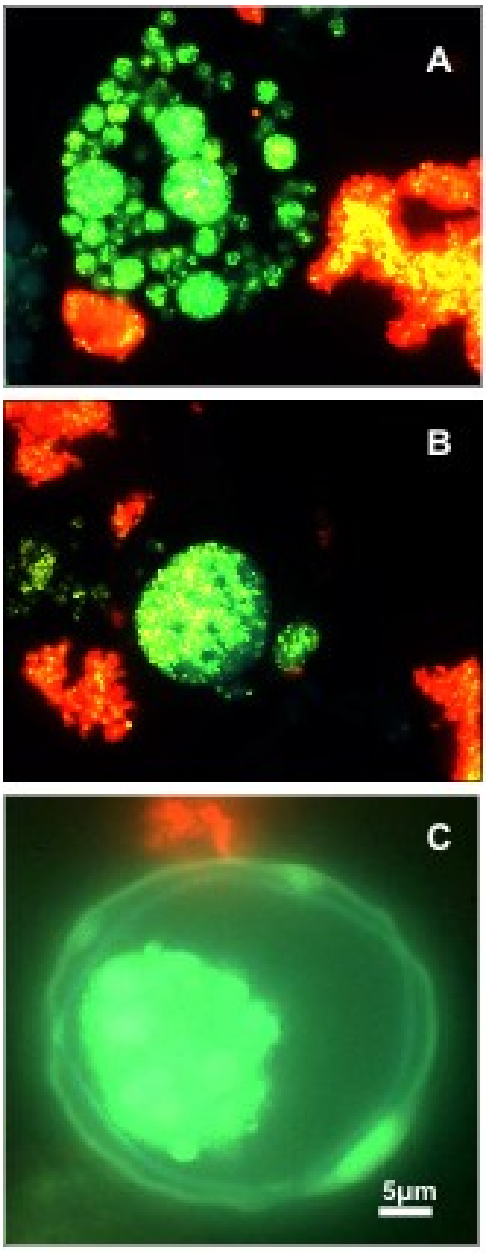

Fig. 7. Differentiation between extracellular and intracellular *F. tularensis* by monoclonal antibodies. Viable intracellular *F. tularensis* LVS/GFP/AVS cells expressing GFP appear green, while extracellular *F. tularensis* appear red after treatment with labelled antibodies specific for *F. tularensis*. A. Disintegrating *A. castellanii* trophozoite containing *Francisella*-filled vesicles (day 18). B. Individual vesicle containing viable *F. tularensis* GFP/LVS/ASV (day 18). C. *A. castellanii* cyst containing viable *F. tularensis* inside the cyst double-wall (day 40).

4. 1. 4 Intracellular localisation of F. tularensis in A. castellanii - Electron microscopy analysis

Electron micrographs confirmed that *F. tularensis* cells were located within vacuoles in *A. castellanii* (Fig. 8). The vacuoles containing bacteria seemed to attract amoebal organelles such as mitochondria and rough endoplasmatic reticulum (Fig. 8C and Fig. 8D) As shown in Fig. 8E, *F. tularensis* cells could be seen lining up between an emerging double-wall, a sign of encystation. However, another outcome was also observed in some cases (Fig. 6F, 7C, and 8F), in which all the bacteria seemed to be located inside the double wall of the cyst.

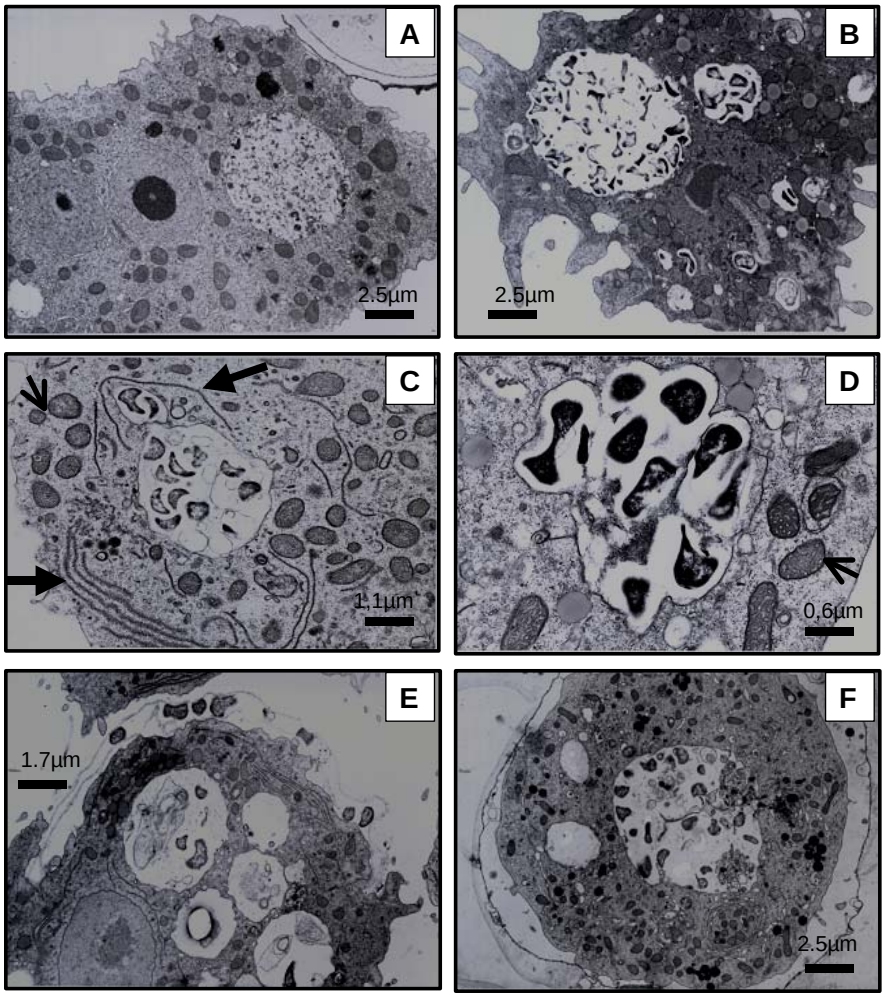

Fig. 8. Electron microscopy analysis. A. *A. castellanii* trophozoite without intracellular *F. tularensis* (day 0). B. *A. castellanii* trophozoite with *Francisella*-filled vacuoles (day 9). C and D. Recruitment of mitochondria (short arrows) and rough endoplasmic reticulum (long arrows) to the vacuole containing bacteria. E. *A. castellanii* trophozoite undergoing encystation with *F. tularensis* cells lined up between the two layers of the emerging double-wall (day 16). F. *A. castellanii* cyst containing *F. tularensis* on the inside of the double-wall (day 16).

4. 1. 5 Growth and survival of singly, co-cultured and intracellular F. tularensis

Viable counts of co-cultured bacteria increased in the presence of *A. castellanii* from 2 x 10^6 cfu/ml at day 0 to 2 x 10^8 cfu/ml at day 20. In contrast, viable counts of singly cultured bacteria decreased from 2 x 10^6 cfu/ml at day 0 to non-detectable levels at day 15 (Fig. 9).

