universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Environmental chlamydiae as new emerging pathogens:

Characterization of two putative TTSS substrates and serological analyses of environmental chlamydiae

verfasst von

Irina Kolarov

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 444
Studienrichtung lt. Studienblatt:	Diplomstudium Ökologie
Betreut von:	Univ.-Prof. Dr. Matthias Horn

To my Dad

A. Introduction

A.1. The order *Chlamydiales*

The order *Chlamydiales* is an independent major line of the domain *Bacteria* with a distant relationship to the order *Planctomycetales*.

The *Chlamydiales* represents an order of obligate intracellular bacteria which need a eukaryotic host for their multiplication (Fig. A.1.). They comprise the families *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* (Everett et al., 1999) (see Fig. A.2.) and have a wide host range including largely mammals but also invertebrates like arthropods (Corsaro et al., 2006), protozoa (Greub and Raoult, 2004; Vannini et al., 2005), fish (Draghi et al., 2004; Meijer et al., 2006) and molluscs (Harshbarger and Chang, 1977). Especially the family of the *Chlamydiaceae* represents important human pathogens. The *Chlamydiaceae* consist of the genera *Chlamydia* and *Chlamydophila*. Members of these genera are known to trigger a variety of diseases in humans and animals (Schachter, 1999; Campbell and Kuo, 2003). *Chlamydia trachomatis* is known to be the most frequently sexually transmitted pathogen of humans (Mabey et al., 2003). It is estimated that around 10% of all community-acquired pneumonia cases are caused by *Chlamydophila pneumoniae* (Marrie et al., 1996), and there is indication of a connection between *Chlamydophila pneumoniae* infections and artheriosclerosis (Grayston, 2000; Belland et al., 2004). Chlamydiae are also associated with arthritis (Taylor-Robinson et al., 1992), Alzheimer (Renvoize et al., 1987) and heart diseases (Saikku et al., 1988).

All members of the *Chlamydiales* show a biphasic developmental cycle (Abdelrahman and Belland, 2005) that is unique among the bacteria. The elementary bodies (EB) are the infectious, metabolically inactive form with a diameter of 0.2 to 0.6 µm, which show an electron-dense appearance in ultrastructure studies. The infectious EBs display a robust cell wall which shows an unusual composition with a low content of peptidoglycan. The stability of the cell wall is due to the high proportion of cysteine- and methionine-rich proteins cross-linked by disulfide bonds (Hatch, 1996; Hackstadt et al., 1997b).

The reticulate bodies (RB, up to 1.5 µm in diameter) are the metabolically active form able to multiply by binary fission. This form is also capable of incorporating host cell's metabolites (Abdelrahman and Belland, 2005).

Chlamydial infection (Fig. A.1.) is initiated by the attachment of EBs to the host cell's surface, which are ultimately taken up by the host via phagocytosis. So far, the mechanisms of bacterial attachment and uptake have not been completely elucidated.

The EBs within the host cell are enclosed by cytoplasmatic vesicles, the so-called inclusion, where they differentiate into RBs. RBs are metabolically active and capable of cell division. Bacterial multiplication is followed by a re-differentiation of RBs to EBs. In pathogenic chlamydiae, the EBs then trigger the lysis of the host cell resulting in their release into the environment and the start of a new infection cycle (see Fig. A.2.) (Everett, 2000). Environmental chlamydiae on the other hand, are able – under favourable environmental conditions – to establish a symbiosis with its host cell and maintain a stable host-parasite ratio (Amann et al., 1997; Horn et al., 2000).

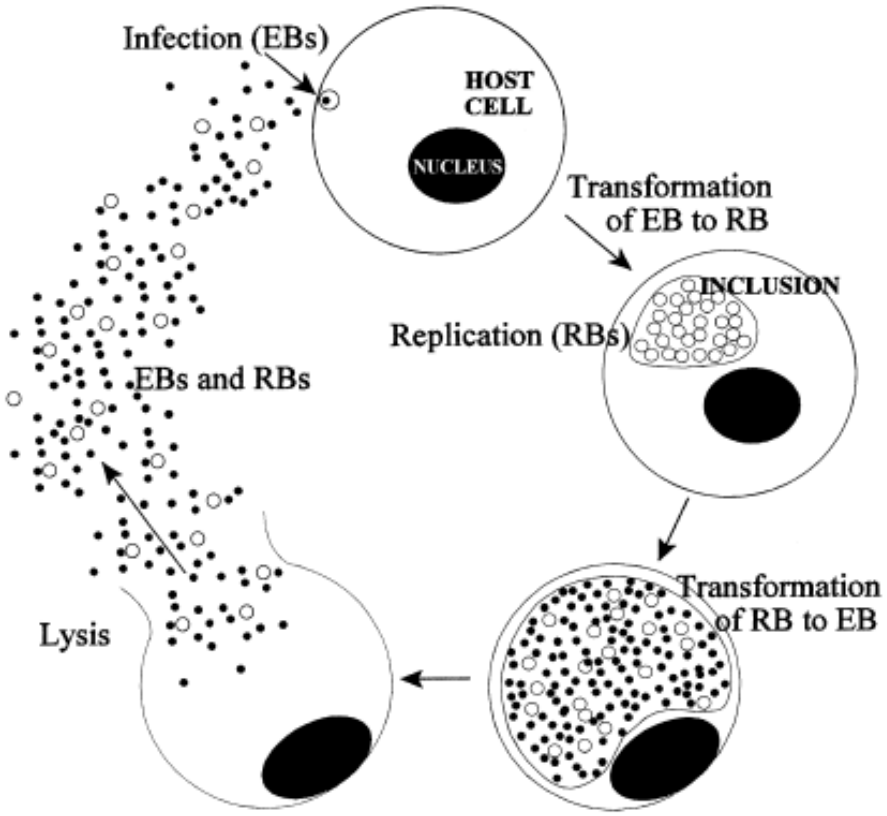

Fig. A.1. The chlamydial developmental cycle (Everett, 2000).

A.2. Environmental chlamydiae

Birtles and Amann could show for the first time the presence of chlamydia-like organisms in protozoa (Amann et al., 1997; Birtles et al., 1997). 16S rRNA analyses demonstrated that the organisms found within these protozoa belonged to the *Chlamydiales* but displayed

a new phylogenetic lineage forming the new family of the *Parachlamydiaceae* with the genera *Neochlamydia*, *Parachlamydia* and *Protochlamydia* (Amann et al., 1997; Fritsche et al., 2000a; Horn et al., 2000; Collingro et al., 2005a). Beside the *Parachlamydiaceae*, other chlamydia-related organisms belonging to the order *Chlamydiales* and forming the new families of the *Waddliaceae* and *Simkaniaceae* were described. *Waddliaceae* were isolated from a bovine fetus (Rurangirwa et al., 1999), while *Simkaniaceae* were first discovered as a cell culture contaminant (Kahane et al., 1999).

Other important environmental chlamydiae are *Rhabdochlamydia* spp. (Corsaro et al., 2006) found as pathogens of cockroaches and *Piscichlamydia salmonis* which was discovered to infect fish gills (Draghi et al., 2004) (Fig. A.2.).

Environmental chlamydiae could be isolated from their natural hosts and some of them could also be transferred into *Acanthamoeba* spp. in cross-infection experiments (Gautom and Fritsche, 1995).

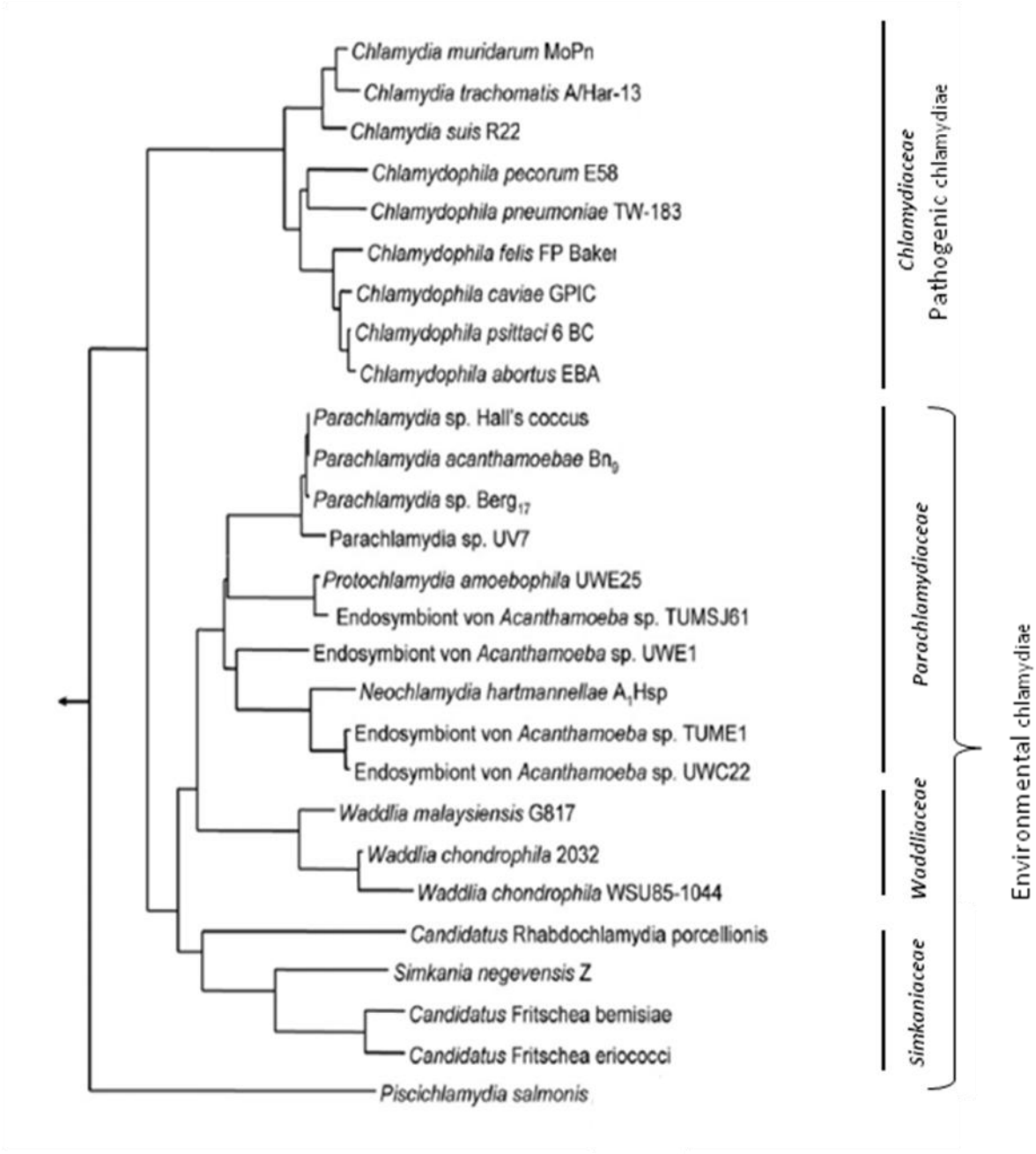

Fig. A.2. The order *Chlamydiales*. 16S rRNA-based tree. Modified after (Collingro and Horn, 2005).

A.2.1. Amoebae as hosts for environmental chlamydiae

The majority of environmental chlamydiae found so far thrive in free-living amoebae (FLA) (Greub and Raoult, 2004), namely *Acanthamoeba* spp. FLA are ubiquitous and could be isolated from different habitats. Amoebae feed on bacteria and other microorganisms like fungi, algae and other protozoa and have an important role for the ecosystem as predators of the microbial community (Rodriguez-Zaragoza, 1994).

Acanthamoebae display a biphasic life cycle. The trophozoites represent the active stage during which the cells multiply by dichotomy. Trophozoites are characterized by the locomotive acanthapodia, a large contractile vacuole, as well as the centrally localized nucleus. Lack of nutrients or other stress factors can trigger encystation of the amoebal cells. The cyst being a survival form is enclosed by a double cell wall, non-motile and extremely resistant to temperature- and pH-changes as well as chemical substances.
Although acanthamoebae feed on prokaryotes, some bacteria are able to evade phagocytosis and multiply in the amoebal cells (Greub and Raoult, 2004). Among these bacteria are some important human pathogens like *Legionella pneumophila* (Rowbotham, 1980) or *Chlamydophila pneumoniae* (Essig et al., 1997).

In this study, two members of the *Parachlamydiaceae* and one member of the *Simkaniaceae* were analysed with respect to pathogenicity and their effects on the human immune system. These organisms are described below.

A.2.2. *Parachlamydiaceae*

One important member of the *Parachlamydiaceae* is the amoebal endosymbiont *Protochlamydia amoebophila* UWE25. The originally symbiont-free amoebal strain *Acanthamoeba* spp. UWC1 (University of Washington clinical isolate 1, C1) was isolated from samples of the human cornea. The intracellular bacteria *P. amoebophila* were isolated from the strain *Acanthamoeba* spp. UWE25 (University of Washington environmental isolate 25) and transferred into the C1-amoebal strain in a cross-infection experiment. It could be shown that the chlamydia-like endosymbiont *P. amoebophila* was able to completely lyse some amoebal strains while in others a stable symbiotic interaction was maintained which resulted in an increase of the cytotoxic potential of the amoebal cells (Gautom and Fritsche, 1995).
The genome of *P. amoebophila* is twice as large as that of pathogenic chlamydiae (Kalman et al., 1999) and shows little indication of recent lateral gene acquisition. Only a few pseudogenes mostly affecting transposases were found. This indicates that the genome of *P. amoebophila* is not in the process of becoming smaller but has instead reached a stable size. Out of the 2031 predicted genes, 938 show significant homologies to other chlamydial genes. Overall, 711 genes are present in all chlamydial genomes encoded so far and represent the core of chlamydial genes. The fact that pathogenic chlamydiae have a smaller genome and have lost several genes when compared to *P. amoebophila* is mainly

due to the fact that their environment is less affected by fluctuating conditions than that of the environmental chlamydiae.
Phylogenetic analyses of the genome data revealed that the last common ancestor of pathogenic and environmental chlamydiae lived 700 million years ago and its genome already encoded for some virulence factors like the Type III secretion system (TTSS) (Horn et al., 2004) which are still present to day in *P. amoebophila* and pathogenic chlamydiae.

Co-cultivation of amoebae with samples from a wastewater treatment plant in Plattling, Germany, resulted in the transfection of the amoebae with the environmental isolate *Parachlamydia acanthamoebae* UV-7 (University of Vienna isolate 7). The bacteria were able to survive and grow in the *Acanthamoeba* sp. UWC1 and maintain a stable symbiosis (Collingro et al., 2005b). The genome of *P. acanthamoebae* is even larger than that of *P. amoebophila* (Collingro et al., 2011). The presence of additional genes compared to the gene set of *P. amoebophila* will help to gain further insight into the phylogenetic relationship of *P. acanthamoebae* to other members of the *Chlamydiales* and the interaction with its amoebal host.

A.2.3. *Simkaniaceae*

Simkania negevensis strain Z, the only member of the new family of *Simkaniaceae* discovered to-date, was found as cell culture contaminant in a laboratory strains of Vero epithelial cells (Kahane et al., 1993). *Simkania negevensis* could be associated with pneumonia in adults and acute bronchitis in infants (Kahane et al., 1998) and is able to survive and multiply in amoebae.
The developmental cycle of *Simkania negevensis* strain Z was found to be longer than that of environmental chlamydiae. There is indication that not only the electron-dense forms of *Simkania negevensis* stain Z comparable to the EBs of pathogenic chlamydiae are able to infect epithelial cells but also the other, RB-similar form which is able to multiply. Since the genome of *Simkania negevensis* is nearly twice the size of the *Chlamydia trachomatis* genome (Kahane et al., 1999), it is possible that additional genes permitting infectivity of all morphological forms are present in *Simkania negevensis* (Kahane et al., 2002).

A.3. Pathogenicity of environmental chlamydiae

The interactions of chlamydia-like organisms with their amoebal hosts are similar to those of pathogenic bacteria and include uptake, survival and multiplication of the bacteria in the host. Due to the comparability of these processes, bacterial infection of protozoa allows the analysis of chlamydial virulence factors and mechanisms and could allow conclusions which are eventually also true for the infection of human cells (Abu Kwaik et al., 1998).
P. acanthamoebae as well as the isolate "Hall's coccus" could be detected in patients with respiratory diseases by immunofluorescence assays. Apart from that, *P. acanthamoebae*-related 16S rDNA sequences could be amplified from histology specimens of patients suffering from bronchitis (Ossewaarde and Meijer, 1999).
Fritsche *et al.* reported that the cytopathic effect on human fibroblasts was enhanced when *Acanthamoeba* spp. contained the *P. amoebophila* endosymbiont (Fritsche et al., 1998b).
Simkania negevensis was found to be able to infect human cell cultures of various tissue origins and to induce a host cell inflammatory response. Previous studies demonstrated its association with respiratory tract infections in infants and adults (Kahane et al., 1998; Lieberman et al., 2002). Apart from that, it could be shown that some environmental chlamydiae are able to infect mammalian cells in vitro (Greub et al., 2003)(Greub et al., 2003).
Besides the growing evidence for the clinical relevance of environmental chlamydiae one must also consider the fact that they are often found as endosymbionts of FLA which are ubiquitously distributed and also capable of surviving under adverse conditions. Due to this, environmental chlamydiae are not only protected by the host in which they can multiply but can also be distributed to different habitats by the amoebal cell. Therefore, amoebae were suggested to play a significant role as Trojan horses of the microbial world (Barker and Brown, 1994).

The most important factors of the human immune response are the major histocompatibility complexes I and II (MHC) (1999). It is known that pathogenic chlamydiae are able to block the immune system by degrading two transcription factors essential for the expression of the MHC (Zhong et al., 1999). This is done by the chlamydial protease-like activity factor (CPAF). CPAF has a protease-activity and is secreted into the host cell cytoplasm (Zhong et al., 2001). CPAF-homologues were found to be encoded on the *P. amoebophila*-genome and these genes are actively transcribed (Schmitz-Esser, unpublished data). As *P. amoebophila* is an endosymbiont of amoebae,

which do not have an immune system comparable to that of higher organisms, the function of CPAF in *P. amoebophila* is yet not clear. On the other hand, the occurrence of CPAF in the *P. amoebophila* -genome could be an indication of a possible role of *P. amoebophila* as a pathogen for humans.

A.4. The Type III Secretion System (TTSS) in chlamydiae

Gram-negative bacteria have different ways to transport effector proteins into the cytoplasm of their host. These effector proteins are able to influence molecular processes of the host cell and can thus alter their intracellular environment. The secretion of effector proteins is known to be a major determinant of virulence and pathogenicity of Gram-negative bacteria. Several different protein secretion systems are known in Gram-negative bacteria.

Many proteins that play a significant role in the virulence of gram-negative bacteria are not only secreted across the bacterial membranes but are also translocated across the host cell membrane into the host's cytoplasm and can there interfere with different cellular processes and suppress host defence mechanisms (Angot et al., 2007). The proteins delivered into the eukaryotic cell pass through the host cell membrane by a proteinaceous transmembrane channel known as the Type III secretion translocon. This translocon is of bacterial origin and composed of several structural proteins that are largely conserved among different bacteria (Buttner and Bonas, 2002).
It is known that chlamydiae – among other pathogenic gram-negative bacteria – use a Type III secretion to deliver effector proteins to the host cell (Hueck, 1998).
The TTSS of chlamydiae was originally identified based on homologues of structural components between chlamydiae and other pathogenic bacteria studied in more detail, for instance *Salmonella* and *Yersinia* spp. (Hsia et al., 1997). Contact with a eukaryotic target cell is required as a trigger for the secretion.
The TTSS displays a complex structure and is composed of at least 20 different proteins most of which are localized in the inner membrane or in the periplasm (Hueck, 1998). One of these proteins contains an ATP-binding motif and provides the energy for the secretion process (Koster et al., 2000). Kubori and colleagues discovered a so-called "needle-complex" of the TTSS of *Salmonella typhimurium*. Under the electron microsope, this complex displays a cylindrical structure (Kubori et al., 1998). The basal structure is anchored in the cytoplasmic membrane and is similar to the flagellar basal body (Kubori et

al., 1998). The needle is approximately 50 nm long and spans the bacterial cell surface (Buttner and Bonas, 2002).

In contrast to the Type II Secretion Systems, TTSS substrates do not have conserved N-terminal signal peptides which function as recognition signals and are removed during secretion. Apart from that, TTSS substrates are not only secreted out of the bacterial cell but are also tranlocated into the cytosol of the eukaryotic cell. Due to the fact that most of the inner membrane proteins of the TTSS are homologues to components of the flagellar synthesis-apparatus, a common origin of these two systems is assumed (Hueck, 1998).

While in other pathogenic bacteria the genes for the Type III secretion apparatus are clustered together in the genome or on a plasmid, in chlamydial genomes these genes are dispersed. Compared to *Proteobacteria*, the genes of the TTSS are located on three islands on the chlamydial genome (Horn et al., 2004). In contrast, the genes of the TTSS of *Proteobacteria* are located on one operon in so-called "pathogenicity islands" (PI) or on plasmids (Parsot et al., 2003). Gene clusters are an indication for horizontal gene transfer, while the dispersion of genes across the whole genome implies a more ancient development. Therefore it is hypothesized that the TTSS found in pathogenic chlamydiae as well as in *P. amoebophila* has developed earlier than that of other bacteria (Subtil et al., 2000; Kim, 2001).

A.5. Aims of this work

In 2005, Subtil *et al.* could show the role of several chlamydial proteins as TTSS substrates. Homology analyses revealed that one of these proteins was homologous to two predicted proteins of *Protochlamydia amoebophila* UWE25 (Subtil et al., 2005).
In this study, these two unknown proteins of *P. amoebophila* were analyzed using different methods. The first aim was to characterize their properties based on the amino acid sequence using bioinformatical tools. The presence of homologous proteins in other organisms as well as predictions on their function, structure and physicochemical properties were the questions addressed here. Apart from that, their transcription in *P. amoebophila* should be analysed. The genes encoding these proteins should be cloned in *E. coli* allowing a heterologous protein expression. Finally, the expressed proteins should be purified and used for antibody generation. Immunofluorescence analyses were meant to show the localization of these two bacterial proteins in the *P. amoebophila*-endosymbiont

within its amoebal host. As these proteins were homologous to TTSS substrates of pathogenic chlamydiae, the aim of this study was to assign their localization and to gain more information about their role as putative TTSS substrates and their intracellular fate. The possibility to localize the proteins would also allow assumptions on their function and importance in symbiont-host-interactions.

Another aim was the establishment of an efficient Microimmunofluorescence (MIF) assay and its optimization for the screening of human blood sera.

Different human sera should be screened and titre levels determined against three different environmental chlamydiae. Blood sera of randomly chosen patients for which no former chlamydial infection was reported should be analysed for the presence of antibodies against selected environmental chlamydiae. Another interesting question assigned in this study was whether volunteers of the environmental chlamydiae-group at our department would show elevated antibody titres resulting from work with the infectious elementary bodies of these organisms. The optimized MIF protocol can be used as fast and powerful tool in future clinical studies.

B. Materials and methods

All buffers and solutions in this study were produced under utilization of double distilled and filtered water (H_2O_{bidist}), using a water purification facility (UltraClean^{TM}, Barsbüttel, Germany). Usage of plainly deionised water (H_2O_{dist}) is stated accordingly. Chemicals were purchased and used in *p. a.* quality, if not stated otherwise. All buffers and solutions were sterilized in a watervapour-high pressure autoclave (Varioklav 135S, H+P, München, Germany) for 20 min at 121°C and $1.013x10^5$ Pa pressure, if not stated otherwise. Substances and solutions unstable at high temperatures like antibiotics were filtered sterile (0.22 µm pore size, Qualilab®, Merck Labor und Vertrieb GmbH, Bruchsal, Germany) and added after autoclaving. All centrifugation steps were performed using a table-top centrifuge (Eppendorf 5804R, Eppendorf AG, Hamburg, Germany) at room temperature (RT) if not stated otherwise.

B.1. Software

Table B.1. Software used.

Program	URL	Reference
ARB software package	http://www.arb-home.de/	(Ludwig et al., 2004)
Basic Local Alignment Search Tool (Blast)	http://www.ncbi.nlm.nih.gov/BLAST/	(Altschul et al., 1990)
Chromas	http://www.technelysium.com.au/chromas.html	Technelysium Pty Ltd
Clone manager		Scientific & Educational Software
e-seq	http://www.licor.com/bio/eSeq/DNASeq1.jsp	Licor Inc., Lincoln, NE, USA
Expasy Proteomic Tools	http://www.expasy.org/tools/	Swiss Institute of Bioinformatics
Finch TV		Geospiza Inc., Seattle, USA
Predict Protein	http://www.predictprotein.org	(Rost et al., 2004)
RDP II (Ribosomal Database project)	http://rdp.cme.msu.edu/	(Cole et al., 2005)
Sigma plot	-	Systat Software Inc., Richmond, CA, USA
SOSUI	http://bp.nuap.nagoya-u.ac.jp/sosui/	(Mitaku and Hirokawa, 1999)

B.2. Technical equipment

If not mentioned otherwise, the equipment shown in table B.2. was used during this research.

Table B.2. Technical equipment used.