The viability of the *F. tularensis* cells within *A. castellanii* was analysed by taking viable counts of the bacteria after gentamicin treatment to kill extracellular bacteria followed by deoxycholate-treatment of the *A. castellanii* cells to release the intracellular bacteria. No bacteria were recovered from gentamicin-treated medium, but in *A. castellanii* cells, an increase in *F. tularensis* LVS/GFP/ASV viable counts was observed over time from 0 at days 0, 2, 4 and 8 to 2 x 10^6 cells/ml at days 10 and 15 (Fig. 9).

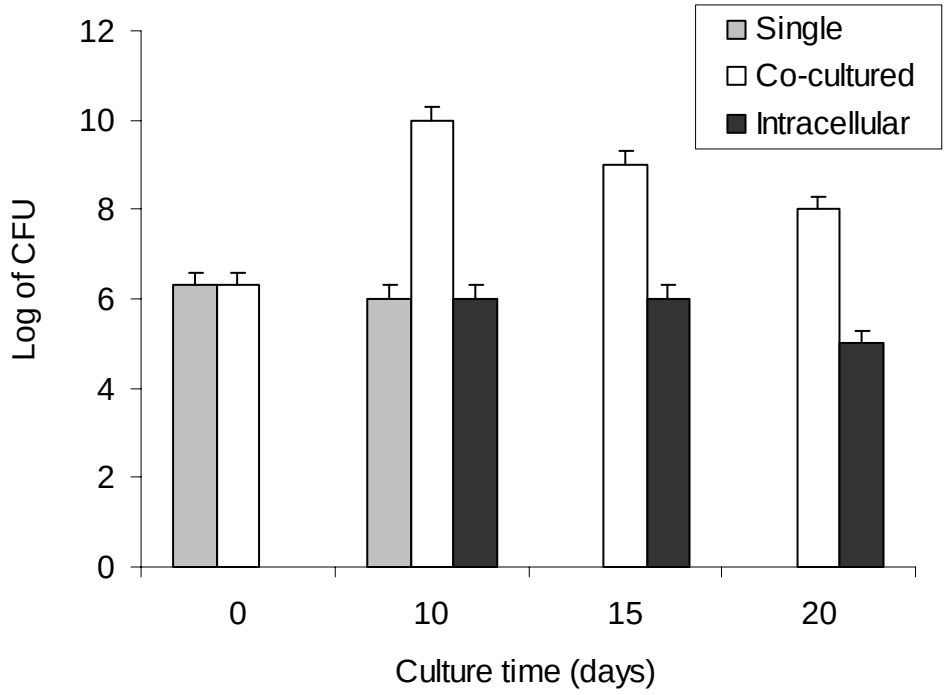

Fig. 9. Viable counts of *F. tularensis* LVS/GFP/ASV. Grey staples indicate singly cultured, white staples co-cultured bacteria with *A. castellanii* and black stables indicate intracellular bacteria. Data indicate mean value ± SD of double experiments. Student's *t*-test was performed for comparison between singly cultured and co-cultured *F. tularensis* with amoebae, $p = 0.016$.

4. 1. 6 Growth and survival of singly and co-cultured A. castellanii

Differences between the numbers of *A. castellanii* in cultures with or without *F. tularensis* LVS/GFP/ASV were also apparent. The total counts, according to Bürker chamber determinations, showed that the numbers of singly cultured *A. castellanii* increased from 4 x 10^5 cells/ml at day 0 to 8 x 10^5 cells/ml at day 20, and decreased from 4 x 10^5 cell/ml at day 0 to 3 x 10^5 cell/ml at day 20 when co-cultured with *F. tularensis* LVS/GFP/ASV (Fig. 10).

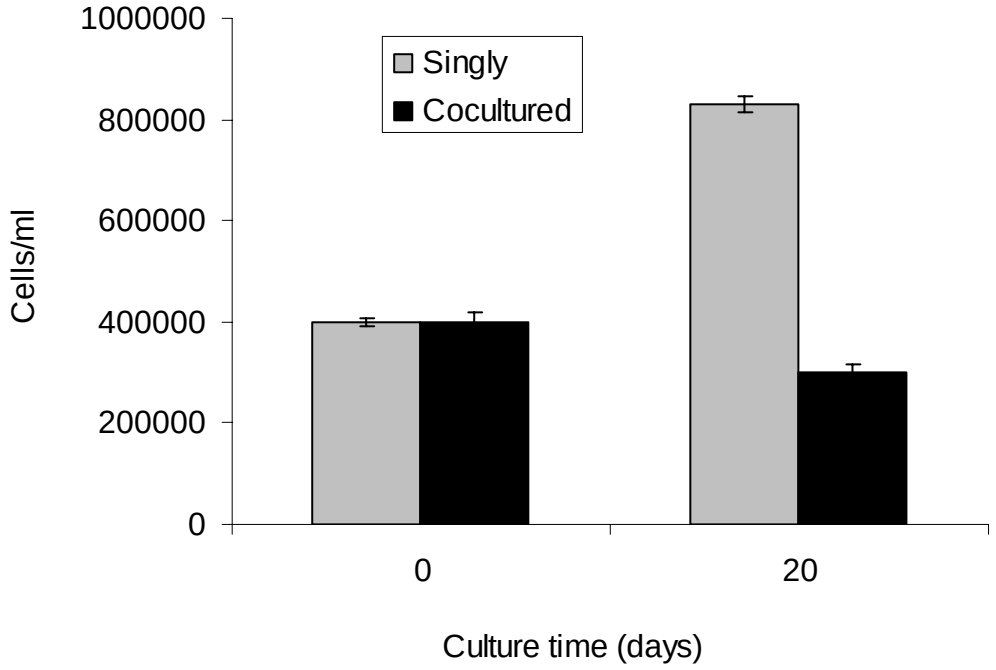

Fig. 10. Counts of *A. castellanii*. Grey staples indicate singly cultured, black staples co-cultured with *A. castellanii* with *F. tularensis* LVS/GFP/ASV. Data indicate mean value ± SD of double experiments. Chi-square test was performed for comparison between singly cultured and co-cultured amoebae, $p < 0.05$.

4. 2 *Pseudomonas aeruginosa* utilises its type III secretion system to kill the free-living amoeba *Acanthamoeba castellanii* (Manuscript) - Paper II

4. 2. 1 Growth and survival of singly and co-cultured A. castellanii - Cell count

Co-cultivation of each *P. aeruginosa* PA103 possessing ExoT, ExoU proteins as well as exotoxin A and its exotoxin A negative, TTSS proteins mutant, a mutant producing ExoS, and a mutant producing ExoY with *A. castellanii* resulting in the death of amoeba populations. ExoT and ExoU proteins killed amoeba population within 3 days, ExoS protein within 4 days and ExoY protein within 10 days, whereas, P. aeruginosa PA103 lacking TTSS proteins killed amoebae within 11 days. Number of viable *A. castellanii* cells co-cultured with *P. aeruginosa* PA103 possessing ExoT, ExoU proteins, counted in Bürker chamber was $2x10^5$ cell/ml (day 0), which decreased to $1x10^5$ cell/ml (day 1), to $2x10^4$ cell/ml (day 2), and no viable A. castellanii cells were seen 4 days post-infection. In comparison, the number of singly cultured *A. castellanii* increased from $2x10^5$ cell/ml to $2x10^6$ cell/ml at day 12 (Fig. 11).

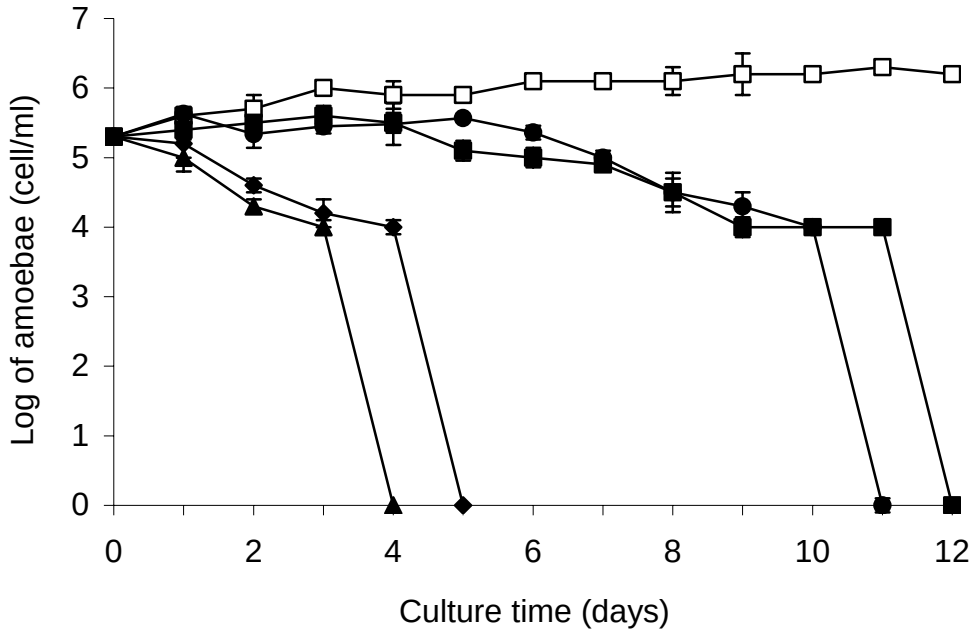

Fig. 11. Counts of *A. castellanii*. Number of *A. castellanii* co-cultured with *P. aeruginosa* 103 possessing the following proteins: Exo T and Exo U (▲), Exo S (♦), Exo Y (●), Exo T and Exo U mutant (■), and singly cultured *A. castellanii* (□). Data indicate mean ± SD values of double measurements.

4. 2. 1. 1 Data analysis

Chi-square test showed a statistical significance between the numbers of singly cultured and co-cultured amoebae ($p < 0.001$). Furthermore, Student's t-test showed statistical significance between the numbers of singly cultured and co-cultured amoebae with each bacterial strain, $p \leq 0.01$ for each strain.

4. 2. 1. 2 Electron microscopic analysis

Electron microscopic analysis using the wild type strain PA103 disclosed that the number of co-cultured *A. castellanii* decreased over time and thus no amoeba cells were seen day 3 post infection indicating cell lysis. The analysis confirmed that 21% of amoeba cells had undergone necrosis at day 1 of co-cultivation and the percentage of necrotic amoeba cells increased to 72% at day 2. The amoeba cells undergoing necrosis characterized by disappearing of nuclei and rapid lysis. The pictures show large number of extracellular bacteria and no intracellular existence in amoeba cells indicating their extracellular nature (Fig. 12).

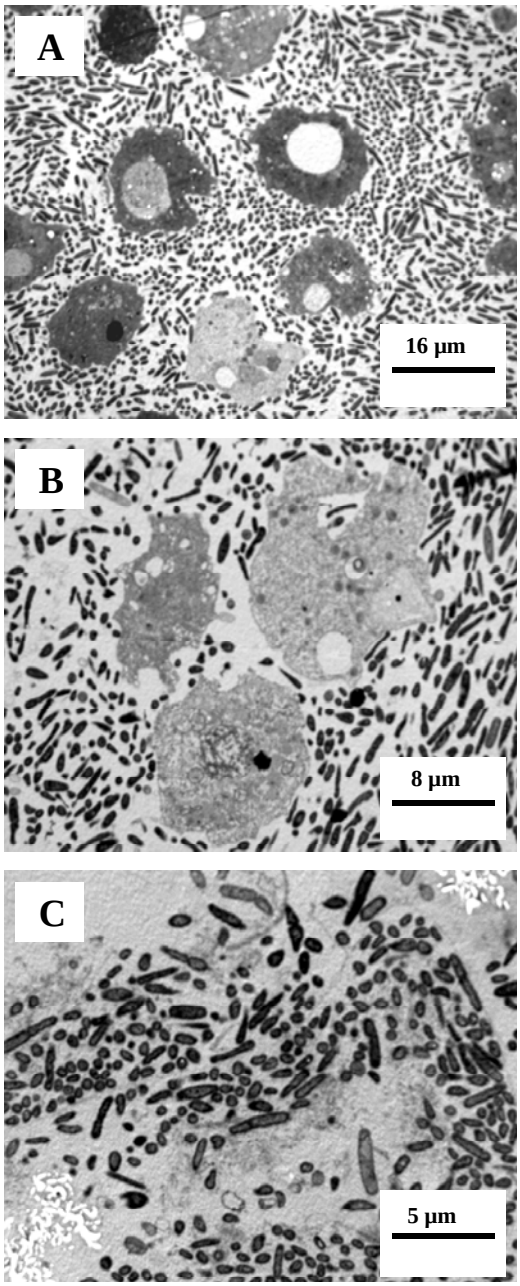

Fig. 12. Electron microscopic analysis showing cytotoxic affects of *P. aeruginosa* 103 possessing Exo U and Exo T proteins on *A. castellanii*, which was characterised by disappearing of nuclei from several amoeba cells indicating necrosis at day 1 and day 2 (A and B), and lysis of all amoeba cells at day 3 (C).

4. 2. 1. 3. Staining of dead A. castellanii cells

100 µl of singly and co-cultured *A. castellanii* with wild type *P. aeruginosa* PA103 was diluted with 100 µl 0.5% basic eosin solution. *A. castellanii* cells were counted in Bürker chamber within 15 min. The percentage of stained cells (dead) was counted over time. Mean values of four measurements *A. castellanii* numbers are presented in Table 3.