Equipment	Company
Beadbeater BIO 101, Carlsbad, CA, USA	Beadbeater BIO 101, Carlsbad, CA, USA
Centrifuges:	
Mikro 22 R	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Rotina 35 S	Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
Eppendorf 5804 R	Eppendorf AG, Hamburg, Germany
DNA sequencer:	
Li-cor Long Readir 4200	MWG-Biotech, Ebersberg, Germany
Electroporator MicroPulser™	Biorad, Munich, Germany
Gelcarriage:	
Typ H3 (11×14 cm)	Gibco-BRL, Eggenstein, Germany
Sub-Cell GT UV-Transparent Gel Tray (15×15 cm)	Biorad, München, Germany
Gel Dokumentationsystem MediaSystem FlexiLine 4040	Biostep, Jahnsdorf, Germany
Gel electrophoresis:	
GNA-100	Pharmacia, Freiburg, Germany
Sub-Cell® GT	Biorad, München, Germany
Heatblock VWR Digital Heatblock	VWR international, West Chester, PA, USA
Hybridisation oven UE-500	Memmert GmbH, Schwabach, Germany
Laminar flow hood Safe 2010 Modell 1.2	Holten, Jouan Nordic, Allerød, Dänemark
Microscopes:	
Inverse microscope Axiovert 25	Zeiss, Jena, Germany
Epifluorescence microscope Axioplan 2 imaging	Zeiss, Jena, Germany
Confocal Laser Scanning Microscope LSM 510 Meta	Zeiss, Jena, Germany
Mini Protean 3-Cell	Biorad, Munich, Germany
PCR thermocyclers:	
Icycler	Biorad, Munich, Germany
Mastercycler gradient	Eppendorf, Hamburg, Germany
pH-Meter WTW inoLab Level	Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Photometers:	
NanoDrop® ND-1000	NanoDrop Technologies, Wilmington, USA
Spectralphotometer SmartSpec™ 3000	Biorad, Munich, Germany
Platform Shaker Innova 2300	New Brunswick Co., Inc., Madison NJ, USA
Power device for gelelectrophoresis PowerPac Basic	Biorad, Munich, Germany
Scales:	
OHAUS Analytic Plus	Ohaus Corp., NY, USA
Sartorius	Sarotius AG, Göttingen, Germany
Sonicator Bandelin Sonoplus HD2070	Bandelin electronic, Berlin, Germany
Sonotrode Bandelin Sonoplus UW 2070	Bandelin electronic, Berlin, Germany
Transilluminator UST-30M-8E (312 nm)	Biostep GmbH, Jahnsdorf, Germany
Ultraviolet Sterilizing PCR Workstation	Peqlab Biotechnology GmbH, Germany
Vortex Genie 2	Scientific Industries, New York, USA
Water purification facility Ultra Clear™	Barsbüttel, Germany
Waterbaths:	
DC10	Thermo Haake, Karlsruhe, Germany
GFL Typ 1004	Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Watervapour high pressure autoclaves:	
Varioclav 135S H+P	H+P, Munich, Germany
Varioclav 25T H+P	H+P Munich, Germany

B.3. Expendables and kits

Table B.3. Expendables and kits used.

Expendables	
Expandable item	**Company**
24-well-plates	Nunc, Wiesbaden, Germany
Citifluor AF1	Agar Scientific Limited, USA
Cover slips 24×50 mm	Paul Marienfeld, Bad Mergentheim, Germany
Cover slips, diameter 24 mm	Carl Roth GmbH & Co., Karlsruhe, Germany
CryobankTM	Mast Diagonstika Lab. Präparate GmbH, Reinfeld, Germany
Culture flasks 10 ml	Nunc, Wiesbaden, Germany
Culture flasks 150 ml	Nunc, Wiesbaden, Germany
Elektroporations-Küvetten, 0.2 cm	Biorad, München, Germany
Eppendorf Reaktionsgefäße (ERT), various sizes	Eppendorf AG, Hamburg, Germany
Erlenmeyer-Kolben DURAN®, various sizes	Schott Glas, Mainz, Germany
Glass slides	Paul Marienfeld, Bad Mergentheim, Germany
Microseal „A“ Film	MJ Research, Waltham, MA, USA
Mikrotiterplatte MicrosealTM 96, V-Boden	MJ Research, Waltham, MA, USA
Plasticcuvettes, Halb-Mikro	Greiner Bio-One GmbH, Frickenhausen, Germany
Sampling vessels, 15 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Sampling vessels, 50 ml	Greiner Bio-One GmbH, Frickenhausen, Germany
Slides, 10 well	Paul Marienfeld, Bad Mergentheim, Germany
Kits	
Kit	**Company**
ABGeneExtensor Hi-Fidelity PCR Enzyme Mix	ABGene, Surrey, UK
Amersham HisTrap HP Columns	GE Healthcare, Uppsala, Sweden
BCA Protein Reagent Assay Kit	Pierce, Rockford, Illinois, USA
Qiagen Ni-NTA Spin Columns	Qiagen, Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Germany
QIAquick Nucleotide Removal Kit	Qiagen, Hilden, Germany
QIAquick PCR purification kit	Qiagen, Hilden, Germany
RevertAid First Strand cDNA Kit	Fermentas, St.Leon-Rot, Germany
TSA Plus Fluorescence Systems	NEN Life Sciences, Boston, USA

B.4. Chemicals

Table B.4. Chemicals used.

Chemical	Company
1,4-Diazabicyclo(2,2,2)octan (DABCO)	Carl Roth GmbH & Co., Karlsruhe, Germany
3,3’-dihexyloxacarbocyanine-iodide ($DiOC_6$)	Invitrogen Molecular Probes, Carlsbad, CA, USA Cambrex
4’-6’-di-amidino-2-phenylindole (DAPI)	Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria
Albumin Fraktion V	Carl Roth GmbH & Co., Karlsruhe, Germany
Acetic acid	Carl Roth GmbH & Co., Karlsruhe, Germany

Acrylamide/bisacrylamide (AA/BA, 30%)	Carl Roth GmbH & Co., Karlsruhe, Germany
Agar	Fluka Chemie AG, Buchs, Switzerland
Agarose, electrophoresis Grade	Invitrogen Corporation, Carlsbad, CA, USA Cambrex
Ammonium peroxy-di-sulfate (APS)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Ampicillin	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Boric acid	Fluka Chemie AG, Buchs, Switzerland
Bromphenol blue	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Casein	Merck KgaA, Darmstadt, Germany
Chisam	Carl Roth GmbH & Co., Karlsruhe, Germany
Di-ethyl-pyrocarbonate (DEPC)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Di-sodiumhydrogenphosphate (NA_2HPO_4)	J. T. Baker, Deventer, Holland
Di-methylsulfoxid (DMSO)	Fluka Chemie AG, Buchs, Switzerland
Egg yolk	Biotrading, Mijdrecht, Netherlands
Ethanol absolute	Merck KgaA, Darmstadt, Germany
Ethidium bromide (EtBr)	Fluka Chemie AG, Buchs, Switzerland
Ethylene-di-amine-tetra-acetic acid (EDTA)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Evans Blue	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Ficoll® 400	Sigma-Alderich Chemie GmbH, Steinhausen, Germany
Glucose	Merck KgaA, Darmstadt, Germany
Glycerol	Carl Roth GmbH & Co., Karlsruhe, Germany
Hydrochloric acid (HCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Hydrogen peroxide (H_2O_2), 30%	Carl Roth GmbH & Co., Karlsruhe, Germany
Imidazol	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropanol (2-propanol)	Carl Roth GmbH & Co., Karlsruhe, Germany
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Magnesium chloride	Carl Roth GmbH & Co., Karlsruhe, Germany
Methanol	Carl Roth GmbH & Co., Karlsruhe, Germany
Milk powder	Carl Roth GmbH & Co., Karlsruhe, Germany
Mowiol 4-88	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Nile Red	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
N,N,N',N'-tetra-methyl-ethylene-di-amine (TEMED)	Fluka Chemie AG, Buchs, Switzerland
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Peptone	Oxoid LTD., Hampshire, England
Poly-L-lysine solution	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Potassium acetate (KCl)	J. T. Baker, Deventer, Holland
Sodium chloride (NaCl)	Carl Roth GmbH & Co., Karlsruhe, Germany
Sodium Citrate	Fluka Chemie AG, Buchs, Switzerland
Sodium dodecyl sulfate (SDS)	Fluka Chemie AG, Buchs, Switzerland
Sodium hydroxide (NaOH)	J. T. Baker, Deventer, Holland
Sodium-di-hydrogenphosphate (NAH_2PO_4)	J.T. Baker, Deventer, Holland
Sucrose	Carl Roth GmbH & Co., Karlsruhe, Germany
Tricine	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Tris	Carl Roth GmbH & Co., Karlsruhe, Germany
Trizol	Invitrogen, Lofer, Austria
Trypticase Soy Broth	DIFCO, Detroit, USA
Tryptone	Oxoid LTD., Hampshire, England
Urea	USB Corp., Cleveland, USA
Xylencyanol	Sigma-Aldrich Chemie GmbH, Steinhausen, Germany
Yeast extract	Oxoid LTD., Hampshire, England

B.5. Organisms

Table B.5. Organisms used in this study.

Organism	Strain	Symbiont	Source	Reference	Medium
Acanthamoeba sp.	UWC1	--	University of Washington, Seattle, USA	(Fritsche et al., 1998a; Collingro et al., 2005a)	TSY
Acanthamoeba sp.	UWC1	*Protochlamydia amoebophila* UWE25	University of Washington, Seattle, USA	(Fritsche et al., 1998a; Collingro et al., 2005a)	TSY
Organism	**Strain**		**First isolated:**	**Reference**	
Parachlamydia sp.	UV-7	Partially purified elementary bodies*	Wastewater treatment plant, Plattling, Germany	(Collingro et al., 2005b)	
Simkania negevensis	Z	Partially purified elementary bodies*	Ben Gurion University of the Negev, Israel	(Kahane et al., 1999)	

* Elementary bodies of this organism were kindly provided by Dr. Sven Poppert, Universitätsklinikum Ulm, Germany

The here used Acanthamoebae are isogenic pairs. These are genetically identical amoebae, one of the strains harboring endosymbionts, while the other strain lives without a symbiotic partner. Such isogenic pairs can be found in nature, but – as in this case – can also be artificially obtained by the infection of amoebae with endosymbionts. With this, a stable living together of both organisms is achieved, which is maintained over several years. Artificially infected amoebae can be cured from their endosymbionts by the use of antibiotics even several years after infection, which is not possible with naturally infected amoebae.

Partially purified elementary bodies of *Parachlamydia* sp. UV-7 and *Simkania negevensis* Z were kindly provided by Dr. Sven Poppert at the Unversitätsklinikum Ulm, Germany.

Acanthamoeba sp. UWC1, which naturally lacks endosymbionts, as well as its isogenic partners UWC1/*Protochlamydia amoebophila* UWE25, UWC1/*Parachlamydia* sp. UV-7 and UWC1/*Simkania negevensis* Z containing chlamydia-like endosymbionts, were used in this study (see Tab. B.5.).

The chlamydia-like endosymbiont *Protochlamydia amoebophila* UWE25 (University of Washington) was isolated from amoebal cells found in soil samples and naturally infects *Acanthamoeba* spp. (Fritsche et al., 2000a), while the chlamydia-like endosymbiont *Parachlamydia* sp. UV-7 was originally found in activated sludge from the wastewater treatment plant in Plattling, Germany (Collingro et al., 2005b). The endosymbiont *Simkania negevensis* Z was first discovered as a cell culture contaminant at the Ben Gurion University of the Negev in Israel (Kahane et al., 1999).

In this study, *Acanthamoeba* sp. UWC1-cells (C1) infected under laboratory conditions with the above-mentioned environmental chlamydiae were used.

B.6. Buffers and media

For solid media 15g/l agar were added before autoclaving.

B.6.1. General buffers

a) PBS

PBS stock solution (Na_xPO_4)

NaH_2PO_4	200 mM	35.6 g/l
Na_2HPO_4	200 mM	27.6 g/l

pH of NaH_2PO_4 solution was adjusted to 7.2 -7.4.

1x PBS

NaCl	130 mM	7.6 g/l
PBS stock solution	10 mM	50 ml/l
H_2O_{bidist}		ad 1000 ml

pH 7.2–7.4

3x PBS

NaCl	390 mM	22.8 g/l
PBS stock solution	30 mM	150 ml/l
H_2O_{bidist}		ad 1000 ml

pH 7.2 –7.4

B.6.2. Buffers, solutions and standards for gel electrophoresis

a) TBE buffer

10x TBE

Tris	890 mM	162.0 g/l
Boric acid	890 mM	27.5 g/l
EDTA	20 mM	9.3 g/l
H_2O_{bidist}		ad 1000 ml

pH 8.3 – 8.7

1x TBE

10x TBE	100 ml/l
H_2O_{bidist}	ad 1000 ml

b) **Loading buffer**

Ficoll	25% (w/v)
Bromphenol blue	0.5% (w/v)
Xylencyanol	0.5% (w/v)
EDTA	50 mM

c) Ethidium bromide solution

Ethidium bromide stock solution

10 mg/ml Ethidium bromide (EtBr) in H_2O_{bidist}

Ethidium bromide staining solution

EtBr-stock solution diluted 1:10,000 in H_2O_{bidist}

d) DNA ladder (KbL)

GeneRuler™ 1kb (Fermentas, St. Leon-Rot, Germany)

B.6.3. Culture media and buffers for amoebae

a) TSY-Broth (Trypticase Soy Broth with Yeast extract)

Tryptone Soy Broth	30 g
Yeast extract	10 g
H_2O_{dist}	ad 1000 ml
pH 7.3	

b) Page's Amoebic Saline (PAS, 10x concentrate)

NaCl	1.2 g
$MgSO_4$*7 H_2O	0.04 g
$CaCl_2$*2 H_2O	0.04 g
Na_2HPO_4	1.42 g
KH_2PO_4	1.36 g
H_2O_{dist}	ad 1000 ml

c) NNA (Non Nutrient Agar)

Page's Amoebic Saline (10x concentrate)	100 ml
H_2O_{dist}	900 ml

d) SCGYEM-medium (Chang-medium)

Solution A:

Casein (ref. Hammerstein, Merck, Darmstadt)	10 g
Na_2HPO_4	1.325 g
H_2O_{bidist}	800 ml

pH > 8

Solution A was autoclaved at 110°C for 15 min and then cooled at 4°C over night (o/n).

Solution B:

Glucose	2.5 g
KH_2PO_4	0.8 g
H_2O_{bidist}	50 ml

Solution C:

Yeast extract	5 g
H_2O_{bidist}	50 ml

Solutions B and C were filtered sterile (0.22 µm pore size) and added to solution A. Finally the medium was complemented with 50 ml of heat inactivated (water bath at 56°C for 30 min) foetal calf serum (Biochrom, Berlin, Germany).

e) PYG-medium (Peptone-Yeast-Glucose-medium)

Peptone	20 g
Glucose	18 g
Yeast extract	2 g
Sodiumcitrate	1 g
$MgSO_4$*7 H_2O	980 mg
Na_2HPO_4*7 H_2O	355 mg
KH_2PO_4	340 mg
$Fe(NH_4)_2(SO_4)_2$*6 H_2O	20 mg
H_2O_{dist}	ad 1000 ml

pH 6.5

B.6.4. Culture media and buffers for bacteria

a) Luria-Bertani medium (LB-medium)

Tryptone	10.0 g/l
Yeast extract	5.0 g/l
NaCl	5.0 g/l
H_2O_{dist}	ad 1000 ml
pH 7.0-7.5	

b) dYT medium

Tryptone	16 g/l
Yeast extract	10 g/l
NaCl	5 g/l
H_2O_{bidist}	ad 1000 ml

c) SOC medium

Tryptone	2 % w/v
Yeast extract	0.5 % w/v
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM
Glucose	20 mM

B.6.5. Antibiotics and induction solutions

a) Ampicillin

In solid media, final concentration of ampicillin was achieved by adding stock solution to the autoclaved media at ~50°C. Media containing ampicillin were stored at 4°C and used within 4 weeks after preparation. Ampicillin was enclosed to liquid media right before usage.

Ampicillin stock solution (Amp)

Ampicillin 100 mg/ml

Amp was dissolved in 50% $EtOH_{abs}$.

Amp was added to medium reaching a final concentration of 100 µg/µl.

b) IPTG stock solution

Isopropyl-β-D-thiogalactopyranosid (IPTG) 1 M

IPTG was dissolved in H_2O_{bidist}, filtrated sterile (0.22 µm pore size) and kept at minus 20°C.

IPTG was used for induction of *in vivo* transcription of cloned genes of *Protochlamydia amoebophila* UWE25 in different *E. coli* expression strains (see Tab. B.10.). For this, 100 µl of stock solution were added to 100 ml of an actively growing (OD_{600} = 0.3-0.6) *E.coli* culture.

B.6.6. Solutions for plasmid isolation

a) P1 buffer

Tris-HCl, pH 8.0	50 mM
EDTA	10 mM
RNase A	100 µg/ml

b) NaOH/SDS solution

NaOH (2 M)	1 ml
10% SDS	1 ml
H_2O_{bidist}	8 ml

c) Potassium acetate/acetate solution

KCl (5 M)	6 ml
H_2O_{bidist}	2.85 ml
Acetic acid (pure)	1.15 ml

B.7. Cultivation and strain collection

B.7.1. Cultivation and strain collection of acanthamoebae

The axenically growing amoebae were cultivated in the incubator at 20°C or at RT using liquid media in 10 ml and 150 ml culture flasks, respectively (Nunc, Wiesbaden, Germany). Growth and possible occurrence of contamination in the culture flasks were checked regularly using an inverse microscope (Axiovert 25, Zeiss, Jena, Germany). In the case of a strong increase of trophozoites detached from the bottom of the culture flask or

the occurrence of cysts, the medium was carefully poured out of the flask and replaced by fresh one.

For the growth and maintenance of acanthamoebae, several subcultures of each strain were prepared using bacteria-free TSY-medium and were grown at 20°C, while the medium was exchanged approximately every week.

B.7.2. Partial purification of infectious elementary bodies (EBs)

Sucrose-Phosphate-Glutamate (SPG)-Buffer

Saccharose	75 g
KH_2PO_4	0.52 g
$NaHPO_4$*7 H_2O	2.3 g
Glutamic acid	0.75 g
H_2O_{bidist}	ad 1000 ml

Two 150 ml culture flasks containing acanthamoebae harbouring chlamydia-like endosymbionts (UWC1/UWE25) were first vigorously shaken in order to largely detach the amoebae from the bottom of the culture flask. The amoebae suspension was then harvested in 50 ml-falcon-tubes (2x 5000 rpm, 5 min, 4°C) and washed once with 10 ml of 1x PAS. After that, the pellet was resuspended in 10 ml 1x PAS and the amoebal cells were disrupted by freezing at -20°C, followed by rapid defrosting in the water bath at 55°C (freeze-and-thaw). This freeze-and-thaw step was repeated once again. Afterwards, the cell suspension was mixed with glass beads (0.75-1 mm in diameter) and vortexed for 3 min to separate the endosymbionts from amoebal cell remnants. Glass beads and cell debris were pelleted at 2200 rpm, 10 min, 4°C and the pellet was discarded, while the endosymbionts largely remained in the supernatant. Thereafter, the supernatant was centrifuged at 20000 rpm for 40 min at 4°C to pellet the bacterial cells. The supernatant was then discarded and the pellet was suspended in 10 ml SPG-buffer, followed by an additional centrifugation step (20000 rpm, 40 min, 4°C). The EB-pellet was then resuspended in 7 ml SPG-buffer, aliquoted and stored at -80°C.

In order to check for the presence of viable amoebae in the EB-preparation, 500 µl of the obtained suspension were added to a 10 ml culture flask containing TSY medium devoid of amoebal cells. The culture flask was incubated at RT and checked for amoebal growth using an inverse microscope after 1-2 days.

B.8. Culturing and maintenance of recombinant *E. coli* strains

B.8.1. Culturing and cell harvesting

Solutions

LB medium (see Sec. B.6.4.)

Amp stock solution (see Sec. B.6.5.)

Procedure

Recombinant cells were cultured on plates with solid media and in liquid media, respectively. Solid media was used for short-term storage and clone screening, while liquid media was used for cell growth. To avoid growth of cells without plasmid after the cloning procedure (see Sec. B.13.), LB media contained 100 µg/ml Amp. Cells were grown at 37°C.

For culturing cells in liquid media, a test-tube containing 5 ml LB medium was inoculated with a single colony from plates under sterile conditions and incubated at 37°C on an orbital shaker (Innova 2300; New Brunswick Scientific Co., Inc., Madison NJ, USA) o/n at 200 rpm. Bacterial cells were harvested by centrifugation (14000 rpm, 1 min) of 2x 2 ml culture in sterile Eppendorf reaction tube (ERT).

For culturing cells in larger volumes, 200 ml Erlenmeyer flasks with 100 ml of LB medium were used. The medium was inoculated with 5 ml of an o/n culture and incubated at 37°C on an orbital shaker at 200 rpm until required cell density was reached. The optical density (OD) was measured against sterile LB medium using plastic cuvettes (Halb-Mikro, Greiner) in a spectralphotometer (SmartSpec^{TM} 3000, Biorad, Munich, Germany) at a wavelength (λ) of 600 nm.

For the purification of sufficient amounts of *P.amoebophila* proteins expressed by recombinant *E.coli* cells, 2 l Erlenmeyer flasks with 1 l of LB medium were used. The medium was inoculated with 50 ml of an o/n culture. The following steps were performed as described above.

B.8.2. Maintenance

For short term maintenance, single clones were applied to LB masterplates containing Amp, incubated o/n on 37°C and stored at 4°C.

For long term maintenance, cryostocks of single clones were prepared under sterile conditions as follows:

800 µl of o/n culture were transferred into 2 ml screw caps (Cryobank^{TM}, Mast Diagonstika Lab. Präparate GmbH, Reinfeld, Germany) containing cryostock solution and hollow plastic spheres. The caps were inverted several times, thus allowing cells to attach to the inside of the plastic spheres, the supernatant removed and the caps finally stored at -80°C. If required, the plastic spheres can be removed from the cap, be placed in a flask containing LB medium and grown at 37°C, while the remaining cryostocks are again stored at -80°C.

B.9. Methods for isolation of DNA

B.9.1. Isolation of genomic DNA from chlamydial elementary bodies (EBs)

Solutions

Unset lysis buffer:

Urea	8 M
SDS	2%
NaCl	0.15 M
EDTA	0.001 M
Tris	0.1 M
pH 7.5	

Chisam (phenol-chloroform-isoamylalcohol) (Carl Roth GmbH & Co., Karlsruhe, Germany)

95% EtOH

3 M Sodium acetate

H_2O_{bidist}

Procedure

For the isolation of genomic DNA from purified chlamydial elementary bodies (EBs), a modified UNSET (Hugo et al., 1992) procedure, described below, was used.

The EB suspension was pelleted (15000 rpm, 15 min, 4°C), washed with 1x PBS, centrifuged again and the supernatant discarded. The pellet was then resuspended in 500 µl Unset lysis buffer and incubated for 10 min at 60°C in a water-bath. The suspension was then transferred to a screw cap, 1 ml of chisam was added and the cap was inverted repeatedly for 10 min to ensure good mixing. The cap was then centrifuged at 5500 rpm for 10 min at RT and the resulting upper aqueous phase was carefully transferred to a new

screw cap. An equal amount of chisam was added, the cap inverted several times and centrifuged. Again, the upper phase was carefully transferred to a new cap, 1/10 volume of 3 M sodium acetate and 2.5 volumes of ice-cold 95% EtOH were added. The cap was inverted several times and the DNA was precipitated at -20°C for at least 2 h or o/n.
The precipitated DNA was then centrifuged for 30 min at 15000 rpm at 4°C and the supernatant removed. The DNA pellet was washed in 500 µl ice-cold 70% EtOH, centrifuged for 10 min and air-dried at RT. The DNA was then dissolved in 50 µl H_2O_{bidist}, quantified photometrically (see Sec. B.10.1.) and stored at -20°C.

B. 9.2. Isolation of plasmid DNA from recombinant *E. coli* cells

The plasmid isolation was performed according to the principle of alkaline lysis, followed by organic precipitation of proteins and precipitation of plasmid DNA by 2-propanol.

Solutions (see Sec. B.6.6.)
P1 buffer
NaOH/SDS solution
Potassium acetate/acetate solution
2-propanol
70% EtOH

Procedure
2x2 ml of o/n culture were centrifuged in a sterile ERT for 1 min at 13000 rpm and the supernatant discarded. The cell pellet was resuspended in 100 µl of buffer P1 and incubated for 5 min at RT to digest RNA. For cell lysis, 200 µl of NaOH/SDS solution were added. Tubes were inverted several times and incubated for 5 min on ice (meanwhile inverted again). To precipitate proteins, 150 µl of potassium acetate/acetate solution were added, the tubes vortexed briefly, and then put on ice for 5 min. Thenceforth, the proteins were separated by centrifugation (13000 rpm, 1 min) and the obtained supernatant (≤450 µl) transferred to a new sterile ERT. DNA was precipitated by adding one volume of 2-propanol and incubation for 10 min at RT. Hereafter, the tube was centrifuged (13000 rpm, 1 min), the supernatant removed, and the DNA pellet washed by resuspension in 500 µl of ice-cold 70% EtOH. After a final centrifugation step (13000 rpm, 1 min), the supernatant was removed and the pellet air-dried. The DNA was dissolved in 50 µl of DNA-free H_2O_{bidist}, quantified photometrically (see Sec. B.10.1.) and stored at -20°C.

B.10. Quantitative and qualitative analyses of nucleic acids

B.10.1. Quantitative, photometric analyses of nucleic acids

For quantitative measurement (Clark and Swika, 1977), 1.5µl of the nucleic acid solution were pipetted onto the end of the fibre optic cable of a NanoDrop® ND-1000 spectralphotometer (NanoDrop Technologies, Wilmington, USA). The concentration of nucleic acids was measured at λ = 260 nm.

B.10.2. Qualitative analyses of nucleic acids using agarose gel electrophoresis

For qualitative analyses of nucleic acids horizontal agarose gel electrophoresis was used. This technique offers the possibility to separate nucleic acids based on their size-dependent migration through a gel under the influence of an electric field.

Solutions (see Sec. B.6.2.)
1-2.5% (w/v) agarose in 1x TBE buffer
Loading buffer
DNA-ladder (KbL)
EtBr staining solution

Procedure
An appropriate amount of agarose (Invitrogen Corporation, Carlsbad, CA, USA, Cambrex) was weighed, mixed with 1x TBE buffer and melted in a microwave. The concentration of agarose was dependent on the type of analyses. For control of PCR products, agarose concentrations of 1-1.5% w/v were used. For RFLP analyses, concentrations of ≥1.8% were used.
After cooling the agarose to ~50°C it was poured into a gel-tray (Typ H3, 11×14 cm, Gibco-BRL, Eggenstein, Germany) with applied comb. After polymerization, the tray was inserted into the electrophoresis apparatus (SubCell GT, Biorad). The nucleic acid solution to be analyzed was mixed 1:1 with loading buffer and pipetted into the pocket of the gel. Separation of nucleic acids took place by applying a voltage of 100-200 V for 30 min-1.5 h, depending on fragment size and type of analysis. After separation, gels were stained using EtBr staining solution for ~45 min. Detection of nucleic acids was done by placing the gel onto a transilluminator (Biostep GmbH, Jahnsdorf, Germany) emitting UV-light (λ

= 312 nm). Banding patterns were recorded and digitalised with a gel-documentation system (Biostep, Jahnsdorf, Germany).

B.11. In vitro amplification of DNA fragments via Polymerase Chain Reaction (PCR)

DNA regions of interest were amplified with specific primers (Tab. B.6.) and the Polymerase Chain Reaction (PCR) technique. PCR starts with the denaturation of the template DNA and continues with 20-40 temperature cycles of (i) denaturation of the DNA, (ii) primer-annealing and (iii) an elongation phase. The PCR is accomplished with a final elongation step.

B.11.1. In silico design of new PCR primers

The construction of highly specific PCR primers for the full length of the genes *pc0373* and *pc0374* of *Protochlamydia amoebophila* UWE25 was accomplished by using the implemented Probe design and Probe match tools of the software package ARB (Ludwig et al., 2004). The primers were designed under the viewpoint of a following cloning step of the amplified gene into an expression vector. For the PCR, during which the DNA-fragment to be cloned is amplified, primers introducing restriction sites at the 3'- and 5'-ends of the PCR-product were constructed (see Tab. B.6.). The same restriction sites were used for the linearization of the vector. The PCR-product as well as the vector were cut with the according restriction enzymes resulting in complementary ends of the vector and the PCR-product which are later ligated and the PCR-product inserted into the vector.
All primers described in this study were obtained from Thermo Electron GmbH (Ulm, Germany). PCR amplifications were carried out using the iCycler (Biorad) or the Mastercycler gradient PCR cycler (Eppendorf, Hamburg, Germany).

B.11.2. Amplification of target genes

pc0373 and *pc0374* of *Protochlamydia amoebophila* UWE25 were amplified by PCR using the following specific primer pairs:

Table B.6.: Primers used for the amplification of specific target genes

Primer name*	Sequence	Target gene	Specificity	Expected length	Comment	Reference
pc0373F	5'-CGC GGG **CAT ATG** TTG ATT TGG TAT TCC AG-3'	*pc0373*	*Protochlamydia amoebophila* UWE25	252 bp	**NdeI restriction site** for the cloning into the pET-16b vector	this study
pc0373R	5'-GCG GCG **CTC GAG** TTA AAT TTC ATG CCC TTT TTC-3'	*pc0373*	*Protochlamydia amoebophila* UWE25		**NdeI restriction site** for the cloning into the pET-16b vector	this study
pc0374F	5'-CGC GGG **CAT ATG** ATG TAC ACC TTG GAG GTG-3'	*pc0374*	*Protochlamydia amoebophila* UWE25	237 bp	**XhoI restriction site** for the cloning into the pET-16b vector	this study
pc0374R	5'-GCG GCG **CTC GAG** TTA ACC ATT AAA ATC TGC-3'	*pc0374*	*Protochlamydia amoebophila* UWE25		**XhoI restriction site** for the cloning into the pET-16b vector	this study

* F…forward primer, R…reverse primer

B.11.3. Standard and gradient PCR

Solutions

$MgCl_2$ (25 mM) (Fermentas Inc., Hanover, MD, USA)

10 x Ex Taq polymerase-buffer (Fermentas Inc., Hanover, MD, USA)

Nucleotide-Mix (2 mM/dNTP) (Fermentas Inc., Hanover, MD, USA)

Forward primer (50 pmol/µl)

Reverse primer (50 pmol/µl)

Taq DNA-polymerase (5 units/µl) (Fermentas Inc., Hanover, MD, USA)

$H_2O_{\text{bidist PCR grade}}$

Procedure

In order to determine the optimal annealing temperature of the newly designed primer pairs for amplification of *pc0373* and *pc0374* a gradient PCR was performed. For this purpose, a defined and increasing gradient of the annealing temperature was established. Denaturation, elongation and final elongation were identical in all PCR reactions. A negative control containing no template DNA was run under the lowest annealing temperature in order to check for PCR contaminations. All other steps were the same as in the standard PCR approach (conditions of the standard PCR are described in Tab.B.8.).

DNA isolated from partially purified EBs of *P. amoebophila* (see Sec. B.9.1.) was used as template for the amplification. The amount of template DNA used in the gradient PCR was 100 ng.

The standard PCR reaction for the amplification of *pc0373* and *pc0374* was performed as described for gradient PCR at an annealing temperature of 65°C.

Table B.7. Temperature gradient used for gradient PCR

Temperature gradient of the thermocycler								
Row	A	B	C*	D*	E*	F*	G	H
Gradient (°C)	70	68.9	67.1	64.3	60.5	57.9	56.1	55

* only these positions were used for gene amplification.

Table B.8. Standard PCR-conditions for the amplification of *pc0373* and *pc0374*

PCR step	Temp. [°C]	Time	Number of cycles
Denaturation	94	3 min	1
Denaturation	94	20 sec	35
Annealing	65	30 sec	
Elongation	72	30 sec	
Final elongation	72	15 min	1

Standard reaction mix (50µl)

$MgCl_2$	4 µl
Buffer (10x)	5 µl
dNTP-mix	5 µl
Forward primer	1 µl
Reverse primer	1 µl
Taq DNA polymerase	0.2 µl
Template	1 µl
$H_2O_{\text{bidist PCR grade}}$	ad 50 µl

If more than one reaction was performed, a master mix without template was prepared and 49 µl of the mix were pipetted into each ERT. After this, 1 µl of the respective template was added. For every PCR a negative control (without addition of template) was performed.

B.11.4. High Fidelity PCR

After determining the optimal respective annealing temperature for the new primer sets, *pc0373* and *pc0374* were amplified in a high fidelity PCR using the ABGene Extensor Hi-Fidelity PCR Enzyme Mix (ABGene, Surrey, UK). This DNA polymerase has a proof-

reading activity ensuring that the amplified sequence, which is used for ligation and cloning, is copied properly so that the sequence is in frame.

Standard reaction mix (in duplicate for each gene):

Mastermix 1:

10x Extensor Buffer 1 (22.5mM $MgCl_2$)	5 µl
Extensor Hi-Fidelity PCR Enzyme Mix	0.25 µl
$H_2O_{\text{bidist PCR grade}}$	ad 25 µl

Mastermix 2:

20 mM dNTPs (5 mM of each dNTP)	5 µl
Template	1 µl
Forward Primer	1 µl
Reverse Primer	1 µl
$H_2O_{\text{bidist PCR grade}}$	ad 25 µl

The two mastermixes were kept on ice and combined shortly before placing the reaction mix into the thermocycler. This is done in order to prevent unspecific amplification as well as degradation of the primers or the template due to the exonuclease-activity of the proofreading DNA-polymerase. For amplification of *pc0373*- and *pc0374*-genes of *P. amoebophila* the primer pairs pc0373F/pc0373R and pc0374F/pc0374R were used, respectively. These primers were designed to amplify the whole gene thereby introducing specific restriction sites at the 5' end (see Tab. B.6.). DNA isolated from *P. amoebophila* elementary bodies (see Sec. B.9.1.) was used as template. The same amplification conditions as for the standard PCR were used (see Tab. B.8).

After that, the PCR products were run on an agarose gel to check for the presence of amplificates.

B.12. Transcription analyses of *pc0373* and *pc0374* genes

B.12.1. Simultaneous isolation of RNA from amoebae and their bacterial endosymbionts (Bead beater/Trizol-based extraction protocol)

$H2O_{bidest/DEPC}$:

0.1 Vol% DEPC (Diethylpyrocarbonat) was dissolved in 1 l H_2O_{bidist}, incubated o/n at RT and then autoclaved.

Aqueous solutions used for RNA treatment should always be prepared with DEPC-treated H_2O_{bidist} in order to inactivate RNases.

For the isolation of RNA, 20 ml of amoebal culture were harvested at 5000 rpm for 5 min at 4°C, the supernatant was removed and the pellet resuspended in 1.5 ml Trizol (Invitrogen, Lofer, Austria). The cell suspension was then disrupted using a bead beater at an intensity of 4.5 for 30 sec. The disrupted cells were then pelleted down at 14000 rpm for 5 min and the supernatant incubated at RT for 1 min. This was followed by the addition of 0.2 ml chloroform per ml volume of the supernatant; the solution was shaken vigorously for 15 sec, incubated at RT for 2 min and finally centrifuged for 15 min at 12000 rpm.
The aqueous supernatant was then mixed with 0.5 ml isopropyl alcohol per ml volume and incubated at RT for 10 min. After another centrifugation step (12000 rpm, 15 min, RT) the RNA-pellet was washed with 1.5 ml 75% EtOH and centrifuged once more for 5 min at 5,000 rpm and RT. The supernatant was discarded and the pellet air-dried. Finally, the RNA was dissolved in 50 µl RNase-free $H_2O_{bidest/DEPC}$ for 10 min at 55°C in a water-bath.
The isolated RNA was then quantified photometrically (see Sec. B.11.1.).

B.12.2. Storage of RNA

For storage, the nucleic acid solution was filled up to a final volume of 200 µl with $H_2O_{bidest./DEPC}$ and the RNA was precipitated by adding 1/10 volumes of 3M $NaAc_{DEPC}$ and 3 volumes of $EtOH_{abs.}$. To save the RNA from degradation 1 µl RNAse Inhibitor was added to the reaction mix.

B.12.3. DNase digestion of isolated RNA

In order to make sure that the isolated RNA which was later used as template for Reverse Transcription PCR (RT-PCR) was free of DNA, a DNase digestion (Deoxyribonuclease I, Amplification grade; Invitrogen, Lofer, Austria) of the isolated RNA-solution was performed. RNase inhibitor, preventing RNA degradation was also added to the reaction mix.

Standard reaction mix (50 µl):

RNA solution	25 µl
DNase I	15 µl
DNase Buffer (10x)	5 µl
RNase Inhibitor	1 µl
$H_2O_{bidest/DEPC}$	ad 50 µl

The reaction was incubated at RT for 60 min, and finally terminated by the addition of 5 µl 25 mM EDTA and incubation for 10 min at 65°C in a water-bath.

B.12.4. Reverse Transcription PCR (RT-PCR)

The isolated and DNA-free RNA was used in a consecutive RT-PCR. This was performed using the RevertAid^{TM} First Strand cDNA Kit (Fermentas, St.Leon-Rot, Germany) following the manufacturer's instructions.

1 µg RNA per reaction was used for reverse transcription utilizing the gene-specific reverse primers pc0373R and pc0374R, respectively.

The obtained complementary DNA (cDNA) was then further amplified using the standard PCR approach (see Sec. B.11.3.) and consequently the presence of amplificates was checked by gelelectrophoresis.

B.13. Cloning of gene amplificates

Table B.9. Vector used.

Vector	Size	Properties	Reference
pET-16b	5711 bp	PT7, Amp^R, *ori*pBR322, *lac*I, N-term. 10xHis	Novagen, Darmstadt, Germany

Table B.10. Transformation-competent *E. coli* strains used.

Strain	Type of transformation	Genotype	Growth temp., medium
BL21*	electroporation	F– *dcm ompT hsdS*(rB- mB-)*gal*	37°C, LB
BL21 (DE3)	electroporation	F- *dcm ompT hsdS*(rB- mB-) *gal* λ(DE3)	37°C, LB
BL21 Codon Plus (DE3) RIPL	electroporation	F- *ompT hsdS*(rB- mB-) *dcm*+ Tetr*gal* λ(DE3) *endA* Hte [*argU proL*Camr] [*argU ileY leuW* Strep/Specr]	37°C, LB
C43 (DE3)	electroporation	F- *dcm ompT gal hsdSB* (rB- mB-) *lon* _DE3	37°C, LB

All *E. coli* strains described in Tab. B.10. were used as electrocompetent cells. The strain BL21 was used for amplification, preparation and maintenance of vectors and recombinant plasmids. The others are expression strains and served for heterologous protein expression. The expression strains feature a protease-deficiency in order to prevent degradation of the heterologously produced protein. Moreover, the *E. coli* strain BL21 Codon Plus (DE3) RIPL encodes for rare codons and is tolerant to toxic proteins.

B.13.1. Preparation of PCR-products

After the genes of interest had been amplified, the duplicate reactions were pooled and finally purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).
The purified PCR-products were modified at the 3'- and 5'-end by generating sticky ends, which were introduced by a double digestion with the restriction enzymes NdeI and XhoI (Fermentas Inc., Hanover, MD, USA).

Double digest reaction mixture (30 µl)

PCR-product (25-30 ng/µl)	20 µl
10x R buffer	3 µl
XhoI (10 units/µl)	3 µl
NdeI (10 units/µl)	3 µl
H_2O_{bidist}	ad 30 µl

The reaction mixture was incubated for 3 hours in a water-bath at 37°C. Finally, the temperature was shifted to 65°C for 20 min to inactivate the restriction enzymes.

B.13.2. Preparation of vector

5 ml tubes of LB-Amp medium were inoculated with *E.coli* cells containing the pET-16b vector and grown o/n. The vector was then isolated using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

The pET-16b vector was digested with the same restriction enzymes as the PCR products prior to ligation (see Sec. B.13.1).

Double digestion reaction mixture (20 µl)

Vector pET-16b (150 ng/µl)	3µl
10x R buffer	2 µl
XhoI (10 units/µl)	3 µl
NdeI (10 units/µl)	3 µl
H_2O_{bidist}	ad 20 µl

B.13.3. Ligation

After double digestion of both, the PCR-products and the vector, resulting in the introduction of sticky ends, a ligation step using a T4-DNA-ligase (Fermentas Inc., Hanover, MD, USA) was performed. In order to make sure that the PCR-products are present in excess compared to the vector, PCR-product and vector concentrations were determined photometrically and the molecular ratio (mr) of both were calculated. The molecular ratio of PCR-products and vector used in the ligation step accounted for

mr (PCR-product) : mr (vector) = 3 : 1

Standard reaction mixture (20 µl)

- undiluted PCR-product

5x ligase buffer	4 µl
T4 DNA ligase	1 µl
PCR-product	4 µl
vector	4 µl
H_2O_{bidist}	ad 20 µl

- diluted PCR-product (1:8 dilution in $H2O_{bidist}$)	
5x ligase buffer	4 µl
T4 DNA ligase	1 µl
PCR-product	3 µl
vector	1 µl
H_2O_{bidist}	ad 20 µl

The reaction was incubated o/n at 4°C.

In order to prevent a short-curcuit during the following electroporation step caused by salt ions present in the reaction mixture, salt ions were removed by dialysis against H_2O_{bidist} for 20 min using a nitrocellulose filter (pore size 0,025 µm, Φ 22 mm; MF Millipore, Milliopore Corporation Billerica, MA, USA).

B.13.4. Transformation of plasmid DNA

One method for the transformation of plasmid DNA into bacterial cells is electroporation. In this method, the bacterial cell membrane is made permeable for charged molecules by a short electric impulse. For this purpose the cells must be made electrocompetent by the procedure described below.
After ligation, the vector containing the insert was transformed into the non-expression *E. coli*-strain BL21 (see Tab. B.10.) and spread on LB-Amp agar for selection of clones containing a vector (see Sec. B.13.4.2.).

B.13.4.1. Preparation of electrocompetent *E. coli* strains

For the preparation of electrocompetent *E.coli* cells the 1.5 l dYT medium was inoculated with the desired strain at a ratio of 1:1000 and was grown on a shaking platform at 37°C until OD_{600}= 0.5-0.6 was reached. Then the cells were placed on ice for 15 min to cool them down. All further steps were performed on ice. The cells were harvested in 50 ml falcon tubes by centrifugation (5000 rpm, 15 min, 4°C). The cell pellet was washed with an overall volume of 1 l ice-cold $H2O_{bidist}$. The pellet was resuspended in 50 ml 10% aqueous glycerine solution (ice-cold) followed by an additional centrifugation step. After carefully removing the supernatant, the pellet was resuspended in an equal volume 10% glycerine solution, aliquoted à 100 µl in screw caps and immediately stored at -80°C until further use.

B.13.4.2. Transformation using electrocompetent cells

For each reaction two LB Amp agar plates were accommodated to 37°C for ~15 min. The electrocompetent cells were gently thawed on ice, while in the meantime the SOC medium was accommodated to RT and the electroporation cuvette (0.2cm, Biorad) was cooled on ice.

10 µl of the ligation mixtures were added to the competent cells and the mixture was stirred gently. After incubation for 15 min on ice, the whole solution was pipetted into the electroporation cuvette and placed in the electroporator (Micro Pulser^{TM}). Then the electrical impulse was performed with the following parameters: voltage of 2.5 kV, capacity of 25 mF, resistance of 200 Ω, with a time for the electric discharge of 4.5 to 5 msec. After that, 250 µl of SOC medium were added and the suspension was transferred to a screw cap and incubated for 1 h at 37°C on an orbital shaker. Finally, 100 and 150 µl of the suspension were plated, respectively, and incubated o/n at 37°C.

B.13.5. Identificaton of positive clones - Insert screening via T7-PCR

Table B.11. Primers for insert screening used.

Primer name	Sequence (5' - 3')	T_m	target vector
T7 F	TAA TAC GAC TCA CTA TAG GG	56	pET-16b(+)
T7 R	GCT AGT TAT TGC TCA GCG G	56	pET-16b(+)

The cells were grown on agar Amp plates, in order to select for cells harbouring the pET-16b vector, containing an Amp resistance site. After cloning, the recombinant cells were checked for the presence of a plasmid containing the desired PCR-product as insert.

After the cloning reaction, a T7-screening-PCR was performed in order to identify positive clones. T7-primers bind to the flanking regions of the insert site on the vector and therefore PCR allows the amplification of the insert and estimation of the insert size. The reaction mix was the same as for a standard PCR, as well as the conditions, with the exceptions that the annealing temperature was set at 56°C and the reaction volume was 25 µl per well. Apart from that, whole cells containing the constructed vector were used in the PCR-mix.

A part of a colony was picked using a sterile toothpick and suspended in the reaction mix, provided in one cavity of a 96-well microtiterplate (MJ Research, Waltham, MA, USA), which was kept on ice. The microtiterplate was sealed with a thermostable foil (MJ Research, Waltham, MA, USA) and the PCR was started.

PCR products were analysed via 1.5% agarose gel. Clones with an insert of correct size were further analysed.

B.14. Restriction fragment length polymorphism (RFLP)

After the T7-screening PCR, restriction fragment length polymorphism (RFLP) analysis was performed using the obtained PCR-products. In RFLP, restriction endonucleases are used to cut DNA at recognition sites, specific for the respective enzyme. Since distances between the restriction sites vary between different sequences, RFLP can be used for a rough estimation of the sequence diversity of a clone library. For this, the resulting RFLP fragments were analysed by agarose gel electrophoresis.
For all RFLP analyses the enzyme AluI was used.

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Table B.12. Enzyme used for RFLP analysis of PCR-products.

Restriction enzyme	Restriction site[a]	Buffer	T_{inc} [°C][b]	Company
AluI	5'-A G ↓ C T-3'	Tango	37	Fermentas Life Sciences Inc., Hanover, MD, USA

[a] arrow indicates site of restriction

[b] incubation temperature

Standard reaction mix (30 µl)

Restriction enzyme (10 U/µl)	1 µl
Buffer	3 µl
PCR-product	20 µl
H_2O_{bidist}	ad 30 µl

The reaction mix was incubated for 3 h at 37°C. Subsequently, loading buffer was added to the reaction mixture to stop restriction.

Reactions were applied to 2-2.5% agarose gels and run at 100-120V for 1.5-2 h. The plasmid of at least one representative clone of each banding pattern obtained was isolated (Sec. B.9.2), and the inserts of the purified plasmids were sequenced (see Sec. B.15).

B.15. DNA-sequencing

Sequencing was performed following the principle of cycle sequencing, being a combination of the di-deoxy- mediated chain termination method (Sanger et al., 1977) and PCR including a DNA-polymerase (Saiki et al., 1988).

B.15.1. Sequencing using Li-cor Long Readir 4200

The reaction was carried out using the ThermoRequinase Cycle Sequencing Kit (Amersham, Freiburg, Germany) according to the instructions of the manufacturer. For sequencing of vector inserts primers complementary to the vector were used (Tab. B.11.). The primers were obtained from Thermo Electron GmbH (Ulm, Germany) and were labelled with the fluorescent dye IRD800 (heptamethine cyanine) at its 5'-end. The reactions were analysed by an automated DNA sequencer (Li-cor Long Readir 4200, MWG-Biotech) by electrophoretic separation on a polyacrylamid (PAA)-gel. After separation, labelled bands were excited by a laser and detected by a photodiode. The resulting signals were processed and an image was stored. Using the software program e-seq (Licor Inc., Biotechnology Division, Lincoln, NE, USA), sequences were determined automatically and exported in FASTA-format.

B.16. Heterologous expression of proteins

For the structural and biochemical characterisation large amounts of protein are required and usually extraction of sufficient amounts from a homologous system is difficult. Apart from that, the regulation of gene expression of unknown proteins is usually not known either.

In order to overcome these problems, proteins can be expressed using a heterologous expression system, where the protein is expressed in a different host organism. For this purpose the gene coding for the given protein is inserted into the multiple cloning site of a vector which contains a promotor controlling the expression of the inserted gene.

Vectors and strains used for the heterologous expression of proteins are described in Tab. B9. and Tab. B.10. Cloning of genes of interest is described in Sec. B.13.

B.16.1. Heterologous expression of *pc0373* and *pc0374* in different *E. coli* expression strains

Cells harbouring the constructed vector with insert were grown on plates containing solid media as well as in liquid media.

For protein expression, 50 ml of LB-Amp medium were inoculated with an o/n culture of the respective *E. coli* expression strain containing the constructed vector and grown at 37°C on an orbital shaker until an OD of 0.5-0.6 was reached.
After that, protein expression was induced using Isopropyl-β-D-thiogalactopyranosid (IPTG) at an end concentration of 1mM and incubated for another 4 hours. Cultures which had not been induced with IPTG were used as negative control. IPTG binds to the repressor, which is bound to the operon of the gene. IPTG-binding to the repressor results in the dissolution of the repressor from the operon, the gene can be transcribed and finally translated into the corresponding protein.

Cells were harvested by centrifugation (8000 rpm, 15 min, 4°C) and washed with 1x PBS. The cell pellet could then be stored at -20°C until further analysis.

For protein purification (see Sec. B.17.) larger amounts of induced cell culture were required. For this purpose, cells were grown in 1 l of liquid medium.

B.16.2. Protein gelelectrophoresis

After induction with IPTG, the cells were further processed for protein gelelectrophoresis. For this purpose, 400 µl of the cell culture were pelleted down by centrifugation (8000 rpm, 15 min, 4°C) and resuspended in 40 µl 4x SDS-PAGE loading buffer (see below). 1 µl of benzonase (Fermentas, St. Leon-Rot, Germany) was added in order to remove present DNA and the solution incubated on ice for 1 h. After that, gelelectrophoresis was performed.

B.16.2.1. Sodium-Dodecylsulfate - Polyacrylamidgelelectrophoresis (SDS-PAGE)

Solutions for SDS-PAGE

a) SDS-PAGE loading buffer

Tris/HCl, pH 6.8	200 mM	2.42 g
SDS	8%	8 g
Bromphenol blue	0.2%	0.02 g
Glycerine	40%	40 ml
DTT	400 mM	6.2 g
β-Mecaptoethanol		28.5 ml
H_2O_{bidist}		ad 100 ml

b) 10x SDS-PAGE running buffer

Tris	30.2 g
Glycine	144 g
SDS	10 g
H_2O_{bidist}	ad 1000 ml

c) SDS-PAGE upper buffer

Tris	30.3 g
10% SDS	20 ml
H_2O_{bidist}	ad 500 ml
pH 6.8	

d) SDS-PAGE lower buffer

Tris	90.85 g
10% SDS	20 ml
H_2O_{bidist}	ad 500 ml
pH 8.8	

e) Coomassie dye solution

Methanol	50% (v/v)
Acetic acid	10% (v/v)
Coomassie brilliant blue R 250	1.38 g
H_2O_{bidist}	ad 500 ml

f) Destain solution

EtOH	20% (v/v)
Acetic acid	5% (v/v)

SDS-PAGE (12.5%)

a) Separating gel

Lower buffer	2 ml
30% AA/BA	3.33 ml
10 % APS	40 µl
TEMED	8 µl
H_2O_{bidist}	2.66 ml

b) Stacking gel

Upper buffer	0.63 ml
30% AA/BA	0.38 ml
10% APS	17.5 µl
TEMED	10 µl
H_2O_{bidist}	1.63 ml

Procedure

Analysis of expression cultures was done by Sodium Dodecyl Sulfate-Polyacrylamide-gelelectrophoresis (SDS-PAGE) using a buffersystem according to Laemmli (Laemmli, 1970). Proteins bind SDS and form a denaturated, negatively charged complex with a constant charge distribution. During electrophoresis the proteins are separated based on their size.