Table 3. TTSS proteins effect on viability of *A. castellanii* cells

	Dead *Acanthamoeba castellanii* cells %	
Day	Single-cultured	Co-cultured
0	0	0
1	2	12
2	6	47
3	4	64
4	9	100
10	7	100

4. 2. 1. 4 Growth and survival of singly and co-cultured P. aeruginosa PA 103

Both singly and co-cultured *P. aeruginosa* strains grew from 10^3 cells/ml at day 0 to 10^8 CFU/ml at day 1 to day 12. Neither presence nor absence of *A. castellanii* affected the growth of the bacteria (t test, $p > 0.05$).

This finding may explain how a free-living and a strict extracellular bacterium such *P. aeruginosa* can survive in the environment by utilising TTSS proteins to kill its eukaryotic predators such as *Acanthamoebae*.

4. 3 Interaction of *Vibrio cholerae* with *Acanthamoeba castellanii* -Paper III and IV

4. 3. 1 Growth and survival of singly and co-cultured A. castellanii with V. cholerae

Singly cultured A. castellanii and co-cultured with V. cholerae O1 classical, O1 El Tor and O139 strains for 14 days was followed by amoebae cell counts. The number of amoeba cells increased from $2.0x10^5$ cell/ml day 0 to $2.15\ x10^6$, $2.5\ x10^6$, $1.8x10^6$ and $2.0x10^6$ cell/ml, respectively, on day 14 (Fig. 13). The presence of V. cholerae strains did not inhibit the growth of the amoebae.

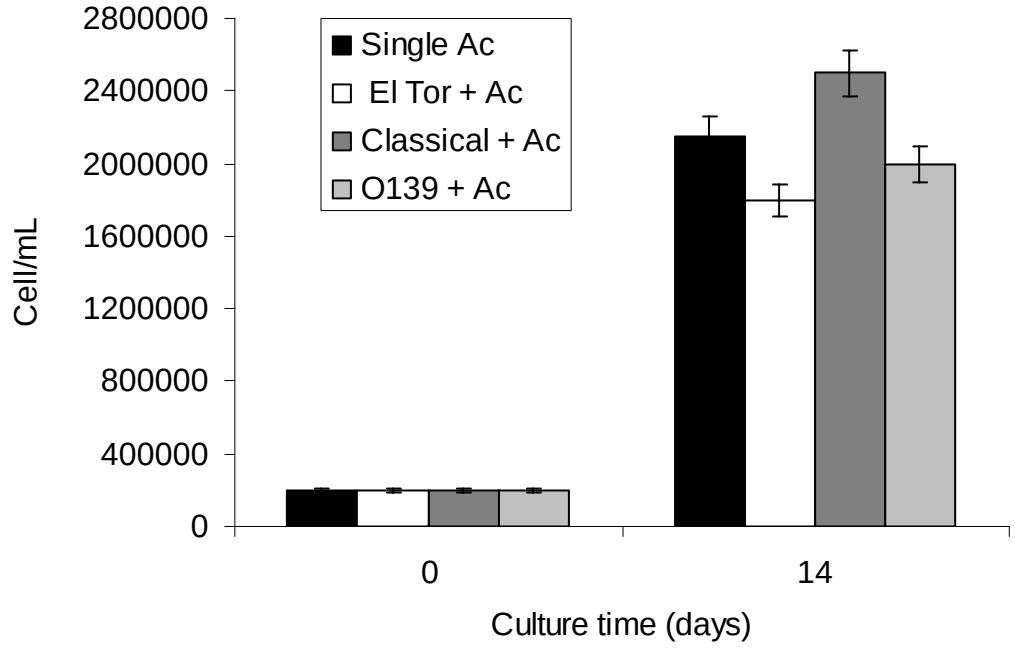

Fig. 13. Counts of amoebae cells. Black staples indicate singly cultured *A. castellanii* and the white *A. castellanii* co-cultured with *V. cholerae* O1 El Tor strains, dark grey with classical strains and light grey with strain O139. Data indicate mean values of double measurements for strain O139 and of six independent experiments for both classical and El Tor strains. Bars indicate standard deviations.

4. 3. 2 Growth of single and co-cultured V. cholerae

The viable counts of co-cultured *V. cholerae* O1 classical and El Tor strains as well as strain O139 with amoebae showed an increase from $2.0x10^6$ CFU/ml day 0 to $3.0x10^7$, $4.0x10^7$, $4.0x10^7$ CFU/ml and to $2.5x10^8$, $3.0x10^8$, $7.8x10^7$ CFU/ml as well as to $2.5x10^{10}$, $3.0x10^9$ and $2.5x10^8$ CFU/ml on day 1, 4, and 14, respectively.

Viable counts of both *V. cholerae* O1 and strain O139 in the absence of amoebae decreased from $2.0x10^6$ CFU/ml day 0 to non-detectable levels by cultivation from day 4 (Fig. 14).

The presence of *A. castellanii* enhanced survival of co-cultured *V. cholerae* O1 and strain O139 during 2 weeks, while the number of singly cultured bacteria decreased to non-detectable levels within 4 days.

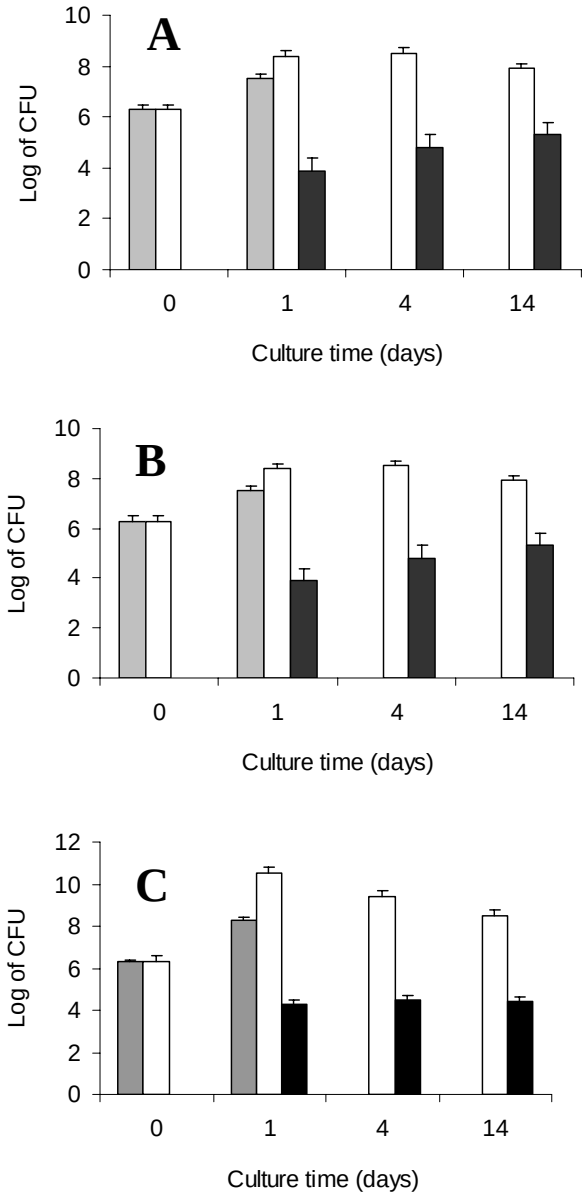

Fig. 14. Viable counts of *V. cholerae* strains. A. Count of *V. cholerae* O1 classical strains, B. count of El Tor strains, and C. count of strain O139. Grey staples indicate singly cultured *V. cholerae*, white staples *V. cholerae* co-cultured with *A. castellanii* and black stables indicate intracellular *V. cholerae*. Data indicate mean ± SD of six independent experiments of O1 strains and double measurements of strain O139. Student's *t*-test was performed for comparison between singly cultured and co-cultured strains with amoebae. For O1 strains $p < 0.001$ and for strain O139 $p = 0.001$.

4. 3. 3 Growth of intracellular V. cholerae strains

After washing, killing of extracellular bacteria from co-culture samples by gentamicin treatment and permeablising of amoebae cells by sodium deoxycholate solution to reach intra-amoebic bacteria viable counts were performed. Viable counts of culturable intracellularly growing *V. cholerae* O1 classical and El Tor as well as strain O139 showed an increase in the number from non-detectable levels on day 0 to $1.25x10^3$, $1.5x10^4$, $1.5x10^5$ CFU/ml and to $8.0x10^3$, $6.0x10^4$, $2.0x10^5$ CFU/ml as well as to $2.0x10^4$, $2.0x10^4$ and $2.5x10^4$ CFU/ml on day 1, 4, and 14 respectively (Fig. 14).

4. 3. 4 Intracellular localisation of V. cholerae

Different methods such as confocal microscopy and electron microscopy were used to confirm the intracellular localisation of *V. cholerae* strains in *A. castellanii*. In addition, a PCR method was utilised to detect *Acanthamoeba* and *V. cholerae* from co-cultures.

4. 3. 4. 1 Confocal microscopy

Samples from co-culture flasks containing *A. castellanii* and each of *V. cholerae* O1 GFP classical strain and *V. cholerae* O1 GFP El Tor strain were washed, mounted and examined by confocal microscopy. Photomicrographs showed an intracellular localisation of *V. cholerae* O1 inside *Acanthamoeba* cells. The bacterial cells emitted green fluorescence, while amoebae cells emitted red autofluorescence (Fig. 15).

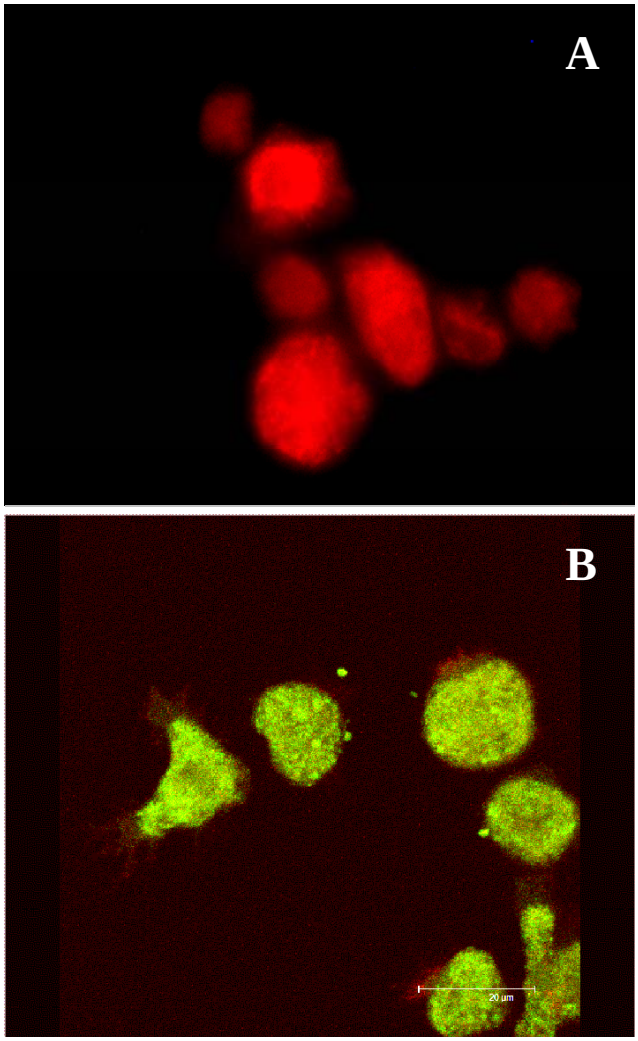

Fig. 15. Confocal microscopy analysis. A. Amoebae cells emitting red autofluorescence as negative control. B. Showing intracullar localisation of *V. cholerae* O1 classical GFP emitting green fluorecsence inside *A. castellanii* trophozoites and cysts at day 3 of co-cultivation.

4. 3. 4. 2 Electron microscopy

Singly cultured *A. castellanii* cells as well as co-cultured with *V. cholerae* O1 and strain O139 were prepared for electron microscopy. Microscopic pictures showed that *V. cholerae* cells were localised intracellularly in vacuoles of *A. castellanii* trophozoites after 3 hours of co-cultivation (Fig. 16B). Multiplication of the bacteria occurred in the cytoplasm of trophozoites 1-7 days after co-cultivation (Fig. 16C and 16D). 3-7 days after co-cultivation *A. castellanii* cysts were heavily loaded with intracellularly located *V. cholerae* cells (Fig. 16E and 16F).