Separating gels with an AcrylAmid/BisacrylAmid(AA/BA)-concentration of 12.5% allow the separation of proteins with a molecular weight of up to 50 kDa.
TEMED and APS are responsible for the polymerisation process and were added immediately before pouring the gel solution into the gel chamber (Biorad, München, Germany). After that, the separating gel was overlayed with isopropanol and left to polymerize. The isopropanol was then removed with filter paper and the stacking gel solution added. The respective comb was inserted immediately.

The polymerized gels were inserted into the gelelectrophoresis chamber (Mini Protean 3 Cell, Biorad) and filled with 1 x running buffer. The prepared samples (see Sec. B.16.2) as well as a protein marker allowing size estimation were pipetted in the slots and electrophoresis was started using 100 V. When the marker reached the gel boarder on the bottom, electrophoresis was stopped and the gel was incubated for 1 h in the commassie staining solution. After destaining for 1 h in destaining solution, protein bands were visible and the gel could be scanned. For more distinct bands and lower background the gel was washed in H_2O_{bidist} o/n.

B.16.2.2. Tricine-Sodium Dodecyl Sulfate-PAGE (Tricine-SDS-PAGE)

For separation of smaller proteins with a molecular weight of 10 kDa and below, the PAGE protocol described by Schägger and Jagow is better suited (Schägger and Jagow,

1987). In this protocol, tricine is used as the trailing ion, which allows a resolution of small proteins at lower acrylamide concentrations than in SDS-PAGE systems. The protocol allows a superior resolution of proteins, especially in the range between 5 and 20 kDa, and overloading effects are largely reduced.

Solutions for Tricine-SDS-PAGE:

a) Gel buffer (3x)

Tris	3 M
HCl	1 M
SDS	3%
pH 8.45	

b) Anode buffer (10x)

Tris	1 M
HCl	0.225 M
pH 8.9	

c) Cathode buffer (10x)

Tris	1 M
Tricine	1 M
SDS	1%
pH ~8.25	

Tricine SDS-PAGE (12.5%)

a) Separating gel

30% AA/BA	3.4 ml
3x Gel buffer	2.7 ml
Glycerine (100%)	670 µl
TEMED	9 µl
10% APS	82 µl
H_2O_{bidist}	1.1 ml

b) Stacking gel

30% AA/BA	340 µl
3x Gel buffer	890 µl
TEMED	3.4 µl
10% APS	55 µl
H_2O_{bidist}	1.4 ml

Procedure

Sample preparation is described in Sec. B.16.2. Gel loading was performed as for SDS-PAGE using the same instruments. For Tricine-SDS-PAGE, two different buffers were used: an anode buffer and a cathode buffer instead of the running buffer for SDS-PAGE. Electrophoresis, staining and destaining of the gels was performed as for SDS-PAGE using the solutions described in Sec. B.16.2.1.

B.17. Purification of His-tagged proteins and quantification

The Histidine tag (His-tag) encoded on the vector and located prior to the N-terminal part of the expressed protein allows the purification of the expressed protein via immobilized metal affinity chromatography.

The purification is performed via columns which contain a prepacked matrix charged with metal ions like Cu^{2+}, Co^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+}or Fe^{3+}. When proteins with surface-exposed amino acids – like a His-tag – are applied to the column, they bind to the metal ions and a complex is formed.

There are several factors which can affect the binding strength between the protein and the metal ions: the length of the affinity tag, the type of metal ions used as a binding matrix, or the pH of the buffers used.

After the desired protein has bound specifically to the column it can be eluted by adding competitors with a strong chelating nature (*e.g.* imidazole), or by lowering the pH.

B.17.1. Purification of His-tagged proteins using the Qiagen Ni-NTA Spin Columns

This protocol is based on the binding of His-tagged proteins to a Ni^{2+} matrix and consecutive elution of the protein by decreasing pH.

Protein purification was performed following the instructions of the manufacturer using 8 M concentration of urea.

B.17.2. Purification of His-tagged proteins using the Amersham HisTrap HP Columns

This protocol is also based on protein binding to a Ni^{2+} matrix via the His-tag. Protein elution is achieved by the addition of increasing concentrations of imidazole, which has a strong chelating nature and competes with the protein for the binding to the matrix.
Protein purification was performed following the instructions of the manufacturer using 8 M concentration of urea.

B.17.3. Desalting of purified proteins

Desalting of the purified proteins prior to antibody generation is crucial due to the fact that urea is toxic for animals and has to be removed before immunisation.
For this purpose, the purified fraction containing the desired protein was filled into a dialysis hose and placed in a beaker containing 1x PBS. Dialysis of urea against 1x PBS was performed o/n under gentle stirring.

B.17.4. Quantification of purified proteins

Quantification of the purified proteins was performed using the BCA Protein Reagent Assay Kit (Pierce, Rockford, Illinois, USA) following the instruction of the manufacturer.

This assay is a formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. It combines the reduction of $Cu^{(II)}$ to $Cu^{(I)}$ by proteins in an alkaline medium with the colorimetric detection of the cuprous ion ($Cu^{(I)}$). The purple-coloured reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentration over a range of 20-2,000 µg/ml.
The photometric measurement at 562 nm was performed with the spectralphotometer SmartSpec^{TM} 3000 (Biorad, München, Germany).

B.18. Antibody generation against purified proteins

Approximately 1 mg/ml of the purified and desalted proteins were used for antibody generation. Primary antibodies against Pc0373 and Pc0374 were obtained from Eurogentech (Seraing, Belgium).

Primary antibodies were raised in both rabbit and guinea pig. Pre-immune sera obtained before the first antigen boost were provided as negative controls. Rabbit and guinea pig sera were obtained after a first and after a consecutive second boost. Final bleed sera were obtained a few weeks after the second boost.

B.19. Bioinformatical analysis and protein prediction

Comparative sequence analysis of the nucleotide sequences of *pc0373* and *pc0374* was performed using the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1990) search engine of the National Institute of Health (USA). The algorithms of this tool search the database for similar sequences and rank them according to their similarity.

Bioinformatical analysis and protein prediction based on the amino acid sequence of Pc0373 and Pc0374 of *P. amoebophila* were performed using the PredictProtein server (Rost et al., 2004).

PredictProtein is an internet service for the analysis and prediction of protein structure and function such as sequence motifs, secondary structure, coiled-coil regions, transmembrane helices and functional annotations.

B.20. Immunofluorescence (IF) assay

B.20.1. Primary and secondary antibodies

All the antibodies used in these studies are polyclonal. The immunisation triggers an increased production of specific antibodies and their enrichment in the animal's serum.

The immunofluorescence (IF) technique used for this study is an indirect assay. This means that the primary antibody binds specifically to the antigen, i.e. the chlamydial protein, while the secondary antibody binds to the non-variable part of the primary antibody. The secondary antibody is conjugated to a fluorescent dye for detection. For example, if the primary antibody was developed in rabbit, the secondary antibody is won through the immunisation of another animal, *e.g.* goat, with the rabbit's non-variable heavy-chain-fraction of the respective antibody (in the present case immunoglobulin G, or IgG).

The optimal dilutions of the primary antibodies were determined empirically using several potentially increasing dilution factors from 1:8 to 1:1024, considering the strongest specific fluorescence signal obtained for the assay.

Table B.13. List of primary antibodies.

Antibody	Antigen	Source of antibody	Modification	Manufacturer	Remark
Pc0373-pi-rab	none	rabbit	none	Eurogentech	pre-immune serum (serum won before immunisation)
Pc0373-pi-gp	none	guinea pig	none	Eurogentech	pre-immune serum (serum won before immunisation)
Pc0373-sb-rab	full length protein Pc0373	rabbit	none	Eurogentech	small bleed (serum won after second antigen boost)
Pc0373-sb-gp	full length protein Pc0373	guinea pig	none	Eurogentech	small bleed (serum won after second antigen boost)
Pc0373-lb-rab	full length protein Pc0373	rabbit	none	Eurogentech	large bleed (serum won after third antigen boost)
Pc0373-lb-gp	full length protein Pc0373	guinea pig	none	Eurogentech	large bleed (serum won after third antigen boost)
Pc0373-fb-rab	full length protein Pc0373	rabbit	none	Eurogentech	final bleed (serum won after fourth antigen boost)
Pc0373-fb-gp	full length protein Pc0373	guinea pig	none	Eurogentech	final bleed (serum won after fourth antigen boost)
Pc0374-pi-rab	none	rabbit	none	Eurogentech	pre-immune serum (serum won before immunisation)
Pc0374-pi-gp	none	guinea pig	none	Eurogentech	pre-immune serum (serum won before immunisation)
Pc0374-sb-rab	full length protein Pc0374	rabbit	none	Eurogentech	small bleed (serum won after second antigen boost)
Pc0374-sb-gp	full length protein Pc0374	guinea pig	none	Eurogentech	small bleed (serum won after second antigen boost)
Pc0374-lb-rab	full length protein Pc0374	rabbit	none	Eurogentech	large bleed (serum won after third antigen boost)
Pc0374-lb-gp	full length protein Pc0374	guinea pig	none	Eurogentech	large bleed (serum won after third antigen boost)
Pc0374-fb-rab	full length protein Pc0374	rabbit	none	Eurogentech	final bleed (serum won after fourth antigen boost)
Pc0374-fb-gp	full length protein Pc0374	guinea pig	none	Eurogentech	final bleed (serum won after fourth antigen boost)
UWE25 serum*	EBs of *Protochlamydia amoebophila* UWE25	rabbit	none	Universitäts-klinikum Ulm	serum won after immunization with partially purified EBs
UV-7 serum*	EBs of *Parachlamydia acanthamoebae* UV-7	rabbit	none	Universitäts-klinikum Ulm	serum won after immunization with partially purified EBs
Sn serum*	EBs of *Simkania negevensis* Z	rabbit	none	Universitäts-klunikum Ulm	serum won after immunization with partially purified EBs

* Sera were kindly provided by Dr. Sven Poppert, Universitätsklinikum Ulm, Germany

Table B.14. List of secondary antibodies.

Antibody	Antigen	Source of antibody	Modification	Manufacturer	Remark
anti-rab-Cy2	rabbit IgG	goat	Cy2	Amersham	Cy2-conjugated
anti-rab-Cy5	rabbit IgG	goat	Cy5	Dianova	Cy5-conjugated
anti-rab-FITC	rabbit IgG	goat	FITC	Dianova	FITC-conjugated
anti-rab-HRP	rabbit IgG	goat	HRP	Sigma	HRP-conjugated
anti-gp-Cy2	guinea pig IgG	goat	Cy2	Dianova	Cy2-conjugated
anti-gp-Cy3	guinea pig IgG	goat	Cy3	Dianova	Cy3-conjugated
anti-gp-HRP	guinea pig IgG	goat	HRP	Sigma	HRP-conjugated

B.20.2. Mounting solution

In order to reduce fluorescence bleaching during microscopy, Mowiol 4-88 (Sigma-Aldrich Chemie GmbH, Steinhausen, Germany) containing 10% 1,4-Diazabicyclo(2,2,2)octane (DABCO, Carl Roth GmbH & Co., Karlsruhe, Germany) was used as the mounting medium. Mowiol is a poly-vinyl alcohol which becomes solid after ~30 min at RT and prevents the dissolution of the cells from the slide surface. DABCO is meant to reduce the bleaching of the fluorescence signal under UV light irradiation.

The mounting solution was prepared as follows: 2.4 g Mowiol 4-88 were mixed with 6 g glycerine and 6 ml H_2O_{bidist}. 12 ml of a 0.2 M Tris solution (pH 8.5) was added and the solution was stirred at 50-60°C to dissolve the Mowiol. After that, the solution was centrifuged at 5,000 rpm for 15 min at RT to remove air bubbles, the supernatant was collected and 10% w/v of DABCO was added. The obtained solution was stored in aliquots at -20°C.

B.20.3. Immunofluorescence on *Acanthamoeba castellanii* UWC1-cells harboring *Protochlamydia amoebophila* UWE25 endosymbionts

The assay was performed with amoebal cultures containing the chlamydial endosymbiont, while amoebae devoid of endosymbionts were used as negative control.

Pre-immune sera obtained prior to immunisation of the animal with the antigens Pc0373 and Pc0374 were used to check for unspecific antibodies.

B.20.3.1. Blocking of antibodies

Due to the fact that polyclonal antibodies were used in this study, there could be the possibility that the serum also contains antibodies triggered against amoebal proteins, thus resulting in an unspecific background fluorescence of the amoebal host cell. In order to

prevent this and to reduce the background, the obtained sera had to be inactivated against host cell proteins.

For this purpose, a well grown amoebal culture containing no endosymbionts was harvested by centrifugation (2,000 rpm, 10 min, 4°C), the cell pellet resuspended in 1 ml blocking solution (2% Bovine Serum Albumin Fraction V (Carl Roth GmbH & Co., Karlsruhe, Germany) in 1xPBS), sonicated 4 times for 30 sec (Sonicator Bandelin Sonoplus HD2070, Sonotrode Bandelin Sonoplus UW 2070, Bandelin Elektronik, Berlin, Germany) at speed 7, frozen at -20°C and thawed at 45°C to disrupt amoebal cells.

This cell lysate was then mixed 1:1 with the antiserum, vortexed briefly and left at 4°C o/n. The following day, the solution was vortexed again and left at 4°C for another 30 min. Amoebal cell debris was then spinned down at 8,000 rpm for 2 min at RT. The supernatant containing only the unbound specific antibodies against the endosymbiont's proteins was collected and aliquoted.

B.20.3.2. Immunofluorescence protocol

Amoebal cells were detached from the bottom of the culture flask by shaking and 1 ml of cell suspension was pipetted into each well of a 24-well-plate (Nunc, Wiesbaden, Germany) containing a round coverslip (12 mm diameter, Carl Roth GmbH & Co., Karlsruhe, Germany) so that the amoebal cells can attach to the surface of the coverslip. The following steps were performed in the wells at RT.

For IF, the medium was aspirated from each well and washed with 1 ml 1x PBS. The amoebal cells were fixed for 10 min with 100% methanol and washed with 1 ml of 1x PBS. After that, blocking reagent was added to each well and incubated for 20 min. The blocked primary antibody (see Sec. B.20.3.1. and Tab. B.13.) was diluted 1:512 in the blocking solution and 300 µl of this solution were added to each well. After that, the plate was incubated on a shaking platform for 30 min at RT.

The primary antibody solution was aspirated and cells were washed three times with 1 ml 1x PBS. The secondary antibody was diluted 1:1000 in the blocking solution and 300 µl of this solution were added to each well and incubated on a shaking platform for 30 min in the dark. After that, the cells were again washed three times with 1x PBS and finally the solution was aspirated.

The coverslips were taken out of the wells and wiped face-down on a towel damped with H_2O_{dist}. After that, 2 µl of mounting medium (10% DABCO in Mowiol) were pipetted onto a glass slide and the coverslips were placed face-down on the glass slide. After

solidification of the mounting medium (for at least 30 min) the slides were ready for microscopy.

B.20.3.3. IF-Tyramide Signal Amplification (IF-TSA)

The specificity of the antibodies against the proteins Pc0373 and Pc0374 were further analysed after amplification of the fluorescent signal using the TSA Plus Fluorescence System (NEN Life Sciences, Boston, Massachusetts, USA).

The tyramide signal amplification step is performed after incubation of the secondary antibody, which is not fluorescently labelled but is conjugated with a horseradish peroxidase (HRP). HRP catalyzes the deposition of numerous fluorophore-bound tyramides immediately adjacent to the immobilized HRP enzyme, thus resulting in an amplification of the IF signal intensity.

The localisation of the fluorescence signal and its intensity was compared to signals obtained without the additional TSA step. Apart from that, amoebal cells lacking endosymbionts were run as negative control.

Endogenous peroxidases were quenched by incubating the sample with 0.15% H_2O_2 in methanol before addition of primary antibodies. HRP-labelled secondary antibodies were diluted 1:2000 in TNT buffer (see manufacturer's instructions). All other steps were performed following the instructions of the manufacturer.

B.20.4. MicroImmunoFluorescence (MIF) with partially purified chlamydial elementary bodies (EBs)

The MIF assay is used for the detection and titre determination of human sera against whole bacterial cells. In order to optimise the protocol, specific antibodies derived from animals immunised with EBs of the given host were used. The optimised protocol was later used for the screening of human sera and for titre determination.

In order to receive optimal results, the standard MIF protocol described below was optimised by changing different conditions like the washing step and blocking reagents. The aim was to establish a protocol which would provide the strongest specific fluorescent signal and the lowest background fluorescence possible.

The optimal dilution factor for the primary antibody was determined in a dilution series ranging from dilution factor 1:8 to 1:1024.

B.20.4.1. Production of a 4% paraformaldehyde (PFA)- solution

33 ml H_2O_{bidist} were heated to 60-65°C. After addition of 2 g PFA, 1 M NaOH was added drop by drop until the PFA had solved and the solution cleared. Subsequently, 16.6 ml 3x PBS were added and the solution cooled to RT. pH was adjusted to 7.2-7.4 by the addition of 1 M HCl. The solution was sterile filtered (0.22 µm pore size), cooled and finally stored at -20°C.

B.20.4.2. DAPI-staining of EBs

At the beginning, the optimal dilution factor of the obtained partially purified EBs was determined using DAPI-staining. For this purpose, several dilutions (1:1, 1:10, 1:100, 1:1000) were prepared and 1 µl of the respective solutions were pipetted onto the wells of a 10-well slide (Paul Marienfeld, Bad Mergentheim, Germany). After the suspension had dried, the cells were fixed for 20 min using 10 µl 4% PFA and washed with 1x PBS.
A 1:1000 dilution of 4'-6'-di-amidino-2-phenylindole (DAPI, Lactan Chemikalien und Laborgeräte GmbH, Graz, Austria), known to bind double-stranded DNA, was prepared and 10 µl applied to each well. After incubation for 7 min in the dark, DAPI was removed and the cells were washed with 1x PBS and dried at RT in the dark. Finally, the slides were mounted with Citifluor AF1 (Agar Scientific Limited) and a coverslip, and analysed under the epifluorescence microscope Axioplan 2 imaging (Zeiss, Jena, Germany).
The optimal dilution factor of the EB-solution was determined for each organism considering a good dispersal of cells on the well and the possibility to distinguish single cells.

B.20.4.3. The standard MIF protocol

The standard MIF protocol used in our laboratory is based on the MIF assay described by Wang *et al.* (Wang and Grayston, 1970) and was modified as follows:
1 µl of EB-suspension was diluted in 1x PBS using the optimal dilution factor determined by DAPI-staining (see Sec. B.20.4.1.), fixed for 20 min in 10 µl 4% PFA and washed with 1x PBS. The primary antibody was diluted in blocking reagent (2% BSA in 1x PBS) according to the determined optimal dilution factor and incubated at 37°C for 30 min. The cells were washed in 1x PBS and air-dried. After that, the secondary antibody was applied at a dilution of 1:1000, incubated for 30 min at 37°C in the dark and washed again. The dry slides were then mounted with Mowiol and observed under the epifluorescence microscope.

B.20.4.4. Optimisation of the standard MIF protocol

One of the aims of this study was the determination of antibody titres of different human sera against the environmental chlamydiae *Protochlamydia amoebophila* UWE25, *Parachlamydia acanthamoebae* UV-7 and *Simkania negevensis* Z. For optimisation of the MIF protocol, polyclonal antibodies derived after immunisation of rabbit against partially purified EBs of these organisms were used. The optimised MIF protocol was then used for titre determination whereby human sera were applied to EBs of the above mentioned organisms and checked for the presence of specific antibodies.
In order to determine the best conditions for the MIF assay, and to optimise the obtained results, different steps of the standard protocol were variegated:

B.20.4.4.1. Slide treatment

Different slides were compared: slides coated with poly-L-lysine (Sigma-Aldrich Chemie GmbH, Steinhausen, Germany), SuperFrost slides (Fisher Scientific, Vienna, Austria) and uncoated slides. These were 10-well slides (Paul Marienfeld, Bad Mergentheim, Germany) which were cleaned with 70% EtOH prior to cell application.

B.20.4.4.2. Fixative

Two different fixatives were tried: fixation with 4% PFA (as described above), and with 100% methanol. 10 µl of methanol were added to the dried cells and left to evaporate. No washing step was performed.

B.20.4.4.3. Blocking reagent

Primary and secondary antibodies were diluted in different blocking reagents in order to prevent unspecific antibody binding to the slide surface. For this purpose, different blocking reagents were compared:

- 0.25% egg yolk (Biotrading, Mijdrecht, Netherlands) in 1x PBS
- 5% milk powder (Carl Roth GmbH & Co., Karlsruhe, Germany) in 1x PBS
- 1%, 2%, 5%, 10% BSA (Carl Roth GmbH & Co., Karlsruhe, Germany) in 1x PBS

B.20.4.4.4. Washing conditions

Washing of the cells after antibody incubation was performed in three different ways:

- On-slide washing: 10 µl of 1xPBS were pipetted onto the well and then removed

- whole-slide washing: the slide was dipped into 1x PBS
- washing the slide in blocking reagent

B.20.4.4.5. Mounting medium

Several mounting media were compared during this study. These were Mowiol, 10% DABCO in Mowiol and CitiFluor AF1.

B.20.4.4.6. Counterstaining with Evan's Blue

Counterstaining of the slide surface with the fluorescent dye Evans Blue (Sigma-Aldrich Chemie GmbH, Steinhausen, Germany) is meant to reduce the general background signals resulting from autofluorescence. Under irradiation with UV-light, the slide surface appears in a reddish glow in contrast to the green fluorescent signal of the fluorescein-iso-thio-cyanate (FITC)-labelled secondary antibodies.
15 µl of an aqueous 0.1% solution of Evans blue were added to each well and incubated for 5 min. After that, the slides were washed for 10 min with three changes of 1x PBS and were dried on air. Finally, the slides were mounted with a 10% solution of DABCO in Mowiol.

B.20.4.4.7. Cross-reactivity of specific antibodies

One crucial step of this assay is to determine the cross-reactivity of the antibodies derived after immunisation with the EBs of the according organism. This allows checking for the specificity of the antibodies against each organism and to know, whether antibodies specific for one organism also give positive signals for another closely related organism.
For this purpose, EBs of each organism were incubated with antibodies derived against each of these organisms. Thus, antibodies specific against EBs of each of the three environmental chlamydiae mentioned above were checked for their binding to each – *P. amoebophila*, *Parachlamydia sp.* and *Simkania negevensis* EBs. Apart from that, a mixture of all three organisms was used as comparison as well as for optimisation of the assay. EBs of the different organisms, primary and secondary antibodies were mixed and used in this assay.
The previously determined optimal conditions and dilutions factors for each organism and serum were applied.

B.20.4.5. Blood serum preparation

Blood sera of volunteering members of the Department for Microbial Ecology (DOME) were prepared as described below.

Blood samples were taken by a professional nurse using Sarstedt S-monovette® (Sarstedt, Nümbrecht, Germany). S-monovettes containing the blood samples were placed in a 15 ml falcon tube and centrifuged for 10 min at 1000 rpm and 4°C to pellet down blood particles and to obtain the blood serum. Consequently, the cover of the S-monovette was screwed off and the supernatant containing the serum was pipetted into a fresh ERT. Sera were aliquoted and stored at -20°C until use.

B.20.4.6. Optimised MIF protocol and titre determination of human sera

After determining the optimal conditions of the MIF assay, human sera of 15 randomly chosen patients from the Universitätsklinikum Ulm, Germany were screened and their titres against each of the three organisms mentioned above were determined. Apart from that, human sera from the members of the environmental chlamydia group of the Department of Microbial Ecology (DOME) at the University of Vienna, Austria were screened as well.

For these studies several dilutions of the human sera were prepared, applied to fixated EB-cells of each organism and the occurrence of positive signals was determined, while the signal intensity was estimated as well. The following dilutions were checked for titre determination:

- 1:1
- 1:8
- 1:16
- 1:32
- 1:64
- 1:128
- 1:256
- 1:512
- 1:1024

The highest dilution factor which still gives a positive signal is the determinant of the titre concentration of the given serum.

Antibody titres of human sera against the three environmental chlamydiae were determined using the optimised MIF protocol which was performed as follows:
1 µl of EB-suspension of each organism or of the EB-mixture was applied to the well and left to dry. The cells were fixed with 4% PFA for 20 min at RT and washed with 1x PBS on-slide. The human sera (optimal serum dilution factor for each serum was 1:512), diluted in blocking reagent (2% BSA in 1x PBS), was incubated for 30 min at 37°C. The cells were then washed on-slide with the blocking reagent and the secondary antibody was added. The slide was incubated for 30 min at 37°C in the dark. The cells were then washed once in blocking reagent and twice in 1x PBS and the slides left to dry. The dry slides were mounted with 10% DABCO in Mowiol and were ready for microscopy after the Mowiol was left to harden for ~30 min.

B.21. Lipophilic membrane dyes

Two different membrane dyes were used in this study: 3,3'-dihexyloxacarbocyanine-iodide ($DiOC_6$, Invitrogen Molecular Probes, Carlsbad, CA, USA, Cambrex) and Nile Red (Sigma-Aldrich Chemie GmbH, Steinhausen, Germany).
Both are cell-permanent lipophilic dyes that are selective for internal cell membranes. These dyes were used in order to determine the amoebal inner structure and to localize the antigen-specific IF-signal in the amoebal host cell.
Both membrane dyes were applied to the fixed amoebal cells after the addition of the secondary antibodies and the following washing step. The following steps were performed at RT.
$DiOC_6$ was used at a concentration of 2 µg/ml in 1x PBS and incubated for 3 min followed by a washing step and mounting of the coverslip with 10% DABCO in Mowiol.
Nile Red was diluted in 1x PBS to a working concentration of 2 µg/ml and incubated for 5 min. The slides were then washed, mounted and observed under the microscope.

C. Results

C.1. Bioinformatical analyses of the two *Protochlamydia amoebophila* UWE25 proteins Pc0373 and Pc0374

Search for homologous proteins in the *Protochlamydia amoebophila* UWE25 genome were performed using the protein blast tool of NCBI (Tab. B.1). Protein prediction based on the amino acid sequence of the two ORFs *pc0373* and *pc0374* found in the blast search was performed using the Predict Protein Server of the Columbia University (see Sec. B.19).

C.1.1. Comparative sequence analysis

In 2005, Subtil and colleagues demonstrated the secretion of several proteins of pathogenic chlamydiae via the Type III Secretion System (TTSS) in a heterologous system (Subtil et al., 2005). In order to find out whether the genome of the environmental chlamydia *P. amoebophila* contains homologues on amino acid level, a protein BLAST search was performed. Two predicted proteins (Pc0373 and Pc0374) were found, which were highly homologous to the unknown *Chlamydophila pneumoniae* protein Cpn0725 found to be secreted via the TTSS by Subtil *et al*. These two proteins had not been identified as homologues to known TTSS substrates during the annotation of the *P. amoebophila*-genome. Pc0373 (with a length of 75 amino acids) and Pc0374 (with a length of 70 amino acids), showed an amino acid (aa)-homology of 43% (over a length of 64 aa) and of 39% (over a length of 48 aa), respectively, to Cpn0725. No homologues to the three other proteins of *Chlamydophila pneumoniae* found to be secreted via the TTSS by Subtil *et al.* could be found (Tab.C.1.).

Cpn0725 of *C. pneumoniae* is highly homologous (over 50% aa-homology) to proteins in other pathogenic chlamydiae: the *Chlamydophila caviae* protein CCA00018 and the *Chlamydia trachomatis* protein CT652.1, for which TTSS-dependent secretion was also demonstrated by Subtil *et al.* (Subtil et al., 2005).

These homologous proteins were also blasted against the *P. amoebophila* genome and were as well found to be highly similar to the *P. amoebophila* proteins Pc0373 and Pc0374. CCA00018 shares a 42% homology with Pc0373 over 57 aa and 44% with Pc0374 over 43 aa.

CT652.1 shows a homology of 37% (over 54 aa) with Pc0373 and of 36% (over 47 aa) with Pc0374.

When the two *P. amoebophila* proteins of interest were blasted against the whole protein database, the first hit was CAB018 of *Chlamydophila abortus* with a homology of 46% over a length of 49 aa for Pc0373 and of 40% over a length of 54 aa for Pc0374.
Organisms with proteins showing the highest homology to the two *P. amoebophila* proteins which do not belong to the *Chlamydiales* family were *Shewanella oneidensis* for Pc0373 and *Rhizobium leguminosarum* for Pc0374, respectively.

Table C.1. Protein BLAST results for the *P. amoebophila* -proteins Pc0373 and Pc0374.

Homologous proteins	Pc0373 (length 75 aa)	Pc0374 (length 70 aa)
C. pneumoniae Cpn0725	43% (64 aa overlap)	39% (48 aa overlap)
C. trachomatis 652.1	37% (54 aa overlap)	36% (47 aa overlap)
C. caviae CCA00018	42% (57 aa overlap)	44% (43 aa overlap)
C. abortus CAB018	46% (47 aa overlap)	40% (54 aa overlap)
Simkania negevensis Z	No significant homologue	40% (61 aa overlap)
Parachlamydia acanthamoebae UV-7	59% (42 aa overlap)	47% (55 aa overlap)
First hit to non-chlamydial organisms:		
Shewanella oneidensis hypothetical protein	27.8% (73 aa overlap)	----------
Predicted ORF of *Rhizobium leguminosarum*	----------	39.6% (48 aa overlap)

C.1.2. Protein prediction of Pc0373 and Pc0374

Based on the amino acid sequence, the following alignment was produced by the Predict Protein Server for Pc0373. The proteins in this alignment belong to the pathogenic chlamydiae and are hypothetical proteins whose function is yet unknown. One of these proteins belongs to *P. amoebophila* and represents the other hypothetical protein analysed in this study, Pc0374 (see Fig. C.1.). The alignment shows that the N- and C-terminal parts of the proteins Pc0373 and Pc0374 are highly variable, while the middle sections are rather conserved.

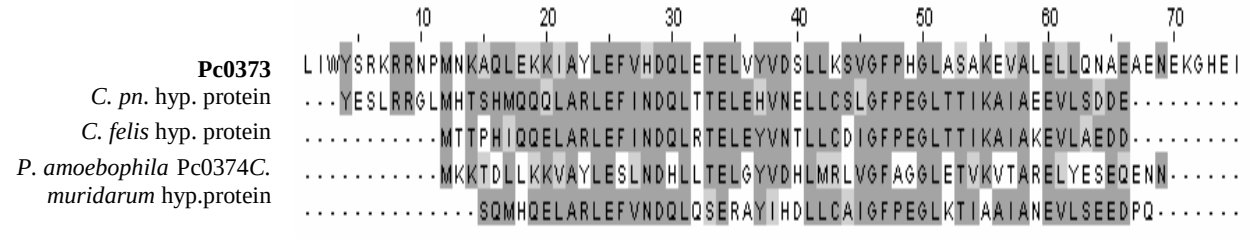


Fig. C.1. Multiple sequence alignment of Pc0373. Hyp.: hypothetical

The alignment produced for Pc0374 by the Predict Protein server includes only hypothetical proteins with unknown function belonging to different pathogenic chlamydiae (see Fig. C.2.).

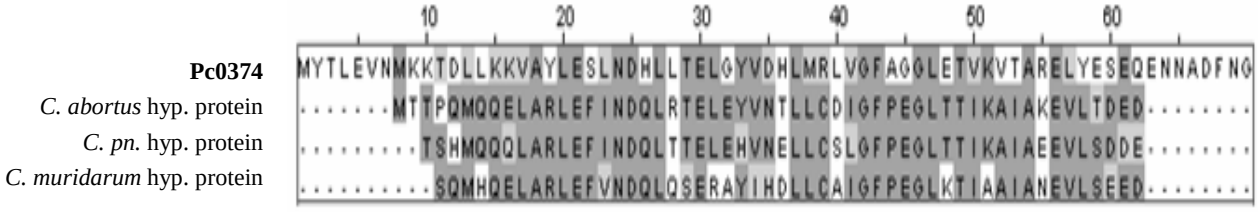


Fig. C.2. Multiple sequence alignment of Pc0374. Hyp.: hypothetical

The predicted secondary structure of Pc0373 shows three helices interrupted by coils (see Fig. C.3.), while the secondary structure of Pc0374 contains only two helices (see Fig. C.4.).

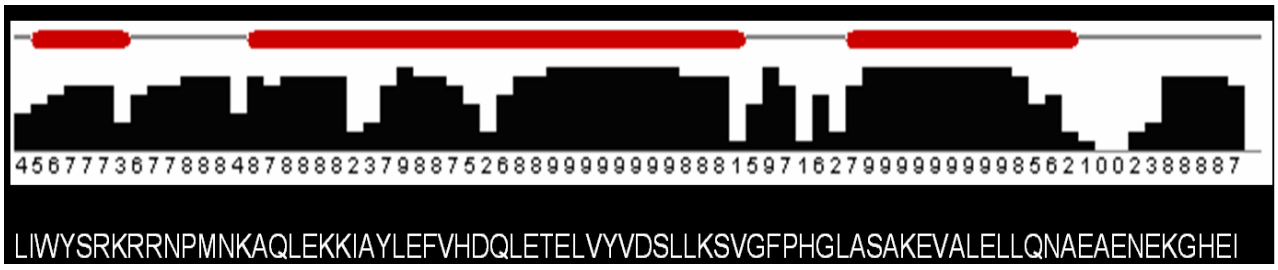


Fig. C.3. Predicted secondary structure of Pc0373. Bars and numbers represent the confidence interval of the predicted structures. Red: helix, grey: coil.

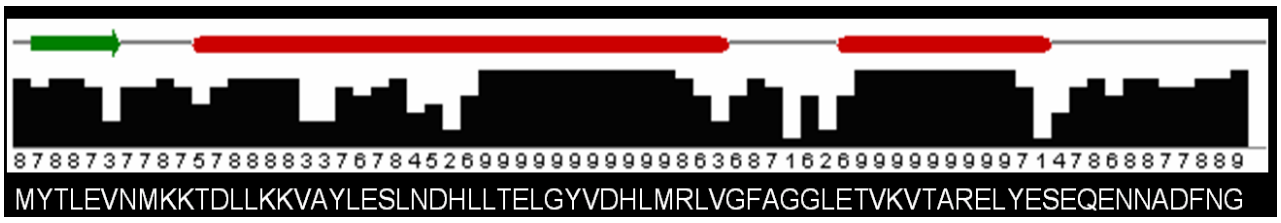


Fig. C.4. Predicted secondary structure of Pc0374. Bars and numbers represent the confidence interval of the predicted structures. Red: helix, grey: coil, green: loop.

Pc0373 and Pc0374 are rather small proteins with a length of 75 and 70 amino acids, respectively. Pc0373 has a molecular weight of 8.7 kDa, while for Pc0374 the molecular weight accounts for 8 kDa. Their isolelectric points are within the acidic range at 5.88 and 4.8, respectively (see Tab. C.2.).

The SOSUI tool (Tab. B.1) was used for calculation of the average hydrophobicity of the two proteins, and for the prediction, whether these are soluble or membrane proteins. The length of the amino acid sequence is also considered in the calculation.
Pc0373 has an average hydrophobicity value of -0.473, while for Pc0374 the value accounts for -0.299. According to the SOSUI tool, both proteins are expected to be soluble (see Tab. C.2.).

The two proteins have no putative conserved domains, no signal peptide and no transmembrane helices. Both are predicted to be non-globular proteins and not to form disulfide bonds due to the absence of cysteine residues.
The motif search performed with the ProSite tool revealed a protein kinase C phosphorylation site, a casein kinase II phosphorylation site and a N-myristoylation site for Pc0373. The same motifs were also found for Pc0374.

Their content of the amino acid leucine is higher-than-average, accounting for 13.3% in Pc0373 and 17.1% in Pc0374, respectively. A search for leucine-rich-repeats (LRR) with the consensus sequence $LxxLxLxx^{N}$ was performed using the ProSite tool. No such repeat was found for neither Pc0373, nor Pc0374 (see Tab. C.2.).

Table C.2. Properties of Pc0373 and Pc0374.

P. amoebophila proteins	Pc0373	Pc0374
function	unknown	unknown
protein length	75 amino acids	70 amino acids
molecular weight	8.7 kDa	8 kDa
isoelectric point	5.88	4.8
signal peptide	no	no
putative conserved domains	none	none
transmembrane helices	none	none
leucine content	13.3%	17.1%
leucine-rich repeat $LxxLxLxx^{N}$	none	none
predicted solubility (SOSUI tool)	soluble	soluble

C.2. Transcription analyses of *pc0373* and *pc0374*

In order to make sure that the *pc0373*- and *pc0374*-genes are functional and are transcribed in *P. amoebophila*, RNA was isolated from amoebal cultures containing the endosymbiont. RNA isolation and RT-PCR were performed three times, each experiment independently at different time points (as described in Sec. B.12).

For transcription analyses, RNA was isolated from an unsynchronised amoebal culture and RT-PCR was performed. DNase digestion was performed right after the RNA isolation in order to make sure that no DNA was present in the preparation. This was confirmed in a standard PCR using the primers specific for each of the two genes. Genomic DNA isolated from *P. amoebophila*-EBs was used as positive control and a PCR-mix without the addition of template was used as negative control (NC). For all three independent RNA-preparations, the control PCR showed that no DNA was present after DNase digestion (see Fig. C.5.).

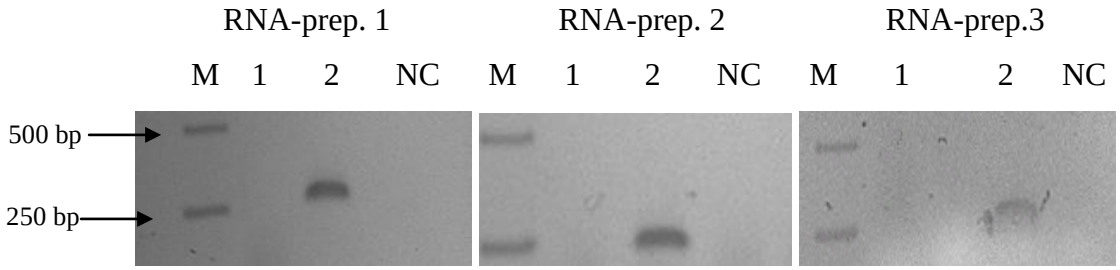


Fig. C.5. Control PCR of isolated RNA after DNase digestion. M: KbL, 1: RNA, 2: DNA, NC: negative control (no template added). Control-PCR was performed using the primer pair 0373F/R under standard conditions. Genomic DNA of *P. amoebophila* used as positive control (2) resulted in an amplicon of the expected size of 271 bp for the *pc0373*-gene.

All three independent RNA-preparations were transcribed back into complementary-DNA (cDNA) in a Reverse Transcription PCR (RT-PCR) step using the primer pairs pc0373F/R and pc0374F/R, respectively. The cDNA was then amplified in a standard PCR using the same primer pairs. cDNA-concentrations of 100 and 300 ng/µl were used and for each concentration, 1 µl and 3 µl were used as template, respectively. The PCR-products were visualized by gelelectrophoresis (see Fig. C.6.).

In the first experiment, no amplificates could be obtained with the primer pair pc0373 F/R. Amplification of cDNA with the primer pair pc0374F/R resulted in amplicons of the right size for all cDNA-concentrations (Fig. C.6.).

In the second and the third experiment, both primer pairs produced the desired amplicon and demonstrated the transcription of both genes at the time of RNA-isolation by the amoebal endosymbiont *P. amoebophila* (Fig. C.6.).

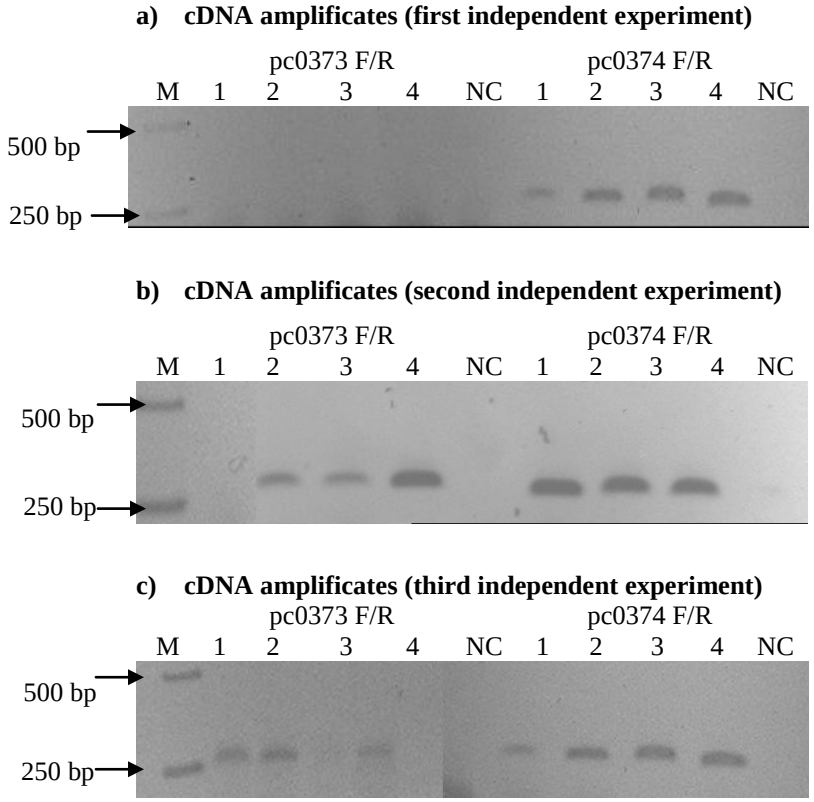


Fig. C.6. cDNA amplificates obtained from the three independent experiments. M: KbL, **1**: 0.5 µg of cDNA and **2**: 1.5 µg of cDNA used for amplification, **3**: 1 µg of cDNA, **4**: 3 µg of cDNA used for amplification, **NC** – negative control, no template was added.

C.3. Amplification of the ORFs *pc0373* and *pc0374*

After the design of specific primers (Tab. B.6.) for the ORFs of interest – *pc0373* and *pc0374*, the best annealing temperature was determined in a gradient PCR.

A high-fidelity PCR using a proof-reading DNA-polymerase was performed at an annealing temperature of 65°C for each ORF and amplificates with the expected length (pc0373F/R – 252 bp; pc0374F/R – 237 bp) were obtained for both ORFs.

C.4. Cloning and clone screening

After restriction digestion of the obtained PCR-products and the vector, cloning was performed as described in Sec. B.13, resulting in the following constructs: *pc0373* in pET-16b (with diluted and non-diluted PCR-product used in the ligation step, designated D3 and

N3 in the following) and *pc0374* in pET-16b (diluted and non-diluted PCR-product used in the ligation step, indicated as D4 and N4) (see Sec. B.13.3.).
After transformation into the *E. coli* non-expression strain BL21, the cells were grown on LB-Amp plates. 20 clones of each construct (D3, N3, D4, N4) were picked and used in a T7-screening-PCR using the primers T7F/R (see Sec. B.13.5). The expected length of the amplicon for *pc0373* accounted for 472 bp, while for *pc0374* the expected amplicon had a length of 457 bp.
For cells containing the D3-construct, 5 out of the 20 picked clones contained an amplicon of the expected length, and for N3, 7 out of 20 clones. For the D4-construct, no amplicons were obtained in the T7-screening PCR, while for the N4-construct, 6 out of 20 clones resulted in the expected amplicon.

The obtained T7-screening-PCR-amplicons with the expected length were used for RFLP using the restriction enzyme AluI. For the *pc0373*/pET-16b-constructs four fragments are expected after RFLP of the T7-screening-PCR-amplicon: 228/152/55/36 bp. For *pc0374*/pET-16b, five fragments with the following length are expected: 231/110/84/33/10 bp.
The expected pattern and length of the fragments after RFLP was obtained for 4 of the 7 positive clones of the N3-constructs, all 5 of the D3-construct and all 6 of the N4-constructs. The patterns of the other clones indicated an incorrect orientation of the insert.

RFLP results indicated that the clones contained the right orientation of the full-length ORFs of *pc0373* and *pc0374*, respectively. In order to make sure that the insert was in-frame, the T7-screening-PCR-products of one clone for each gene (clone N3.10 for *pc0373* and clone N4.1 for *pc0374*) were sequenced and the obtained sequences were 100% identical when compared to genomic data of *pc0373* and *pc0374*. The obtained sequences of both, N3.10 and N4.1, showed that the construct contained the full-length of the ORFs and that these were in-frame and would be transcribed correctly. Therefore, crystocks of N3.10 and N4.1 were prepared (see Sec. B.8.2.) and these clones were used for further protein expression studies.

C.5. Protein overexpression in different *E. coli* strains

After checking the transcription of the genes *pc0373* and *pc0374* in *P. amoebophila*, the vector containing the correct insert of each ORF was isolated and transformed into different expression strains of *E.* coli (see Tab. B.10.) followed by protein PAGE analysis.

Protein expression is achieved by induction with IPTG. First, protein expression was performed using the *E. coli* strain C43 (DE3). Protein bands of the desired size could be obtained after IPTG induction, while non-induced cultures showed no prominent band. Nevertheless, protein expression of both, Pc0373 and Pc0374 was not very strong and bands obtained after SDS-PAGE were rather weak. The proteins Pc0373 and Pc0374 including the His-tag are expected to have a molecular weight of 11.3 and 10.7 kDa, respectively. Protein expression was therefore also visualized by Tricine-SDS-PAGE, which was demonstrated to be more suitable for smaller proteins (Schägger and Jagow, 1987). Indeed, protein gels obtained with the Tricine-SDS-PAGE-protocol showed more distinct bands than SDS-PAGE. Nevertheless, protein expression in the C43 (DE3) strain was very low (data not shown) and protein expression was performed in other strains of *E. coli.*

The *E. coli* strain BL21 Codon Plus (DE3) RIPL was chosen for further expression studies due to its capability to express rare codons and to be more resistant to possible toxic proteins. While Pc0373 was again only expressed at a low level, Pc0374 was not expressed at all in this strain (see Fig. C.7.).

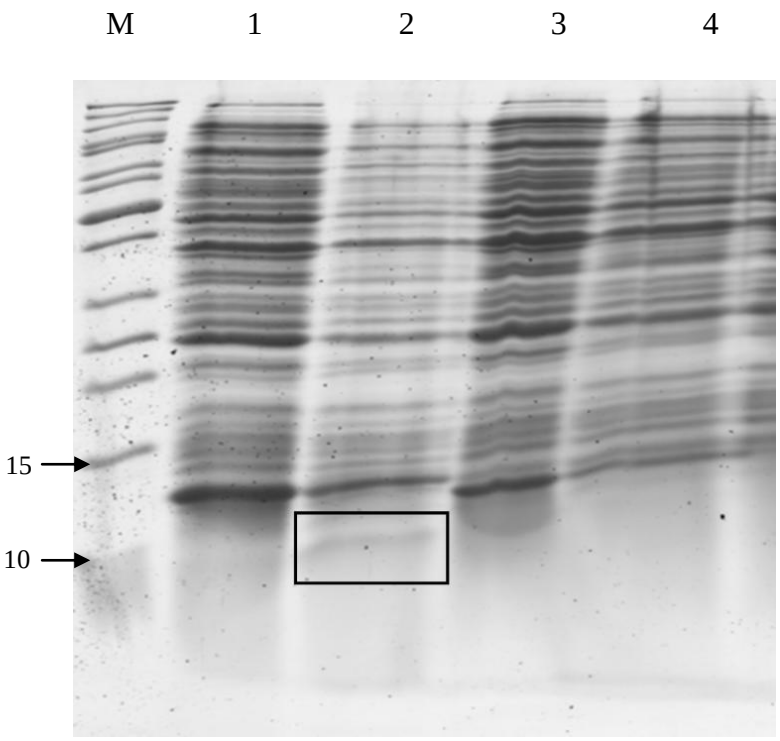


Fig. C.7. Protein overexpression of Pc0373 and Pc0374 in *E. coli* BL21 Codon Plus (DE3) RIPL.
M: protein marker (kDa), **1**: Pc0373 non-induced culture, **2**: Pc0373 induced culture, **3**: Pc0374 non-induced culture, **4**: Pc0374 induced culture.

Protein expression was then analysed using the BL21 (DE3)-strain. Both proteins were readily expressed in this strain, and the expression level for both proteins was higher than in the strains mentioned above. Due to the fact that this expression strain possesses no repressor, no difference could be observed between IPTG-induced and non-induced cultures. Therefore, a vector without insert was transformed into this strain and functioned as a negative control. Different protein volumes (2.5 and 5 µl) were analysed by 12.5% Tricine-SDS-PAGE. Higher protein volumes resulted in a more prominent band for both proteins, while the negative control, namely the pET-16b vector without insert, showed no prominent band of the expected size even after IPTG-induction for none of the used protein volumes (see Fig. C.8.). Therefore, protein expression at large scale and consecutive purification via the His-tag was performed using the *E. coli* BL21 (DE3)-expression strain.

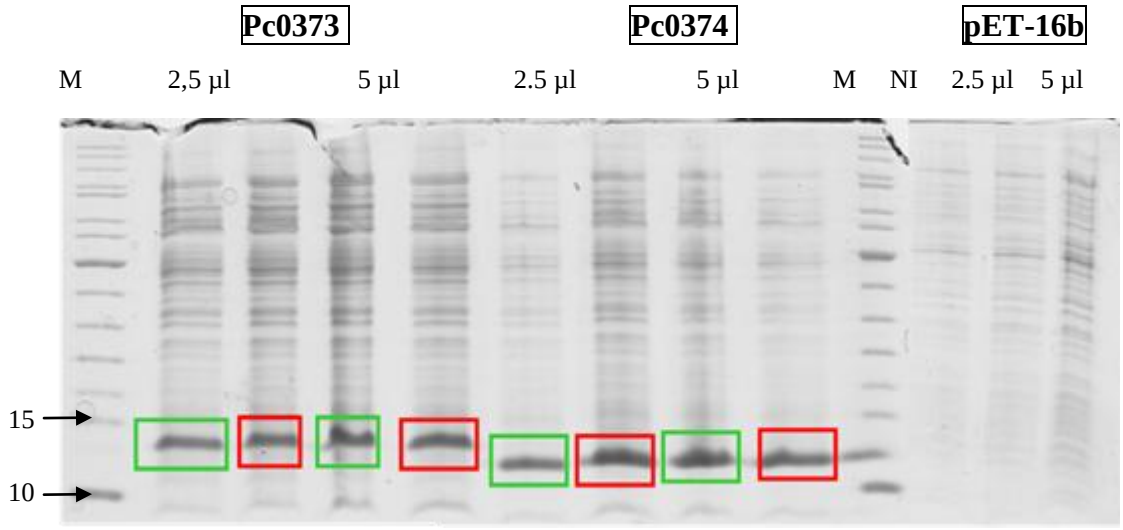


Fig. C.8. Protein expression of Pc0373 and Pc0374 in E. coli BL21 (DE3). Green boxes: protein band after induction with IPTG, red box – non-induced culture. **M** = protein marker (kDa); for each protein, a protein volume of 2.5 µl and 5 µl were used for Tricine-SDS-PAGE. pET-16b-vector containing no insert was used as negative control without (NI=non-induced) and after induction with IPTG (protein volume of 2.5 and 5 µl).