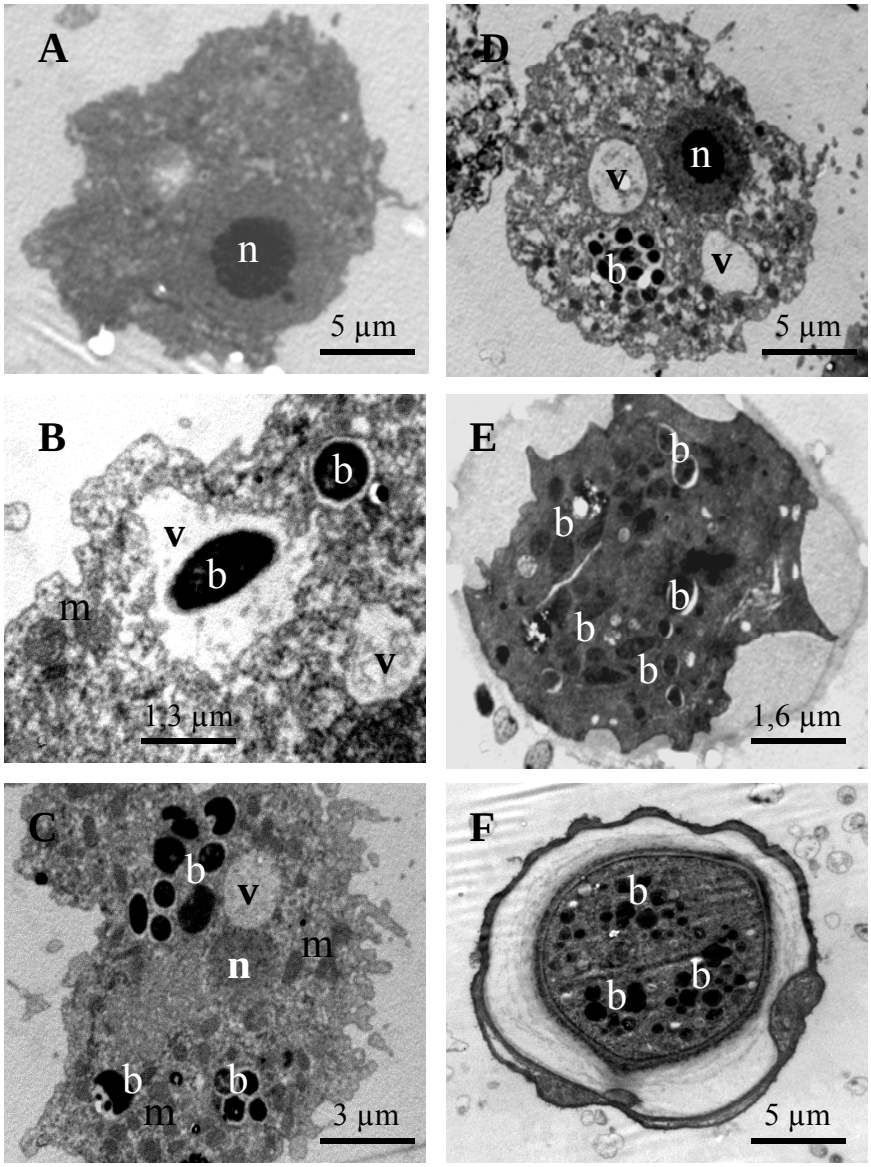

Fig. 16. Electron microscopy analysis of intracellular localisation of *V. cholerae* in *A. castellanii*. The letters in micrographs indicate the following: b; bacterium, m; mitochondria, n; nucleus, and v; vacuole. (A) *A. castellanii* trophozoite without intracellular *V. cholerae*. (B) *A. castellanii* trophozoite showed only few *V. cholerae* O139 cells localised intracellularly in vacuoles (3h post-infection). (C) *A. castellanii* trophozoite with many *V. cholerae* O139 cells localised intracellularly in cytoplasm (1 day post-infection). (D) *A. castellanii* trophozoite with many *V. cholerae* O1 El Tor cells localised intracellularly in cytoplasm (3 days post-infection). (E) *A. castellanii* cyst heavily loaded with intracellularly located *V. cholerae* O1 El Tor (3 days post-infection). (F) *A. castellanii* cyst heavily loaded with intracellularly located *V. cholerae* O1 classical (7 days post-infection)

4. 3. 4. 3 PCR

It is possible to detect presence of *Acanthamoeba* species and their bacterial endosymbionts (toxigenic *V. cholerae*) in co-culture samples by utilising molecular biological analysis. Cholera toxin gene as well as amoebic18S rRNA gene from co-cultured samples after gentamicin killing and washing of extra-amoebic *V. cholerae* was detected by a PCR method (Fig. 17).

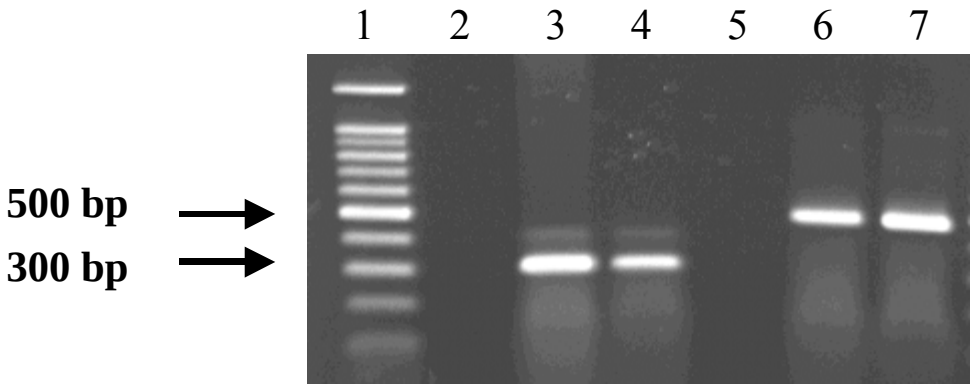

Fig. 17. Agarose gel electrophoresis of PCR products of cholera toxin gene (*ctxA*) from *V. cholerae* O1 classical strain C-19385 and *Acanthamoeba* 18S rDNA gene. Lane 1 is molecular mass marker (1500 bp), 2 bacterial negative control, 3 positive control (308 bp), 4 bacterial sample, 5 amoebic negative control, 6 amoebic positive control (approximately 450 bp) and 7 amoebic sample.

4. 3. 5 Stability of intracellular survival of Vibrio cholerae O1

V. cholerae O1 strains were co-cultivated with *A. castellanii* for 2 weeks. Number of extracellular and intracellular grown *V. cholerae* was estimated by viable counts before and after gentamicin killing of extracellular bacteria. E-test was used to examine susceptibility of the bacteria to several antibiotics before and after their intracellular growth. Viable count, fluorescence microscopy and UV light were used to detect the continuous viability of intracellular grown bacteria, which are able to produce green fluorescent proteins. Antibiotic assay was used to differentiate between extracellular and intracellular bacteria is used in this study.

MIC values of ciprofloxacin, gentamicin and tetracycline before and after intracellular growth of *V. cholerae* for two weeks were not significantly differentiated indicating a stable antibiotic susceptibility of intracellular grown bacteria. Student's t-test was used for comparison between MIC values before and after intracellular growth and *p* values were 0.71 and 0.95 for classical and El Tor strains, respectively (Table 4).

Green fluorescence emission from the intracellular bacteria detected by fluorescence microscopy and UV light showed a stable viability of the intracellular bacteria. Antibiotic assay differentiated between extracellular and intracellular bacteria. Gentamicin and tetracycline killed only the extracellular bacteria, while ciprofloxacin killed both. This assay can be used to differentiate between extracellular and intracellular bacteria.

Table 4 A. MIC mean values before and after 2 weeks of intra-amoebic survival of six classical strains.

Antibiotic	Mean of MIC value µg/ml	
	Before	After passage
Gentamicin	1.10	1.10
Tetracycline	0.025	0.121
Ciprofloxacin	0.023	0.002

Table 4 B. MIC mean values before and after 2 weeks of intra-amoebic survival of 6 El Tor strains.

Antibiotic	Mean of MIC value µg/ml	
	Before	After passage
Gentamicin	1.3	1.2
Tetracycline	0.4	0.4
Ciprofloxacin	0.1	0.1

5 DISCUSSION

The waterborne pathogens cause global problems to the public health. The present project examines the interaction between *A. castellanii* and *F. tularensis*, *P. aeruginosa* and *V. cholerae* in order to find out whether a symbiotic or predatory relation between *A. castellanii* and the waterborne bacteria exists. The interaction was studied by different methods.