C.6. Protein purification

Cells of the *E. coli* expression strain BL21 (DE3) containing the constructs *pc0373*/pET-16b (clone N3.10) and *pc0374*/pET-16b (clone N4.1) were grown in liquid medium and the overexpressed proteins were then purified via the His-tag using the Qiagen Ni-NTA Spin Columns. The proteins are bound to the Ni^{2+}-matrix of the columns via the His-tag and can be eluted by using buffers with decreasing pH.

PAGE-analyses of the protein eluate showed too many unspecific protein bands, indicating that the fraction contained several other proteins and the purity of the overexpressed proteins was not high enough (Fig. C.9.).

Therefore, another protein purification assay was tried out. The Amersham HisTrap HP Columns also contain a Ni^{2+}-matrix to which the His-trap binds, but the proteins are eluted by an increasing imidazole concentration in the elution buffers.

Pc0374 was eluted in the 60 to 300 mM imidazole fraction, while prominent bands of Pc0373 were seen in the 300 mM and 500 mM imidazole fraction. The eluate contained a marginal amount of other proteins, but the obtained purified proteins were pure enough for the following antibody generation (Fig. C.10.).

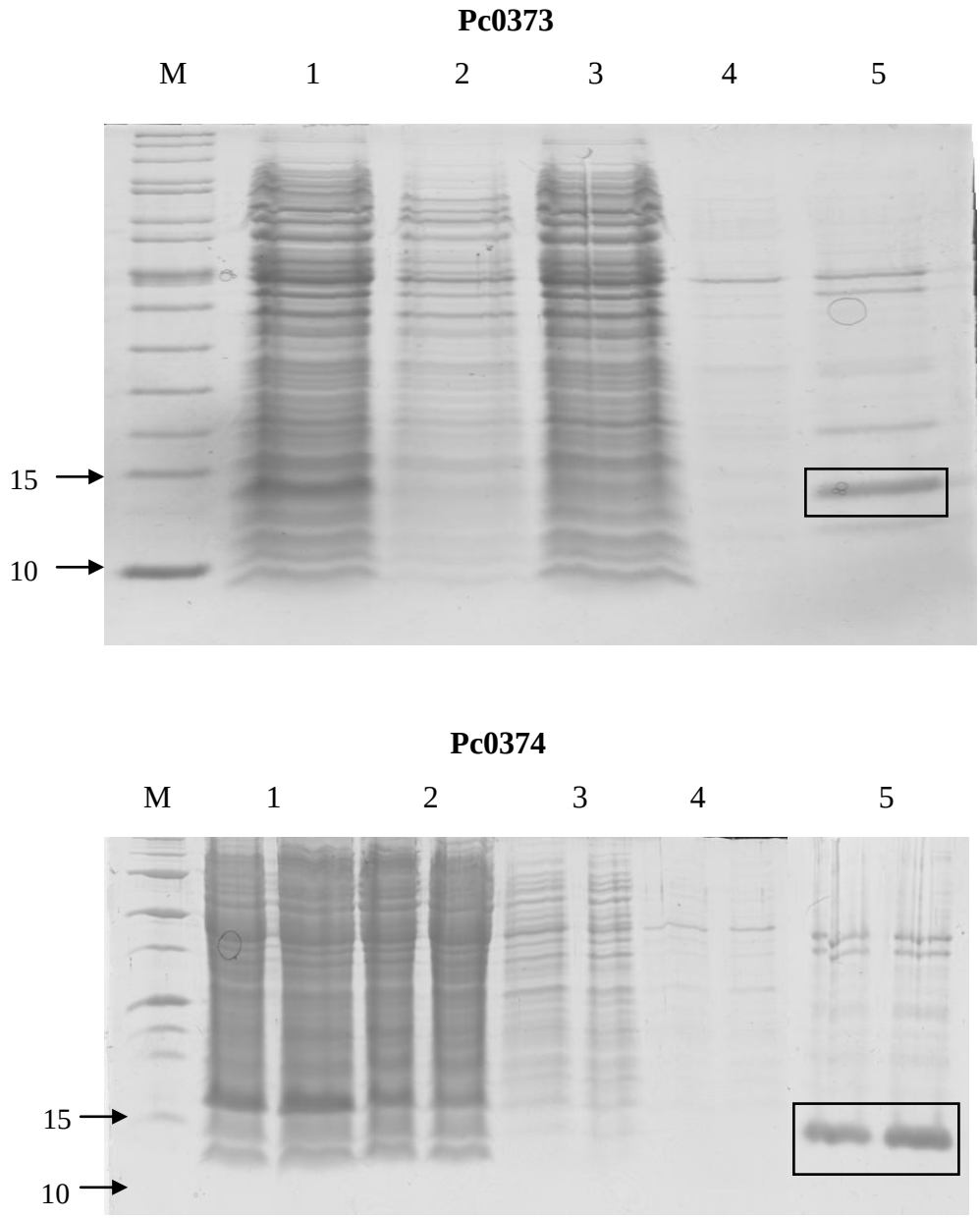


Fig. C.9. Protein purification of Pc0373 and Pc0374 with Qiagen Ni-NTA Spin Columns.

M: protein marker (kDa), **1**: cell lysate, **2**: buffer B, **3** and **4**: buffer C, **5**: protein eluate; The flow-through of all fractions was analysed by Tricine-SDS-PAGE, as well as the protein eluate. The protein eluate shows prominent bands of the desired protein, but the amount of other proteins is too high and an efficient amount of purity could not be achieved.

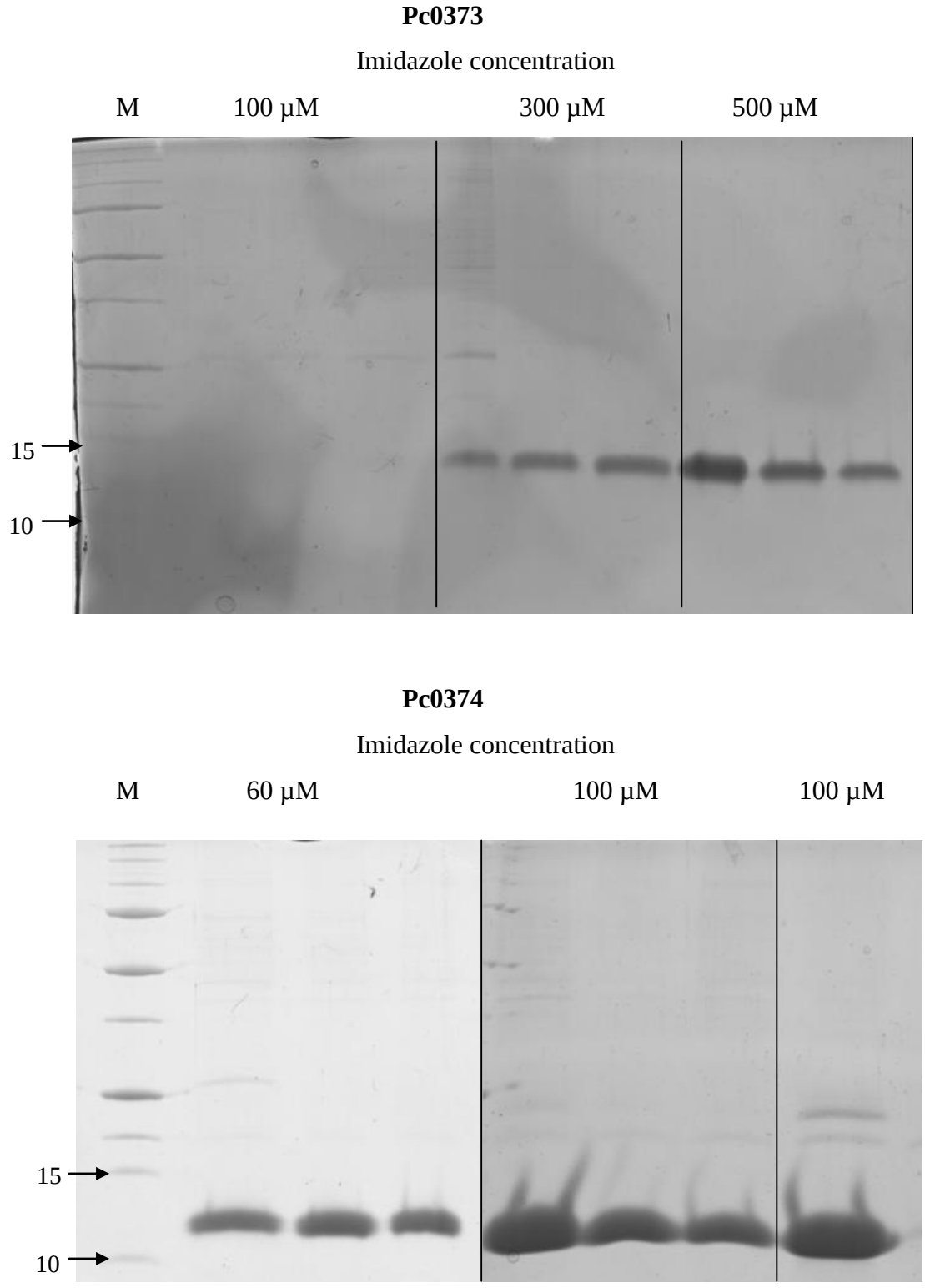


Fig. C.10. Protein purification of Pc0373 and Pc0374 using Amersham HisTrap HP Columns.
M: protein marker (kDa); Prominent bands of Pc0373 are visible in the 300 and 500 mM imidazole fractions, while Pc0374 is eluted at imidazole concentrations between 60 and 300 mM. Note that there are hardly any other proteins present in the eluate and the degree of purity is very high.

After desalting the purified proteins in order to remove the urea, which is toxic for living organisms, ~1 mg total weight, obtained from 1.5 l of expression cultures, for each protein was sent to Eurogentech for antibody generation.

C.7. Antibody generation

Antibodies triggered against Pc0373 and Pc0374, respectively, were raised in both, guinea pig and rabbit by Eurogentech (Seraing, Belgium).
Pre-immune sera drawn before the first immunisation were obtained, as well as sera from a small, a large and a final bleed after several antigen boosts.
The immunization timetable and the dates of bleeding are shown below:

Table C.3. Immunisation timetable of Pc0373- and Pc0374-antibodies.

Antigen	Source of antigen	Immunisation schedule	Bleeding schedule	Serum
Pc0373	rabbit	First immunisation: 17.07.06	Pre-immune bleeding: 17.07.06	Pc0373-pi-rab
Pc0373	rabbit	First boost: 31.07.06		
Pc0373	rabbit	Second boost: 14.08.06	Small bleeding: 24.08.06	Pc0373-sb-rab
Pc0373	rabbit	Third boost: 11.09.06	Large bleeding: 21.09.06	Pc0373-lb-rab
Pc0373	rabbit	Fourth boost: 14.10.06	Final bleeding: 21.10.06	Pc0373-fb-rab
Pc0373	guinea pig	First immunisation: 17.07.06	Pre-immune bleeding: 17.07.06	Pc0373-pi-gp
Pc0373	guinea pig	First boost: 31.07.06		
Pc0373	guinea pig	Second boost: 14.08.06	Small bleeding: 24.08.06	Pc0373-sb-gp
Pc0373	guinea pig	Third boost: 11.09.06	Large bleeding: 21.09.06	Pc0373-lb-gp
Pc0373	guinea pig	Fourth boost: 14.10.06	Final bleeding: 21.10.06	Pc0373-fb-gp
Pc0374	rabbit	First immunisation: 17.07.06	Pre-immune bleeding: 17.07.06	Pc0374-pi-rab
Pc0374	rabbit	First boost: 31.07.06		
Pc0374	rabbit	Second boost: 14.08.06	Small bleeding: 24.08.06	Pc0374-sb-rab
Pc0374	rabbit	Third boost: 11.09.06	Large bleeding: 21.09.06	Pc0374-lb-rab
Pc0374	rabbit	Fourth boost: 14.10.06	Final bleeding: 21.10.06	Pc0374-fb-rab
Pc0374	guinea pig	First immunisation: 17.07.06	Pre-immune bleeding: 17.07.06	Pc0374-pi-gp
Pc0374	guinea pig	First boost: 31.07.06		
Pc0374	guinea pig	Second boost: 14.08.06	Small bleeding: 24.08.06	Pc0374-sb-gp
Pc0374	guinea pig	Third boost: 11.09.06	Large bleeding: 21.09.06	Pc0374-lb-gp
Pc0374	guinea pig	Fourth boost: 14.10.06	Final bleeding: 21.10.06	Pc0374-fb-gp

C.8. Immunofluorescence with *Acanthamoeba castellanii* UWC1 harboring *Protochlamydia amoebophila* UWE25 endosymbionts

After antibody generation, the obtained sera were blocked against amoebal proteins (see Sec. B.20.3.1.) in order to reduce unspecific background signals and immunofluorescence (IF) was performed on acanthamoeba cells harboring *P. amoebophila* endosymbionts. Amoebal cells containing no endosymbiont were use as negative control. Pre-immune sera drawn before the first antigen boost were also used as negative control in order to determine the specificity of the antibodies.

DAPI-staining was performed in order to visualize the intracellular bacterial cells (B.20.4.2.).

C.8.1. Localisation of Pc0373 in *Acanthamoeba castellanii* UWC1 cells harboring *Protochlamydia amoebophila* UWE25 endosymbionts

Blocked antibodies against Pc0373 from guinea pig and rabbit were used in an IF assay on fixed amoebal cells containing *P. amoebophila* and on amoebal cells devoid of endosymbionts.

Pre-immune sera from both, rabbit and guinea pig showed no specific fluorescence signals and the unspecific background fluorescence was rather low, indicating that no unspecific antibody binding occured.

Small-bleed-sera from guinea pig and rabbit won after the first immunisation and two consecutive antigen boosts showed rather high background fluorescence for both, amoebal cells with and without endosymbionts. No specific antibody binding could be seen for amoebal cells containing *P. amoebophila* with the small-bleed-sera. When compared to the pre-immune sera, small-bleed-sera resulted in even stronger unspecific background fluorescence. The background fluorescence had approximately the same intensity throughout the cytoplasm of the amoebal cells and no intracellular structures showing unambiguous fluorescence signals could be recognized.

Stronger blocking of the small-bleed-sera with a higher amount of amoebal cell lysate could only slightly reduce the background fluorescence, while specific signals could still not be observed.

Antibodies derived from guinea pig showed higher background fluorescence intensity than antibodies developed in rabbit. On the other hand, the difference of background fluorescence intensity between amoebal cells containing *P. amoebophila* and those devoid of endosymbionts was more evident for the guinea pig-serum than for the rabbit-serum. The small-bleed serum from guinea pig (sb-gp) showed a stronger unspecific background for amoebal cells containing an endosymbiont, while these signals were slightly weaker for amoebal cells devoid of endosymbionts. For the rabbit-small-bleed sera (sb-rab), background fluorescence had a comparable intensity in amoebal cells with and without endosymbionts.

The large bleed sera from guinea pig (lb-gp) won after a third antigen boost showed very specific IF-signals for amoebae containing endosymbionts, while cells without symbiont

showed no signals. The background fluorescence of blocked lb-gp-sera was quite low, comparable to the unspecific background observed with pre-immune sera. This indicates that the signals obtained with lb-gp-sera for amoebal cells containing endosymbionts were specific. On the other hand, large-bleed-sera from rabbit (lb-rab) still showed no signals even after a third antigen boost.

The lb-gp-sera clearly showed a ring-shaped fluorescence signal within the amoebal cells containing endosymbionts. Overlay of the fluorescence image with a phase-contrast image of the amoebal cells containing *P. amoebophila* showed that this ring-shaped signal is localized within the nucleus of the amoebal cell, while the nucleolus in the centre gave no signal. This was not the case for amoebal cells devoid of endosymbiont, where no specific signal could be observed (Fig. C.11.).

This experiment was repeated several times with unsynchronized amoebal cultures in order to demonstrate that the fluorescence signals are specific and the results reproducible. Some of the experiments resulted in weaker fluorescence signals, yet the specific signal seen in amoebal cells containing *P. amoebophila* was always significantly stronger than unspecific signals observed for amoebal cells with no endosymbionts or the background fluorescence.

The same results were obtained with secondary antibodies conjugated with Cy3 and with Cy2, respectively. For both, the ring-shaped signal around the nucleolus could be observed in amoebal cells containing *P. amoebophila*, while cells without endosymbionts showed no specific signals. For both differently labelled secondary antibodies, the background fluorescence in cells with and without *P. amoebophila* was comparable and showed approximately the same intensity when compared with the pre-immune sera. This is another strong indication that the observed fluorescence signals are specific and are not due to any artefacts of the IF-assay.

Apart from that, sera blocked with different amounts of amoebal cells lysate in order to reduce antibody binding to amoebal proteins also resulted in a fluorescence signal around the nucleolus of the amoebal cell containing *P. amoebophila*, while amoebal cells devoid of *P. amoebophila* showed no such signal.

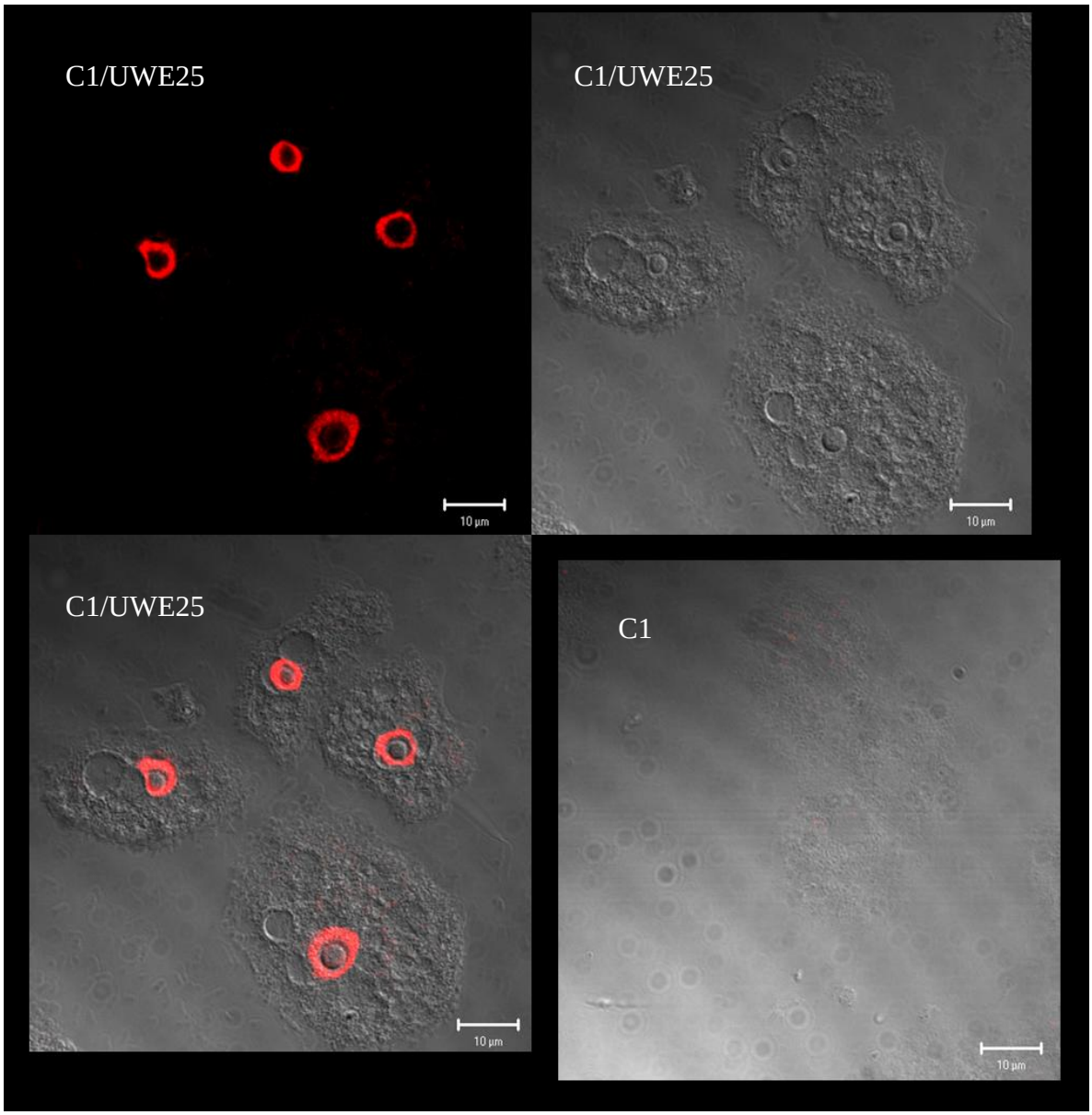


Fig. C.11. Localisation of Pc0373 in amoebal cells containing endosymbionts (C1/UWE25), and cells without endosymbionts (C1) as negative control. Primary antibodies: guinea-pig-large-bleed serum 1:512 dilution, secondary antibodies: anti-guinea pig-Cy3; C1 shows an overlay of the fluorescence and the phase-contrast-image; pictures of C1/UWE25 and C1 were taken with exactly the same software and microscope settings.

Final bleed sera won after a fourth antigen boost were obtained from guinea pig and rabbit. The final-bleed-sera from guinea pig (fb-gp) also showed fluorescence signals around the nucleolus of amoebal cells containing endosymbionts but the fluorescence intensity was weaker than for lb-gp sera. Amoebal cells containing no endosymbionts showed no fluorescence signals (data not shown).

Final bleed sera from rabbit (fb-rab) failed to produce positive signals even after the fourth antigen boost for amoebal cells containing an endosymbiont and cells devoid of endosymbionts as well.

In order to demonstrate that the specific fluorescence signal is located within the amoebal nuclear membrane, the membranes were made visible using the fluorescent lipophilic membrane dyes $DiOC_{(6)}$ and Nile Red. While Nile Red treatment failed to result in fluorescing signals for the membranes, $DiOC_{(6)}$ treatment showed fluorescing signals for the amoebal outer membrane and all intracellular membranes including the ring-shaped signal of the bacterial membrane as well as the adjacent inclusion membrane of the endosymbionts scattered throughout the amoebal cytoplasm (Fig. C.12.).

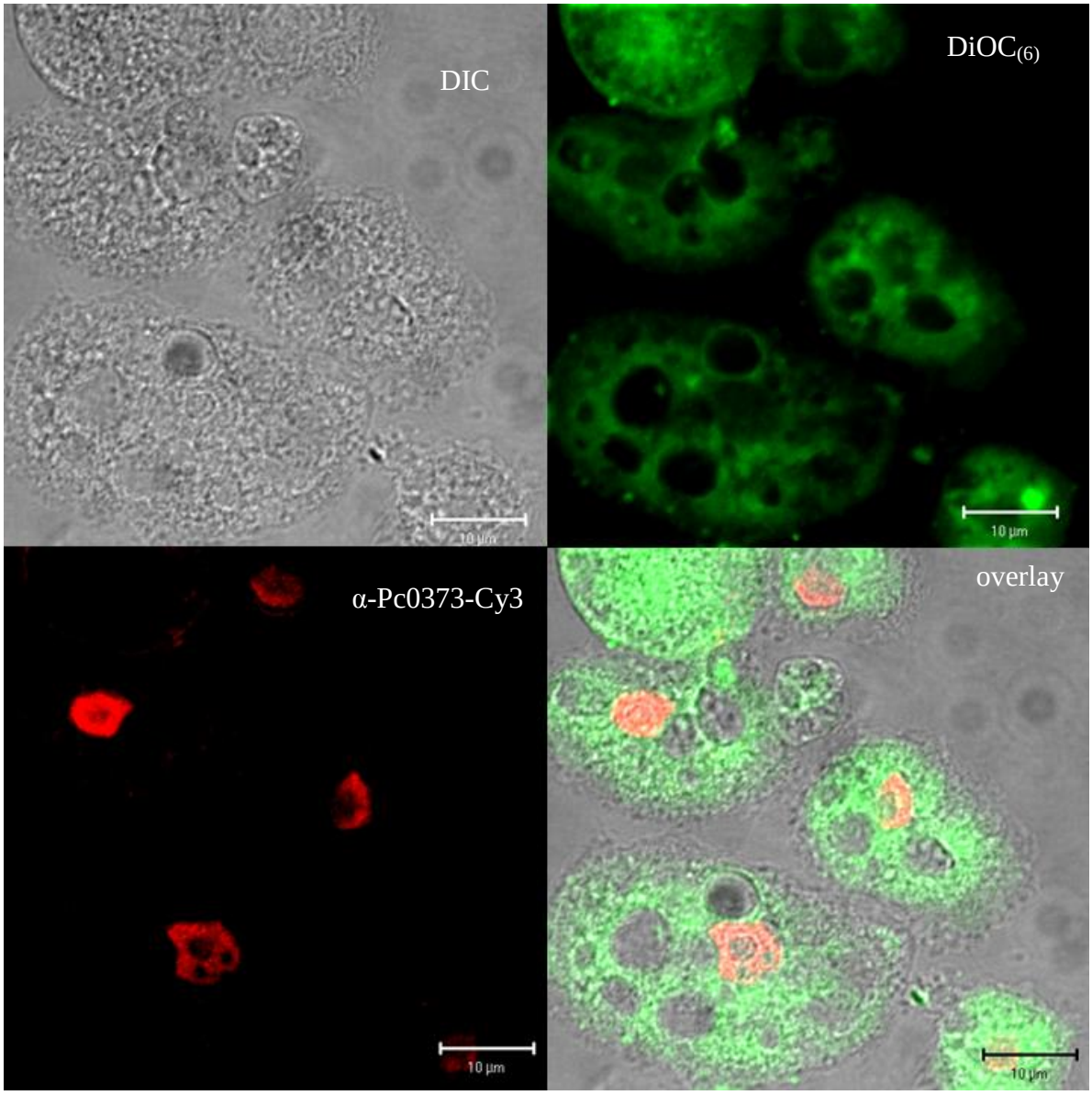


Fig. C.12.: Localisation of Pc0373 in C1/UWE25 and simultaneous visualisation of membranes. $DiOC_{(6)}$: visualisation of membranes with the lipophilic dye $DiOC_{(6)}$. α-Pc0373: localisation of Pc0373-specific signal visualised by the use of a 1:512 dilution of large-bleed sera from guinea pig and Cy3-conjugated secondary antibodies. DIC: phase conrast image. Overlay: overlay of the images taken for $DiOC_{(6)}$ with the fluorescence image showing Pc0373-specific signals and the phase-contrast image. Bar: 10 µm. Note the localisation of the red specific signals around the amoebal nucleolus and the localisation of this signal within the amoebal nuclear membrane.