The results of Paper I show that the facultative intracellular *F. tularensis* can survive and grow intracellularly in *A. castellanii*. The infection process begins when trophozoites engulfing *F. tularensis* cells, which replicate and grow in vacuole structures inside the trophozoites. Thus, the infection process in amoeba shows resemblance to *Francisella* infection in macrophages (5, 48). Electron microscopy analysis shows that cell organelles such as mitochondria and endoplasmatic reticulum are recruited to the vacuoles containing bacteria (Fig. 8C and 8D). Infected trophozoites are found both intact, filled with vacuoles containing *F. tularensis* and in the process of cytolysis, excreting vesicles containing *F. tularensis*. Some infected trophozoites are also seen undergoing encystation and *F. tularensis* cells are found in precyst and in mature cyst (Fig. 8E and 8F). The infection cycle of *F. tularensis* in *A. castellanii* seems to display many features in common with *Legionella* infection in *A. castellanii* (60).

Viable counts of co-cultured *F. tularensis* show that the presence of *A. castellanii* enhanced its growth. This finding is in accordance with previous results (59) and is probably due to the *F. tularensis* using CO_2 produced from live *A. castellanii* cells and nutrients derived from dead ones. In addition, intracellular *F. tularensis* may escape into the culture media after lysis of the *A. castellanii* cells.

Counts of *A. castelanii* cells show that there are 25% fewer amoebae when co-cultured with *F. tularensis*, than when grown alone, apparently because *F. tularensis* kills a substantial number. Furthermore, the increase of fluorescence inside *A. castellanii* cells with time shows that the number of *F. tularensis* per amoeba increases over time. Accordingly, viable counts of *F. tularensis* release from *A. castellanii* by deoxycholate treatment also increase over time.

In this study the attenuated vaccine strain *F. tularensis* LVS was used. Compared to virulent strains of *F. tularensis*, this strain obviously is not highly virulent for humans.

However, it is still highly virulent for mice and guinea pigs (118). It is possible that the LVS strain compared to fully virulent *F. tularensis* strains could have a different toxic effect on the protozoan host.

The ability of *F. tularensis* to survive in trophozoites of *A. castellanii* and cysts demonstrated in this study may have implications for the mode of transmission of the bacteria. The close connection of tularaemia with water (101) and the isolation of the bacterium from water samples used for domestic purposes, as well as from natural water systems, as the causal agent of outbreaks of the disease (101) support the hypothesis that amoebae may play a role in the natural transmission of *F. tularensis.*

The extracellular and free-living *P. aeruginosa* produces several extracellular enzymes and toxins that may be used for its survival in natural environments in order to inhibit or kill competing or predatory eukaryotic cells.

Paper II examined the effect of different TTSS proteins as well as exotoxin A on free-living amoeba by co-cultivation of *A. castellanii* cells with wild type *P. aeruginosa* producing ExoT and ExoU as well as exotoxin A or isogenic mutant strains, followed by counting of amoeba cells, electron microscopy and statistical analysis. The analysis showed that all bacterial strains used in the study killed the amoeba cell populations at different time intervals and confirmed that co-cultured amoeba cells had undergone necrosis. Moreover, the analysis showed large number of extracellular bacteria and no intracellular existence in amoeba cells indicating their extracellular nature and neither presence nor absence of *A. castellanii* affected the growth of the bacteria.

Vallis et al. (129) observed that Exo U and Exo S are cytotoxic causing irreversible damage in morphology and cell membranes of eukaryota as well as necrotic death, while Exo T and Exo Y have no cytotoxic effects. Shaver et al. (120) assessed effects of TTSS proteins on a mouse model of acute pneumonia by measurements of mortality, as well as bacterial persistence in the lung and he found that secretion of ExoU had the greatest impact on virulence while secretion of ExoS had an intermediate effect and ExoT had a minor effect. It has been shown that ExoT possesses only 0.2% of the enzymatic activity of ExoS (136) and Exo U was required to kill the soil free-living amoeba *Dictyostelium discoideum* (109). Therefore, it can be concluded that Exo T has no remarkable affect on the killing of *A. castellanii*, and that killing was caused mainly by Exo S and Exo U, while ExoY and exotoxin A did not kill the amoeba. The statistical significance estimated by t-test (p= 0.004) between growth of singly cultured amoebae and co-cultured with *P. aeruginosa* PA103 lacking the known four TTSS proteins may indicate presence of additional TTSS or non-TTSS toxic products, which could be identified in the future.

Several virulence factors of *P. aeruginosa* such as proteases, rhamnolipids, pyocyanin, and exotoxin A were produced under control of two quorum-sensing systems Las and Rhl

(75) when the bacterial cell density reaches a certain threshold (104). It has been shown that factors produced by Las quorum-sensing system are not involved in inhibition or killing of *D. discoideum* (30, 109) while factors produced by Rhl quorum-sensing system inhibited growth of *Dictyostelium* cells (30).

In humans or in nature it is likely that *P. aeruginosa* uses various survival strategies by utilising its virulence factors such as cell components, extracellular products, and TTSS proteins. Previous studies have shown that *P. aeruginosa* utilises its TTSS proteins to kill macrophages and epithelial cells (27, 113). Moreover, Pukatzki et al. (109) demonstrated that TTSS proteins are necessary for *P. aeruginosa* to kill the amoeba *D. discoideum* and that the ExoU protein played a key role. The result of paper II confirmed this role of TTSS proteins, and found a possible role for ExoS in the used experimental system.

P. aeruginosa is a free-living bacterium in nature and adapted as a strict extracellular bacterium that can be easily killed by phagocytosis. Therefore, it utilises its TTSS proteins to kill phagocytic cells, both amoebae and macrophages, to avoid being ingested.

Cholera is a severe diarrhoeal disease caused by *V. cholerae* O1 or O139, which are waterborne bacteria (111). The disease is a major public health problem in many parts of the world causing large numbers of deaths during pandemics (116). The infective dose of *V. cholerae* is very high; therefore, the bacterium would require a biological reservoir for amplification of its numbers to high concentration in the environment. The reservoirs for survival and multiplication of *V. cholerae* are far from completely known (68).

V. cholerae and *A. castellanii* inhabit aquatic environments (9) and it has been found that *V. cholerae* survives when associated with zooplankton (123) and attached to various freshwater plants (66) as well as algae (68). Fritsche et al. (50) has found that 25% of environmental and clinical *Acanthamoeba* species isolates contain obligate bacterial endosymbionts. Moreover, Thom et al. (126) showed that *V. cholerae* could survive and multiply during 24 h in microcosms pre-inoculated with trophozoites of freshwater amoebae. Intracellular behaviour of *V. cholerae* O139 and O1 as well as their ability to grow and survive in *A. castellanii* were investigated in Paper III and IV. It was found that *V. cholerae* O1 and O139 strains grew and survived intracellularly inside *A. castellanii* for more than 2 weeks.

Intracellular pathogens use different mechanisms to survive and multiply within their phagocytic host cells such as amoebae and mammalian macrophages. *L. pneumophila* survives and multiplies intracellularly in specialized phagosomes, which have neutral pH and do not fuse with lysosomes (11). *Shigella* escapes into the cytoplasm avoiding lysosomal

digestion (94). *Francisella* survives within phagosomes in macrophages (56) and within intracellular membrane limited vacuoles in *A. castellanii* (Paper I).

It is well known that amoebae use fusion of lysosomal granules with phagosomes to ingest food and bacteria by phagocytosis in acidic vacuoles (39, 99). *V. cholerae* is sensitive to killing in acidified media at pH 5.0 but survives at pH 6.0 (133). During phagocytosis in amoebae the endosomal pH decreases at the first 20 min from 5.4-5.8 to 4.6-5.0, following an increase within the next 20-40 min to pH 6.0-6.2 and the digested nutrients become more alkaline and diffuse out to supply the cell parts (7, 39, 99). It has been shown that *V. cholerae* strains prefer neutral or slightly alkaline conditions for their growth (16). Thus, *V. cholerae* cells find the suitable conditions for their intra-amoebic growth in the cytoplasm, which has a pH around 7.2.

Previous studies showed that *V. cholerae* needs 10^8 to 10^9 cells to cause cholera (71, 116). Therefore, the bacterium needs an environmental host to grow to high numbers to be able to infect humans.

Presence of *A. castellanii* cells in co-cultures enhanced growth and survival of *V. cholerae* strains, while the presence of *V. cholerae* strains neither inhibited nor stimulated the growth of *A. castellanii*. Therefore, the relationship between *V. cholerae* strains and *A. castellanii* is symbiotic. Interestingly, *V. cholerae* strains, which are believed to be extracellular bacteria (58), occurred as facultative intracellular bacteria in study III and IV. The facultative intracellular behaviour of *V. cholerae* and their symbiotic interaction with *A. castellanii* shown in our studies could, in part explain the ability of *V. cholerae* to avoid the amoebic phagocytosis and may justify why *V. cholerae* loses to predators in environment as mentioned by (32).

Aeromonadaceae, Pseudomonadaceae and Vibrionaceae are families belonging to Gram-negative bacteria, which co-exist with each other in aquatic environments. The opportunistic bacterial pathogens detected in the water include *Aeromonas hydrophila* and *P. aeruginosa* (115) as well as *V. cholerae* (9).

Aeromonas, *Pseudomonas* and *Vibrio* species are extracellular and free-living bacteria. Both *A. hydrophila* (25) and *P. aeruginosa* (91) possess type III secretion system (TTSS) while toxigenic *V. cholerae* does not, although several strains of non-O1/O139 *V. cholerae* strains have recently been shown to possess genes for TTSS (41).

It has been shown that *A. hydrophila* (unpublished data) and *P. aeruginosa* (Paper II) can kill *A. castellanii* in co-cultures, while *F. tularensis* (Paper I) and *V. cholerae* (Paper III and IV) can grow and survive intracellularly and symbiotically within *A. castellanii*. These

findings indicate that extracellular bacteria like *Aeromonas* and *Pseudomonas* have their virulence factors that are able to kill phagocytic cells because these strictly extracellular bacteria have no antiphagocytic strategies. Therefore, they need TTSS proteins to kill their predators before being ingested in order to live as free-living cells in aquatic environments in contrast to other strict extracellular bacteria such as *Escherichia coli* and *Klebsiella aerogenes*, which have been found to be excellent nutrients to *A. castellanii* and *A. polyphaga* and accordingly they are rapidly killed by amoeba (2).

The toxigenic *V. cholerae* resembles the facultative intracellular bacteria such as *L. pneumophila* (3, 76) and *F. tularensis* (54) since they all lack TTSS and can survive in *A. castellanii* (138). The *icmF* and *icmH* genes were required for intracellular multiplication of *L. pneumophila* in *A. castellanii*. IcmF and IcmH proteins are found in many bacteria such as *Yersinia pestis*, *Salmonella enterica* and *V. cholerae*, which associate with eukaryotic cells (138). It has been shown that *V. cholerae* possesses *icmF* gene (35). All these evidences show that *V. cholerae* differs from strictly extracellular bacteria and instead resembles facultative intracellular bacteria. Therefore, *V. cholerae* species should be considered as facultative intracellular bacteria.

Both differences as well as common properties between *V. cholerae* O1 and *V. cholerae* O139 serogroups have been found. *V. cholerae* O1 serogroup is divided in El Tor and classical biotypes on the basis of biochemical properties and phage sensitivity. It is well documented that *V. cholerae* O139 possess a capsular polysaccharide, whereas O1 strains do not (70). *V. cholerae* O1 El Tor biotype as well as O139 serogroup possesses mannose-sensitive hemagglutinin (MSHA), which is required for colonisation to zooplankton (26). Moreover, the common property of epidemic *V. cholerae* serogroups is the presence of toxin co-regulated pilus (TCP), which is a colonisation factor to the intestine of humans (71).

Although there are differences between toxigenic *V. cholerae* serogroups and biotypes, our studies III and IV show that *V. cholerae* strains O1/O139 grow and survive intracellularly in *A. castellanii*. These findings show that *V. cholerae* strains have a facultative intracellular behaviour as a new common property and a symbiotic relationship with *A. castellanii*.

The intracellular behaviour and symbiotic relationship of *V. cholerae* with *A. castellanii* presented in our studies may support the role of free-living amoebae as trainings grounds for intracellular parasites (92) and as environmental hosts of the epidemic *V. cholerae*.

The stable antibiotic susceptibility and viability of intracellular grown *V. cholerae* in *A. castellanii* may support the endosymbiotic relation between the two microorganisms.

Moreover, antibiotic assay differentiated between extracellular and intracellular bacteria determined which antibiotic that was able to kill both extracellular and intracellular localised bacteria. These findings help to identify an effective antibiotic treatment of the infections caused by the intracellular bacteria, whereas, it has been shown previously that aminoglycosides failed to treat infections caused by strict or facultative intracellular pathogens (84).

6 CONCLUSIONS

Methods used in this project to study the interaction between waterborne microorganisms showed that the extracellular bacterium *P. aeruginosa* killed *A. castellanii*, whereas the facultative intracellular bacterium *F. tularensis* grew and survived in *A. castellanii*.

V. cholerae, which was held to be an extracellular bacterium, could survive and multiply in *A. castellanii* and showed a facultative intracellular behaviour. Thus, the relation between *P. aeruginosa* and *A. castellanii* was predation, whereas, it was symbiosis between *A. castellanii* and each of *F. tularensis* and *V. cholerae*.

The role of free-living amoebae as environmental hosts for *F. tularensis* and *V. cholerae* is possible according to their stable symbiotic relation.

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Does the life really as described before like a walking shadow or a brief candle? I hope that the young generation of our family Jacob, Isac and Aida have a different description for the life.

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