C.8.2. Localisation of Pc0373 in *Acanthamoeba castellanii* UWC1 cells harbouring *Protochlamydia amoebophila* UWE25 endosymbionts after IF-Tyramide Signal Amplification (IF-TSA)

In order to make sure that the positive signals for Pc0373 in amoebal cells containing *P. amoebophila* were indeed localized within the host cell nucleus and the antibody binding is specific, the signals were amplified using the IF-TSA assay (see Sec. B.20.3.3.). The aim was to show that the specific fluorescence signals within amoebal cells containing *P. amoebophila* could be amplified in intensity, while cells without endosymbionts would show no fluorescence even after signal amplification, delivering another strong indication for the specificity of the antibodies.

Fluorescence signals located around the nucleus of amoebal cells containing *P. amoebophila* could be observed with the IF-TSA assay and the signal intensity was remarkably increased when compared to cells treated with the normal IF assay. The background fluorescence was comparably low even after signal amplification. Most importantly, amoebal cells lacking endosymbionts showed no fluorescence signals after TSA treatment (Fig. C.13.).

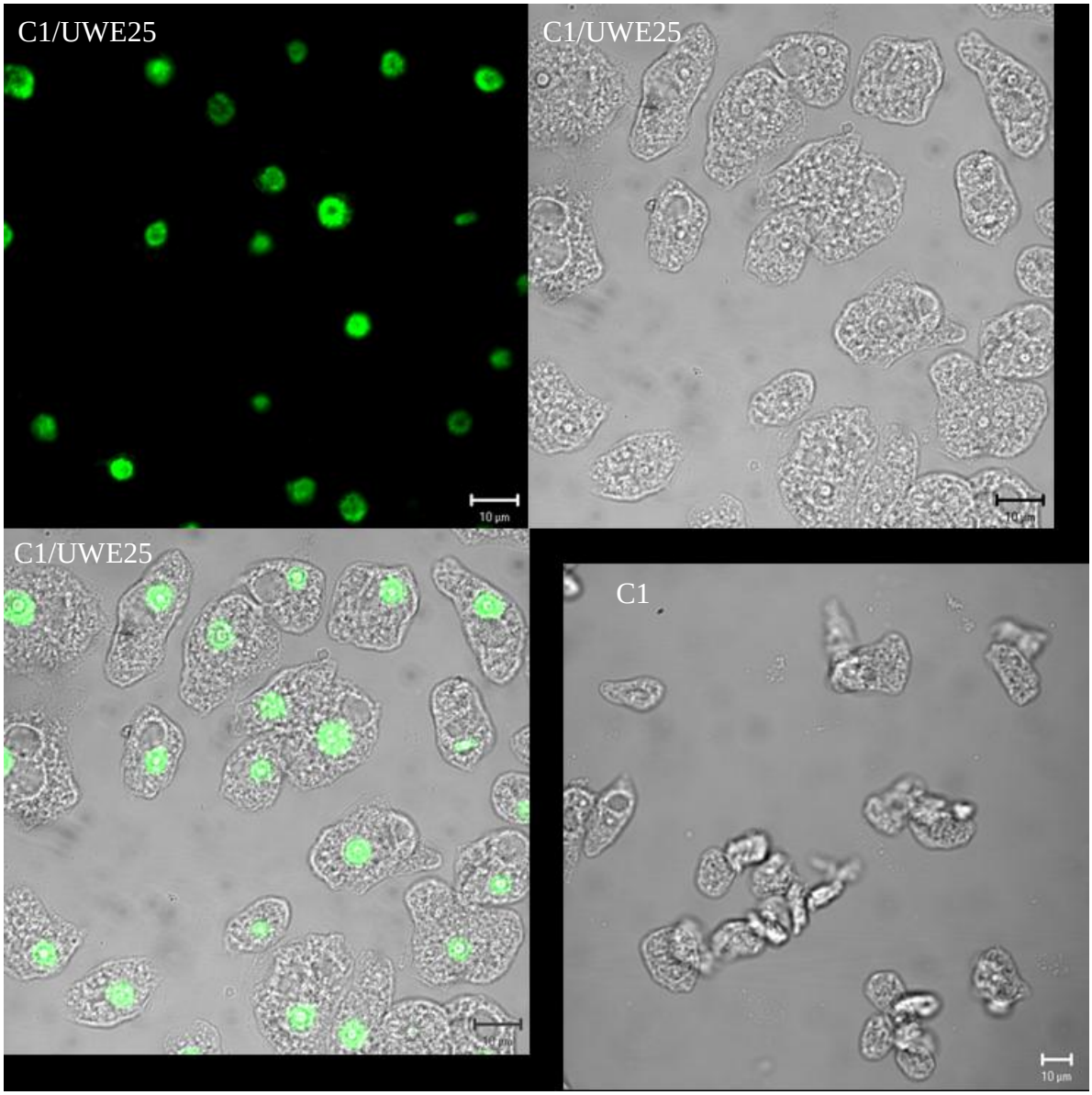


Fig. C.13. Localisation of Pc0373 in amoebal cells containing endosymbionts (C1/UWE25), and cells devoid of endosymbionts (C1) as negative control after IF-TSA. Primary antibodies: guinea-pig-large-bleed serum 1:512 dilution, secondary antibodies: anti-guinea pig-HRP; C1 shows an overlay of the fluorescence and the phase-contrast-image; pictures of C1/UWE25 and C1 were taken with exactly the same software and microscope settings. Note the absence of specific fluorescence signals for cells lacking endosymbionts.

C.8.3. Localisation of Pc0374 in *Acanthamoeba castellanii* UWC1 cells harbouring *Protochlamydia amoebophila* UWE25 endosymbionts

Blocked antibodies against Pc0374 from guinea pig and rabbit were used in an IF-protocol on fixed amoebal cells containing *P. amoebophila* and on amoebal cells free of endosymbionts.

Pre-immune sera from both, rabbit and guinea pig, showed no fluorescence signals and the unspecific background fluorescence was rather low, indicating that no unspecific antibody binding occurred.

Small-bleed-sera from guinea pig and rabbit won after the first immunisation and two consecutive antigen boosts showed rather low background fluorescence for both, amoebal cells with and without endosymbionts. No specific antibody binding could be seen for amoebal cells containing *P. amoebophila* with the small-bleed-sera from guinea pig. When compared to the pre-immune sera, small-bleed-sera resulted in even stronger unspecific background fluorescence. The background fluorescence had approximately the same intensity throughout the cytoplasm of the amoebal cells and no intracellular structures showing stronger fluorescence signals could be recognized.
Stronger blocking of the small-bleed-sera with a higher amount of amoebal cell lysate could only slightly reduce the background fluorescence, while very weak specific signals for the rabbit-small-bleed-sera (sb-rab) could be observed by focusing through the amoebal cells. These specific signals had a ring-shaped structure and were evenly distributed in the amoebal cytoplasm. Nevertheless, the signals obtained with the sb-rab sera were too weak in order to allow any exact conclusions.
Antibodies derived from guinea pig resulted in higher background fluorescence intensity than antibodies developed in rabbit. On the other hand, the difference of background fluorescence intensity between amoebal cells containing *P. amoebophila* and those devoid of endosymbionts was more evident for the guinea pig-serum than for the rabbit-serum. The small-bleed serum from guinea pig (sb-gp) showed a stronger unspecific background for amoebal cells containing endosymbionts, while these signals were slightly weaker for amoebal cells lacking endosymbionts. For the rabbit-small-bleed sera (sb-rab), background fluorescence signals had a comparable intensity in amoebal cells with and without endosymbionts.

The large bleed sera from guinea pig (lb-gp) won after a third antigen boost showed no unambiguous IF-signals for amoebae containing endosymbionts. The background fluorescence of blocked lb-gp-sera was quite low, comparable to the unspecific background observed with pre-immune sera.
The lb-rab-sera clearly showed evenly distributed ring-shaped fluorescence signals within the amoebal cells containing endosymbionts. These signals were observed to be localized

around the bacterial cells which were stained with the DNA-binding dye DAPI (data not shown). This was not the case for amoebal cells devoid of endosymbionts, where no specific signal could be observed (Fig. C.14.).

This experiment was repeated several times with unsynchronized amoebal cultures in order to demonstrate that the fluorescence signals are specific and the results reproducible. Some of the experiments resulted in weaker fluorescence signals, yet the specific signal seen in amoebal cells containing *P. amoebophila* was always significantly stronger than unspecific signals observed for amoebal cells with no endosymbionts or the background fluorescence. These observations indicate that the *P. amoebophila*-protein Pc0374 is located in the bacterial cell envelope and/or the adjacent inclusion membrane or between the bacteria and the inclusion membrane. As *P. amoebophila* is known to make single-cell inclusions (Fritsche et al., 2000b), it is difficult to differentiate between the bacterial and the inclusion membrane using IF-techniques, which is due to the close association of the membranes.

Apart from that, sera blocked with different amounts of amoebal cell lysates in order to reduce antibody binding to amoebal proteins, also resulted in fluorescence signals throughout the cytoplasm of the amoebal cell containing *P. amoebophila*, while amoebal cells devoid of *P. amoebophila* showed no such signals.

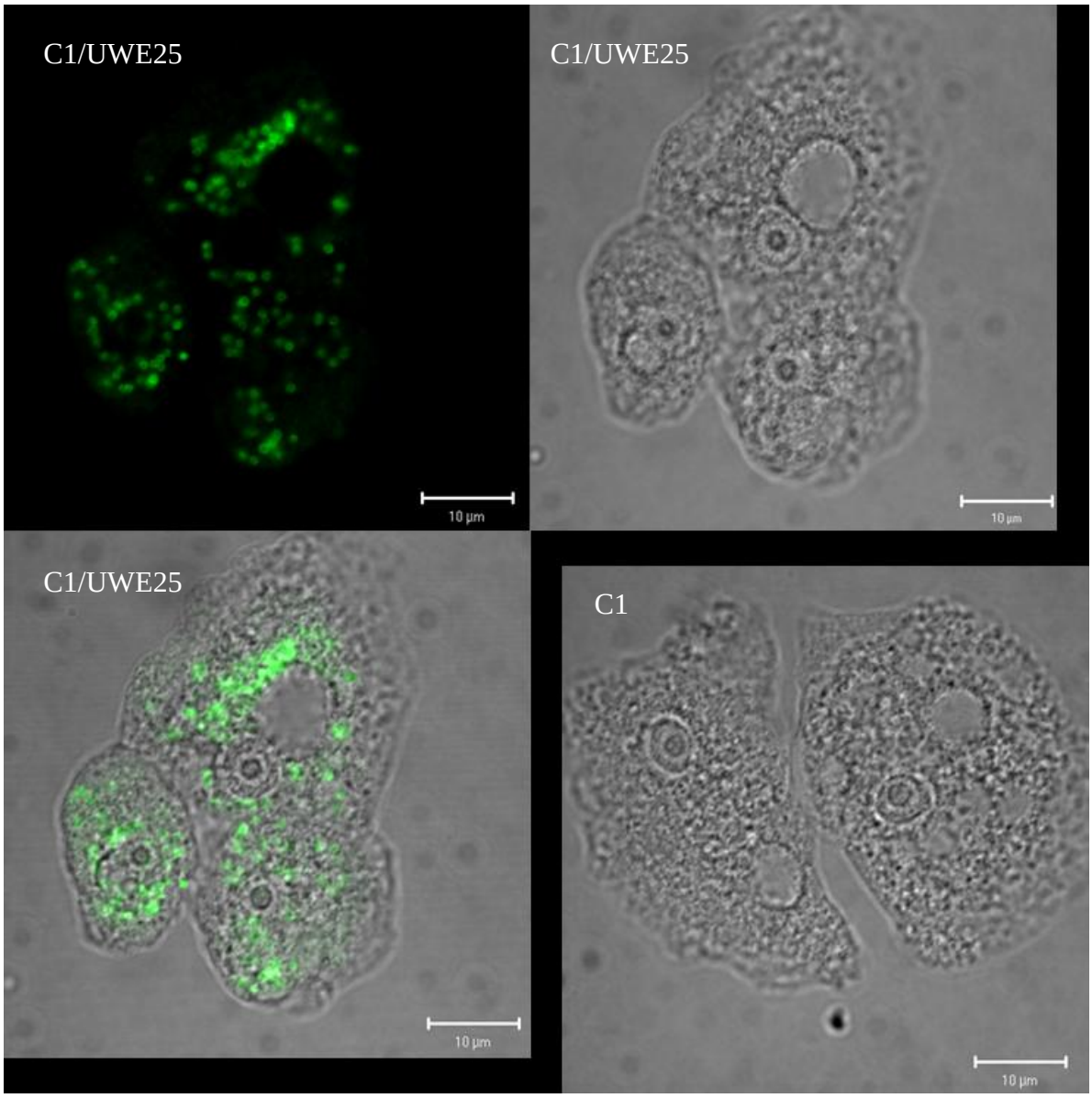


Fig. C.14. Localisation of Pc0374 in amoebal host cells containing endosymbionts (C1/UWE25) and cells lacking endosymbionts (C1) as negative control. Primary antibodies: rabbit-large-bleed serum 1:512 dilution, secondary antibodies: anti-rabbit-Cy2; C1 shows an overlay of the fluorescence and the phase-contrast-image; pictures of C1/UWE25 and C1 were taken with exactly the same software and microscope settings.

C.9. Screening of human sera

C.9.1. Optimisation of a standard MIF protocol

Partially purified EBs of *Protochlamydia amoebophila* UWE25, *Parachlamydia acanthamoebae* UV-7 and *Simkania negevensis* Z were analysed using the standard MIF protocol (see Sec. B.20.4.3.) with specific antibody sera raised against these organisms in rabbit. The obtained results were not satisfactory because of high background fluorescence due to unspecific antibody binding and the comparably low signal intensity, especially for *P. amoebophila* and *Parachlamydia sp*.

Therefore, the standard MIF procedure was modified in different ways in order to optimize the results.

C.9.1.1. Slide treatment

Differently treated slides were used and the results compared considering fluorescence intensity and background fluorescence.

SuperFrost slides, which were not treated prior to EB-fixation failed to provide high fluorescence intensities and to keep the background fluorescence low.

Slides coated with poly-L-lysine showed low fluorescence intensity of specific signals, while the background fluorescence was rather high. Uncoated slides not only showed a better dispersion of the EBs, but also a higher specific and a lower background fluorescence than the poly-L-lysine coated slides.

Uncoated slides were cleaned with different reagents prior to cell fixation and the results were compared. Slides cleaned with absolute ethanol provided the highest fluorescence signals, while the background fluorescence remained low. Cleaning with 70% ethanol and 1% HCl showed lower specific fluorescence intensity and medium background fluorescence.

C.9.1.2. Fixative

Three different cell fixatives were compared: 4% PFA, pure acetone, and pure methanol. Fixation with PFA showed the best results with the lowest background fluorescence.

Acetone fixation showed strong background fluorescence, which was probably due to the disruption of the bacterial cells due to the treatment with acetone.

Methanol fixation showed low background fluorescence, but on the other hand, a stronger formation of cell clusters. This is probably due to a patchy drying of the fixative on the slide.

EBs which were not treated with any of the above mentioned fixatives formed several clusters and did not provide satisfying results. Apart from that, the absence of a fixative reagent led to a smaller number of EBs attached to the slide when compared to EBs treated with a fixative. This indicates that the fixation of EBs is crucial and prevents their detachment.

C.9.1.3. Blocking reagent

Using a blocking reagent is meant to prevent unspecific antibody binding and to reduce the background fluorescence. On the other hand, unattached secondary antibodies are intercepted as too many fluorophore molecules would result in quenching effects, thus resulting in a decrease of fluorescence intensity.

Three different blocking reagents were used, which showed similar results for all three organisms:

5% milk powder, as well as 0.25% egg yolk did not provide optimal results, as they showed too much background fluorescence.

Treatment with egg yolk led to the weakest fluorescence intensities when compared to the other blocking reagents.

BSA was added in different concentrations of 1%, 5% and 10% (the last only for *P. amoebophila*), but the best results were achieved with a BSA concentration of 1% for all three organisms. The abandonment of a blocking reagent resulted in a lower fluorescence intensity when compared to the results obtained with 1% and 5% of BSA.

C.9.1.4. Washing conditions

On-slide washing with 1x PBS provided better results when compared to slides which were dipped as a whole in 1x PBS. This is probably due to the fact that on-slide washing resulted in a faster and more even drying of the slides, which remarkably reduced the background fluorescence.

Addition of blocking reagent to the washing buffer resulted in higher background fluorescence when compared to slides which were washed with 1x PBS.

C.9.1.5. Mounting medium

The mounting medium is meant to fixate the coverslip to the slide and therefore to prevent detachment of the cells from the slide surface, but also to slow down bleaching of the fluorophores under the UV-light.

CitiFluor did not provide satisfying results, as the EBs did not remain attached to the slide surface but instead seemed to float on the bottom of the coverslip. Apart from that, a rapid bleaching of the fluorescence signals could be observed under the microscope.

Mowiol, a polyvinyl-alcohol, which hardens after ~30 min, seemed to be the better alternative, as the EBs did not detach. However, the inhibition of bleaching was not sufficient enough.

Therefore, a combination of Mowiol with 10% DABCO was used. DABCO is added to the mounting medium and is meant to slow down the bleaching of the sample. The best results could be obtained with the Mowiol/DABCO mix, as the cells remained attached to the slide surface and the longevity of the fluorescence signals could be remarkably prolonged.

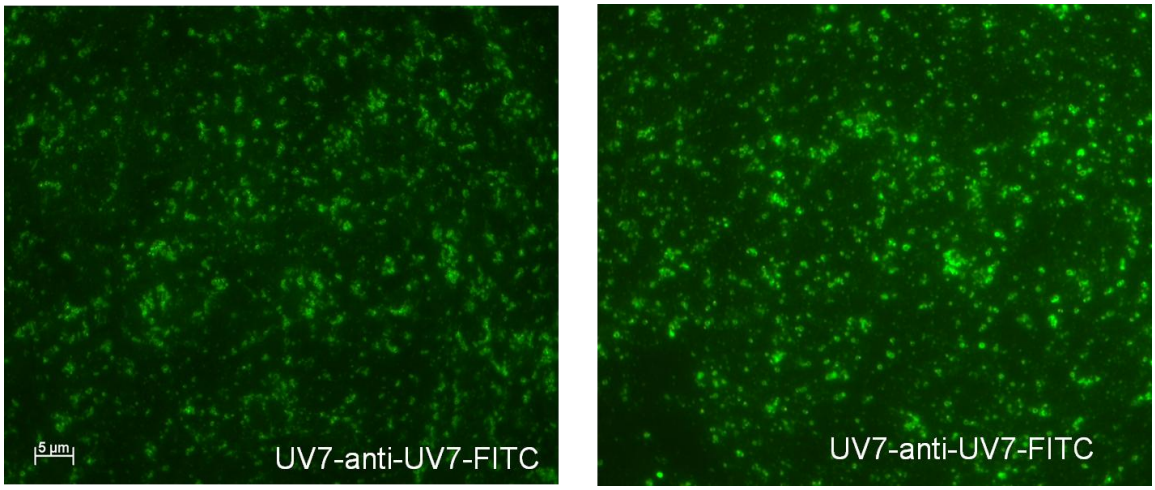


Fig. C.15. Comparison of MIF-images obtained with the standard MIF protocol vs. the optimised MIF protocol. EBs of *Parachlamydia sp*. were incubated with antibodies against *Parachlamydia sp*. (1:512) raised in rabbit and the secondary antibodies used were labeled with fluoresceine-iso-thio-cyanate (FITC, 1:1000).
Left image: standard MIF protocol. right image: optimised MIF protocol. Note the increased signal intensity and the lower background fluorescence obtained with the optimized MIF protocol.

C.9.1.6. Counterstaining with Evan's Blue

Counterstaining of the slide surface with the fluorescent dye Evans Blue is meant to reduce the general background signals resulting from autofluorescence. Under UV-light, the slide surface appears in a reddish glow in contrast to the green fluorescent signal of the secondary antibodies. No positive effects of Evan's Blue staining could be observed for this MIF assay.

C.9.1.7. Cross-reactivity of specific antibodies

One crucial step of this assay was to determine the cross-reactivity of the antibodies derived after immunisation with the EBs of the according organism. This allows to check

for the specificity of the antibodies for each organism, and to examine whether antibodies specific for one organism would also give positive signals for a closely related organism.

Cross-reactivity experiments with EBs of *P. amoebophila*, *Parachlamydia* sp. and *Simkania* sp. showed a strong interaction between antibodies against *Parachlamydia* sp. with *P. amoebophila*-EBs and of sera against *P. amoebophila* with *Parachlamydia* sp.-EBs. Both organisms belong to the *Parachlamydiaceae* and were expected to cross-react to a certain degree. Fluorescence signals resulting from cross-reaction showed only marginally weaker intensities when compared to results obtained with the corresponding organism-specific sera. *Simkania* sp., which belongs to the family *Simkaniaceae* did not cross-react with neither *P. amoebophila*, nor *Parachlamydia* sp. (Tab. C.4.).

Table C.4. Results of cross-reactivity experiments.

		pABs	
EB	UWE25	UV-7	Sn
UWE25	+++	++	-
UV7	++	+++	-
Sn	-	-	+++

UWE25: *Protochlamydia amoebophila* UWE25; UV-7: *Parachlamydia* sp. UV-7; Sn: *Simkania negevensis* Z; pABs: primary antibodies; EB: elementary bodies of the given organism; +++ strong fluorescence signal; ++ medium fluorescence signal; - no fluorescence signal.

C.9.2. Screening and titre determination of 15 human sera derived from randomly chosen patients of the Universitätsklinikum Ulm against *Simkania negevensis* and two *Parachlamydia* spp.

Human sera from 15 randomly chosen patients at the Universitätsklinikum Ulm were kindly provided by Dr. Sven Poppert and checked for the presence of antibodies against the environmental chlamydiae *P. amoebophila*, *Parachlamydia* sp. and *Simkania* sp. All sera were first pre-screened using a mix of all organisms. Those sera which gave a positive signal were then applied to each organism separately and the antibody titres were determined.

Antibody titres were determined in a dilution series of the respective serum, which was applied to each organism solely and to a mix of all three organisms. Due to the significantly smaller size of *Simkania*-EBs, differentiation between *Simkania* sp. and the two *Parachlamydia* spp. was possible for the EB-mix. Due to the finding, that *P. amoebophila*-EBs and *Parachlamydia*-antibodies (and vice versa) strongly cross-react, a differentiation between these two organisms was not possible.

Three out of the 15 sera gave a positive signal when applied to the EB-mix. Out of these three sera, two sera were found positive for the two *Parachlamydia* spp. up to a titre level of 32, while one serum was positive for *Simkania* sp., also up to a titre level of 32.

C.9.3. Screening and titre determination of human sera derived from volunteering members of the Department of Microbial Ecology at the University of Vienna against *Simkania negevensis* and two *Parachlamydia* spp.

Another experiment performed in this study was the screening of human sera derived from people who are often in contact with EBs of environmental chlamydiae. Therefore, we decided to test volunteering members of the environmental chlamydia group in our laboratory for elevated antibody titres against EBs of *P. amoebophila*, *Parachlamydia* sp. and *Simkania* sp. in a blind experiment. Volunteers from other research groups in our laboratory functioned as negative control. Each screening was repeated with almost identical results.

Again, a pre-screening was performed using a mixture of EBs of all three organism and titres were determined against each single organism.

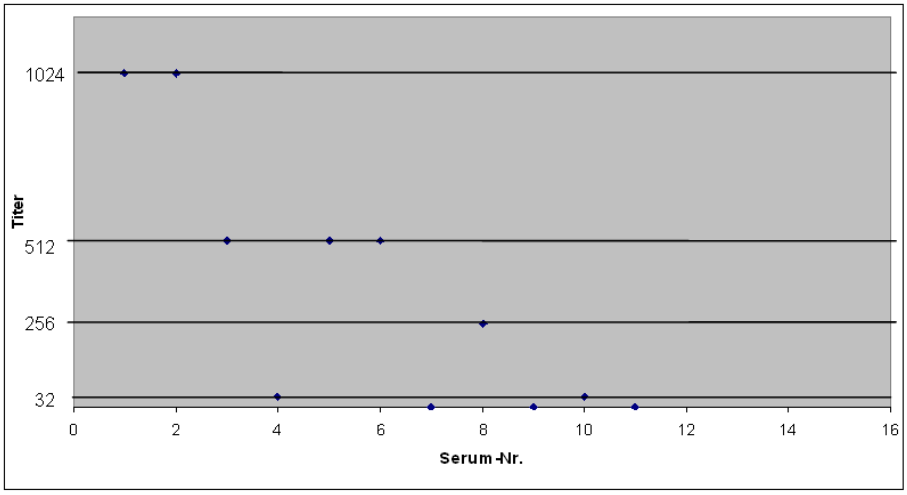
Strongly elevated antibody titres against *Simkania*-EBs could not be observed, the highest antibody titre reaching 32.

This was different for antibody titres against *Parachlamydia* spp., which were significantly elevated. In our group, intense work with EBs of *P. amoebophila* is done and people very often come into contact with *P. amoebophila*-EBs.

Blood samples were taken at two different times, the second ones taken around four weeks later. In the first screening, three people were chosen as negative control, one of them having worked with EBs of environmental chlamydiae some time ago and who was expected to show no elevated titres. Indeed, for this person, antibody titres were zero. Sera from the other negative controls showed no positive reaction as well. One person from the negative control showed antibody titres of 32, as well as one person of the chlamydiae group, who never worked with chlamydial EBs before. The person who only occasionally worked with environmental chlamydiae showed titres of 256, while the three persons who regularly worked with these organisms had elevated titres of 512. Two people had strongly elevated antibody titres reaching 1:1024, which was also the case for the second blood samples taken four weeks later. These two persons had recently extensively worked with *P. amoebophila* and performed several EB-purifications, thus resulting in a close and extensive contact with the infectious elementary bodies.

For the second blood screening, two people were chosen as negative control. One of these two showed antibody titres of 32 while the other remained negative. From the chlamydiae group, six people were tested and the lowest antibody titre determined was 128, the serum obtained from a person, who only rarely uses the chlamydia-research L2-laboratory, and never worked with *P. amoebophila*-EBs. One person, who works in the chlamydia-research L2-laboratory but rarely comes into close contact with *P. amoebophila* –EBs also showed a titre of 128. Three people, who regularly work with *P. amoebophila* showed antibody titres of 512, while two had strongly elevated antibody titres reaching 1:1024. These two persons had recently extensively worked with *P. amoebophila* and performed several EB-purifications.

First blood samples:



Second blood samples:

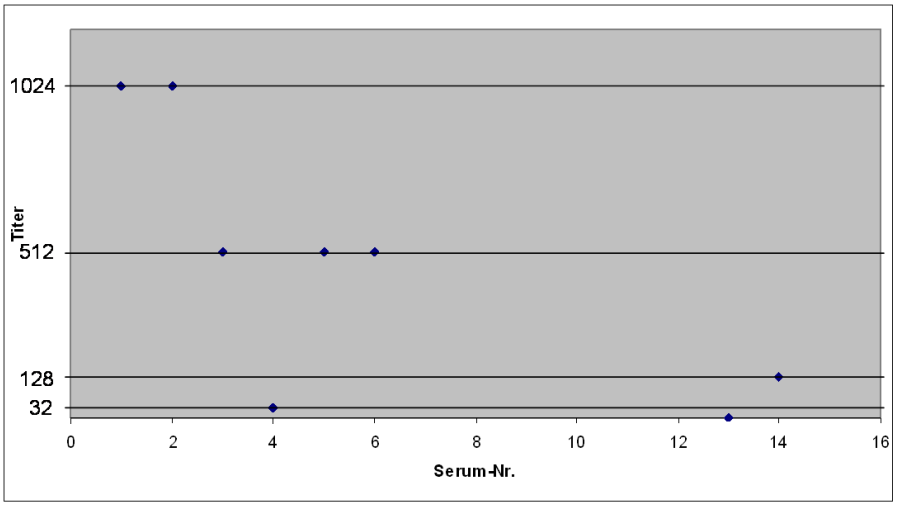


Fig. C.16. Results of the titre determination of the sera derived from the members of the Department of Microbial Ecology, University of Vienna, Austria.

D. Discussion

D.1. Bioinformatical analysis of the ORFs *pc0373* and *pc0374* of *Protochlamydia amoebophila* UWE25

D.1.1. BLAST search

In 2005, Subtil and colleagues could demonstrate the secretion of several proteins from pathogenic chlamydiae via the Type III Secretion System (TTSS) in a heterologous expression system (Subtil et al., 2005). In their study, a screen for putative chlamydial TTSS substrates from *Chlamydophila pneumoniae* and their homologues in *Chlamydia trachomatis* and *Chlamydophila caviae* was performed. The aim of the study was the characterisation of functionally unknown proteins, therefore proteins of the Inc family were excluded from the study. Chlamydial proteins of the Inc family are known to be secreted via the TTSS and then inserted into the host-derived inclusion membrane (Hackstadt et al., 1997a).

Subtil and colleagues could not only show the full-length-secretion of four proteins via the TTSS but also that the ability of the proteins to be recognized by the TTSS was largely conserved among homologues (Subtil et al., 2005). These findings imposed the question whether the genome of the environmental chlamydia *P. amoebophila* also contains ORFs showing definite homology to the proteins found to be TTSS substrates by Subtil *et al.*

The BLAST search for homologues of these proteins in the *P. amoebophila* genome showed two potential candidates of *P. amoebophila*, which showed a high homology on amino acid level to one of the proteins of *C. pneumoniae* and their homologues in *C. trachomatis* and *C. caviae* characterized by Subtil *et al.*

All of the proteins demonstrated by Subtil *et al.* to be TTSS substrates were of unknown function and showed no significant homology to other known TTSS substrates (Subtil et al., 2005). Therefore, amino acid (aa)-homology is not sufficient in order to make predictions on the possible function of an unknown protein. However, aa-homology of an unknown protein to characterized proteins allows a first hint for further analyses of the unknown protein.

D.1.2. Sequence alignment

Bioinformatic analyses of these two candidates – namely Pc0373 and Pc0374 – were performed. Both proteins have no signal peptide which is also the case for other known

TTSS substrates in different organisms (Hueck, 1998). The multiple sequence alignment obtained from the PredictProtein Server shows that the N- and the C-terminal ends are highly variable, while the middle section is rather conserved. It is assumed that the ability of effector proteins to be translocated by the TTSS relies on the universality of the secretion signal recognised by the TTSS machineries. However, this mechanism of recognition has not been elucidated to date. The secretion signal has been proposed to reside within the first 15 residues of the secreted proteins, in the corresponding mRNA-sequence, or even in the use of specific tRNA molecules during translation (see (Ramamurthi and Schneewind, 2003) for a discussion on these models). The fact that the N-terminal part of Pc0373 and Pc0374 are variable over the first 10 amino acid-residues could therefore indicate that either, these proteins are not secreted by the TTSS or that the recognition signal is not solely dependent on the 15 amino acids. This could be shown for several other proteins by the modification of the N-terminal amino acids and subsequent analysis of the minimal N-terminal sequence required for secretion of the protein via the TTSS (Anderson et al., 1999; Mudgett et al., 2000). So, the mRNA could probably also function as a recognition signal in the TTSS. On the other hand, the first 15 amino acids could indeed play a role as a recognition signal by the recognition not being based on the primary but on the secondary or tertiary structure of the protein, which can be similar between proteins even if the primary structure is deviating (reviewed in (Ghosh, 2004)).
Generally, the fact that TTS machines from many organisms are able to secrete proteins from heterologous species suggests a common recognition mechanism. Due to the fact that the N-terminal part of most TTSS substrates is not conserved on amino acid level, the uniformity of the recognition signal could probably occur in the secondary structure, due to biochemically similar amino acids or at mRNA level.

This shows that search of TTSS effectors solely based on sequence analysis is not sufficient and predictions whether Pc0373 and Pc0374 are secreted via the TTSS cannot be made based on this information. More detailed functional analyses are required in order to reveal the role of unknown proteins as putative TTSS substrates.

D.1.3. Secondary structure and hydrophobicity prediction

The secondary structure prediction shows three helices for Pc0373 and only two for Pc0374. The proteins are predicted to have no transmembrane helices which could partly be due to the shortness of the amino acid sequence. According to Mitaku and Hirokawa,

the solubility of a protein is also dependent on its length (Mitaku and Hirokawa, 1999). In general, the authors could show that the longer the protein, the higher its hydrophobicity and higher the probability that this protein is a membrane protein.

For Pc0373 which has a length of 75 aa, the SOSUI tool predicted a hydrophobicity value of -0.473 and for Pc0374 (70 aa) a value of -0.299. This means that these two proteins are largely hydrophilic and expected to be soluble proteins. This is in accordance with the lack of predicted transmembrane helices.

Another indication that these two proteins are not inserted into a membrane is the fact that most of the membrane proteins known so far have an isoelectric point (pI) in the alkaline range. This is not the case for Pc0373 and Pc0374 which have pIs of 5.88 and 4.8, respectively. The fact that Pc0373 and Pc0374 are expected to be soluble proteins supports the assumption that they could be secreted via the TTSS, as during Type III secretion effectors must pass a hollow needle-like structure in order to be translocated into the host cell. This would not be expected to be possible for highly hydrophobic proteins (Mitaku and Hirokawa, 1999).

D.1.4. Motif search

A motif search was performed using the ProSite tool. For both proteins, a protein kinase C phosphorylation site, a casein kinase II phosphorylation site and a N-myristoylation site were predicted. Protein kinases are able to modify other proteins by phosphorylation resulting in a functional change of the protein. Kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction (Litchfield, 2003). The casein kinase II has a vast array of candidate physiological targets and participates in a series of cellular functions. It is hypothesized that the casein kinase II can exert an anti-apoptotic role by protecting regulatory proteins from degradation (Litchfield, 2003). *P. amoebophila* is an endosymbiont which is able to survive within its host cell, so that the occurrence of a casein kinase II binding site and the possible effects of this enzyme would have a positive outcome for the endosymbiont by preventing apoptosis of its host. N-myristoylation is known to promote weak and reversible protein-membrane and protein-protein-interactions. Proteins modified in this way are also involved in signal transduction cascades (Farazi et al., 2001). A possible interaction of the two *P. amoebophila* proteins with other proteins or membranes could also be in support of their possible function as TTSS effectors.

D.1.5. Amino acid composition

Most remarkably, both proteins have a rather high content of the amino acid leucine. The leucine content accounts for 13.3% of all amino acids in Pc0373 and for even 17.1% in Pc0374. The leucine content was also compared to other proteins found to be secreted via the TTSS by Subtil *et al.* and almost all of them had a leucine content ranging from 15 to 20% or even higher. This is remarkable, since comparison with different proteins not secreted via the TTSS show an average leucine content that is considerably below 10%. Perhaps the amount of leucine of a TTSS substrate also plays a role as a secretion signal.
This observation suggested the possible occurrence of a leucine-rich-repeat (LRR) in Pc0373 and Pc0374. All proteins containing this LRR known to date are involved in protein-protein interaction and often also in signal transduction (Kobe and Deisenhofer, 1994). The occurrence of this repeat would therefore be another indication that these two proteins are able to interact with other proteins and be secreted via the TTSS. However, no such motif could be found in the amino acid-sequence of neither Pc0373 nor Pc0374. The fact that all proteins found to have a LRR are involved in protein-protein interactions does not rule out the possibility that proteins without such a motif are nevertheless involved in such interactions. Therefore the absence of a LRR in Pc0373 and Pc0374 does not imply that these are not secreted by the TTSS.

D.2. Importance of a TTSS for chlamydiae

Sequencing of the whole genome of *Protochlamydia amoebophila* UWE25 revealed the presence of a functional TTSS (Horn et al., 2004). The genes required for a functional TTSS were also found in other environmental chlamydiae like *Parachlamydia acanthamoebae* UV-7 (Collingro et al., 2011) and *Simkania negevensis* Z (Peters et al., 2007). The TTSS is known to be a major virulence determinant in pathogenic bacteria which are able to influence the host by the secretion of effector proteins via the TTS apparatus (Hueck, 1998).

While the genes encoding structural proteins of the TTSS are rather conserved between different organisms, genes coding for different TTSS substrates – also called effectors – are highly variable and therefore the search for an uniform recognition signal of these substrates is difficult (Ghosh, 2004).

It has been shown that TTSS substrates translocated into the host cell can have a variety of effects on the host and in this way alter its environment in its own favour. These effects include actin and tubulin dynamics, gene expression, vesicular trafficking, programmed cell death and cell cycle progression. Effectors translocated by the TTSS can even exploit the eukaryotic ubiquitin signalling pathways and by this can alter the properties of host cell proteins, but also block intermediates in the host's innate immunity (Angot et al., 2007). Another remarkable feature of these effector proteins is one of "mimicry" as a central strategy to modulate cellular functions of the host cell. Unlike most other bacterial toxins that exert their function by introducing non-reversible modifications to their target host cell proteins, TTSS substrates rather act by "mimicking" the function of host cell proteins, which could for instance be shown for the *Salmonella* protein SopE. It hast been proposed that bacteria harbouring a TTSS might use "mimicking" as a strategy for the establishment of a close functional interface that is often of symbiotic nature (Galan and Wolf-Watz, 2006).

The fact that the TTSS of pathogenic bacteria and especially of chlamydia is an effective tool for translocation of bacterial proteins into the host cell and that these proteins can therein influence a variety of cellular functions and pathways in its own favour, makes the characterisation of TTSS substrates and the understanding of their function a crucial step in the understanding of host-symbiont-relationships. Knowledge of the function of TTSS substrates would therefore enable us to better understand the degree of dependence of the two organisms on each other, the way that the bacteria can evade the defence mechanisms of the host cell and possible mutual effects on both the host and the endosymbiont.

It is remarkable that there is a homology between the more ancient flagellar system and the TTSS, indicating that the TTSS may have evolved from the flagellar system. Free-living bacteria which do not form flagella also have genes that are known to be components of the flagellar system or close homologues to such genes (Hueck, 1998). For the intracellular chlamydiae this could perhaps be an indication of an early extracellular lifestyle in which the flagellar system played an important role, but which was abandoned when they assumed a symbiotic lifestyle with a eukaryotic host. Perhaps the flagellar system was then modified to the TTSS in order to enable the endosymbiont to manipulate its intracellular environment.

In this study, two proteins of the amoebal endosymbiont *P. amoebophila* that might be possible TTSS substrates were analysed with respect to their localisation in the amoebal host cell using immunofluorescence techniques. These and further analyses hopefully will help to better understand the mechanisms behind the symbiotic lifestyle of *Protochlamydia amoebophila* UWE25 within its amoebal host.

D.3. Protein secretion into the chlamydial inclusion

Immunofluorescence analyses using polyclonal antibodies raised against the protein Pc0374 of *P. amoebophila* resulted in ring-shaped signals located around the bacterial cells which are distributed throughout the amoebal cytoplasm. This was not the case for amoebal cells containing no endosymbionts, showing that the observed ring-shaped signals were specific (Fig. C.14.).

Only antibodies raised in rabbit resulted in specific fluorescence signals, while sera obtained from guinea pig failed to show such signals. Apparently the immune response in rabbit was significantly stronger than in guinea pig after immunization with Pc0374.

Apart from that, the large bleed sera obtained from rabbit after three antigen boosts resulted in stronger fluorescence signals when compared to the final bleed sera obtained after another antigen boost. The fact that sera obtained after the third antigen boost seemed to contain a higher amount of antibodies than those won after one additional boost might be due to the beginning decrease of antibodies in rabbit after the third immunization. Probably the time span between large and final bleed was too long and the antibodies generated by the animal had started to degrade and decrease in number. Another reason could be the adaptation of the animal's immune system to the antigen which would mean that after the amount of antibodies has reached a certain level, further exposure to the antigen did not result in the generation of still more antibodies and another rise in antibody titres produced by the animal's immune system.

Apart from that, the first immune response of an animal results in the production of Immunoglobulin M (IgM) and after a delayed period IgG-production is initiated, finally resulting in the accumulation of IgG. The secondary antibodies used in this study were specific for IgG and this may be the main reason why the sera obtained at earlier time points failed to result in positive signals.

Another observation was the occurrence of strong unspecific background fluorescence for all of the tested sera including the pre-immune sera. This effect could be attenuated by blocking the sera against amoebal proteins. The reason for this is probably due to the fact

that the animals immunized with the *P. amoebophila*-protein had come into contact with amoebal cells at some point of their lives and developed antibodies against amoebal proteins. Although the animals used for antibody generation are kept under sterile conditions, contact with organisms that are as ubiquitous as free-living amoebae cannot be ruled out completely and the fact that blocking against amoebal proteins could minimize background fluorescence is an indication that the animals have come in contact with amoebae at some time.

Fritsche and colleagues could show that *P. amoebophila*, in contrast to other environmental and also pathogenic chlamydiae, forms single-cell inclusions after entry into the host cell instead of large inclusions surrounded by a host-derived membrane harbouring numerous bacterial cells (Fritsche et al., 1998a). This can for instance be observed after staining with the DNA-binding dye DAPI which results in fluorescent signals evenly dispersed throughout the amoebal cytoplasm.

In this study, the ring-shaped fluorescence signals obtained after antibody incubation were similarly distributed in the amoebal cell. Comparison with DAPI-staining demonstrated that the ring-shaped signals were located around the *P. amoebophila* cells. These observations clearly show that the protein Pc0374 is located either in the bacterial membrane, the inclusion membrane or the inclusion itself, whereby a simultaneous occurrence in more than one of these structures could also be possible. Due to the fact that the inclusion membrane is spatially adjacent to the bacterial inner and outer membranes, a clear localization of the fluorescent signal could not be achieved. Counterstaining of the bacterial outer membrane also failed to clarify the localisation of the fluorescence signals specific for Pc0374.

In order to clearly differentiate between the different single membranes and the space between these membranes in the inclusion, observation at a higher resolution is needed. One possibility of increasing the resolution and obtaining more detailed information on the localisation of the Pc0374-specific signal would be the use of immunogold labelling techniques. These combine the high resolution of electron microscopy with the immunological properties of applied antibodies (Herrera, 1992). Under optimal conditions – which would among others be highly specific antibodies, good resin properties, good penetration of the resin into the sample and a good contrast – the localisation of single antibody molecules can be distinguished. However, this technique is rather time-consuming and requires strong expertise with electron microscopy. Nevertheless,

immunogold labelling could help to clearly localize the Pc0374-protein and to allow further conclusion.

Bioinformatical analyses predicted Pc0374 to be a soluble protein and not to be inserted into a membrane. In that case, Pc0374 would either be attached to the surface of the bacterial and/or inclusion membrane, or – which is more probable for a soluble protein – be localized in the chlamydial inclusion between the bacterial and the inclusion membrane. This would be a strong indication that Pc0374 is indeed a TTSS substrate and is secreted outside the bacterial cell. Secretion via the TTSS would also occur if the protein was localized in the inclusion membrane. On the other hand, Pc0374 does not show the properties of an inclusion membrane protein (Rockey et al., 2002). Localisation in the bacterial membrane would mean that the protein is not being secreted and is either inserted in or attached to the bacterial membrane. However, the lack of transmembrane helices and the predicted high hydrophilicity of the protein are strong indicators of its solubility and make the localization of the protein in the inclusion the most probable scenario. There, Pc0374 could probably influence the physicochemical properties of the inclusion or be able to interact with other proteins, perhaps such of amoebal origin. The possible property of Pc0374 to influence its environment – namely the inclusion harbouring the bacterial cells – could be of great importance for the survival and proliferation of *P. amoebophila* in its amoebal host.

D.4. Protein secretion into the nucleus of the amoebal host

Large-bleed sera raised in guinea pig after immunisation of the animal and three consecutive antigen boosts with the *P. amoebophila*-protein Pc0373 showed specific and strong fluorescence signals in amoebal cells harbouring *P. amoebophila*, while this was not the case for cells devoid of endosymbionts. This is a strong indication that the obtained fluorescence signals were specific, as Pc0373 is of bacterial origin and is not expected to be found in amoebal cells lacking *P. amoebophila*.

Antibodies raised against this protein in rabbit failed to result in specific binding to the antigen even after several boosts. Rabbit antisera showed rather high unspecific background fluorescence but failed to result in specific fluorescence signals. This shows that rabbit and guinea pig show different reaction after immunization with Pc0373 and that rabbits failed to produce a significant amount of antibodies triggered against Pc0373. The fact that two different animals immunized with the same protein show different reactions

could also be observed for Pc0374, for which antibodies raised in rabbit resulted in strong specific fluorescence signals, while antibodies raised in guinea pig failed to do so. A difference in the immune reaction between guinea pig and rabbit is to be expected, since the immune system of different species (and even different individuals) is very likely to vary.

As observed for antibodies against Pc0374, the strongest fluorescence signals could be obtained with large-bleed-sera won after a third antigen boost, while a fourth antigen boost failed to stimulate the production of further antibodies in guinea pig. The reason for this could either be the beginning degradation of antibodies after the third boost, a long period between the fourth antigen boost and the final bleed or adaptation to the antigen resulting in decreased antibody production.
It is known that the first immune response of an animal results in the production of IgM. After that, IgG production is initiated and IgG molecules accumulate in the animal's body. The secondary antibodies used in this study were specific for IgG, and this probably is the main reason for the observation that the sera obtained at earlier time points failed to result in positive signals.

Rather strong background fluorescence could be observed for all of the obtained sera including the pre-immune sera which were won prior to immunisation of guinea pig with Pc0373. When the sera were blocked against amoebal proteins, this unspecific background fluorescence could be remarkably reduced. This is an indication that the animals used had come in contact with amoebal cells at some point of their lives and developed antibodies against amoebal proteins. This is no surprise as amoebae are ubiquitous and absolute sterility during the maintenance of the immunized animals can hardly be guaranteed.

Immunofluorescence tests with large-bleed-sera from guinea pig obtained after immunization and three consecutive antigen boosts resulted in strong specific fluorescence signals localized within the amoebal nucleus. Most remarkably, the fluorescence signal was localized around the amoebal nucleolus and surrounded by the amoebal nuclear membrane. This was only the case for amoebal cells harbouring *P. amoebophila*-endosymbionts, while amoebae devoid of bacterial cells failed to show fluorescence signals (Fig. C.11.). These observations show that the fluorescence signals were highly

specific and could only be observed in amoebal cells which contained endosymbionts who possess the genetic information for the synthesis of the Pc0373-protein.
On the other hand, pre-immune sera of guinea pig, which were won before the animal had been immunized with Pc0373, resulted in no specific signals, neither in amoebal cells devoid of endosymbionts, nor – most importantly – in amoebae containing *P. amoebophila*. This clearly shows that the antibodies were developed only after the immunization and were specific for the Pc0373 protein. Amoebae which harbour no endosymbiont and therefore do not contain the organism capable of Pc0373-synthesis do not show fluorescence signals around the nucleolus.
These results strongly indicate a translocation of the bacterial protein Pc0373 into the nucleus of its amoebal host cell.

In order to confirm the specificity of the antibodies and the resulting fluorescence signals, the signal was amplified with the use of the tyramide signal amplification (TSA)-assay in which fluorophore-labelled tyramides react with the horseradish peroxidase (HRP)-enzymes conjugated to the secondary antibodies, resulting in the deposition of a huge amount of fluorophores in the vicinity of the antibodies, yielding a significant amplification of the fluorescence signal as compared to the standard IF assay. Indeed, signal intensity was remarkably increased in amoebal cells containing *P. amoebophila* compared to results obtained without TSA. Despite signal amplification, amoebal cells containing no endosymbionts failed to show such signal (Fig. C.13). This is another strong indication for the specificity of the fluorescence signals and the localization of Pc0373.
These results strongly indicate a translocation of the bacterial protein pc0373 into the nucleus of the amoebal host cell. The fact that pc0373 is predicted to be a soluble protein makes its secretion and translocation even more probable.
Pc0373 is highly homologous to proteins of *C. pneumoniae*, *C. trachomatis* and *C. caviae* shown to be secreted via the TTSS (Subtil et al., 2005). The protein is predicted not to be membranous and is localized around the nucleolus and within the nuclear membrane of the amoebal cell containing *P. amoebophila*-endosymbionts. It is known that the *P. amoebophila*-genome encodes a functional TTSS (Horn et al., 2004). These facts are strong indicators for the possible role of Pc0373 as a TTSS substrate, and its translocation to the nucleus of the amoebal host.

Proteins from other intracellular organisms are known to be secreted into the nucleus of their host cells via the TTSS. In 2001, Toyotome and colleagues could show the secretion of the *Shigella* protein $IpaH_{9.8}$ into the nucleus of mammalian cells via the TTSS (Toyotome et al., 2001). This translocation seems to be dependent on the microtubule network of the host cell, since nuclear accumulation of $IpaH_{9.8}$ is inhibited in cells treated with microtubule-destabilizing agents. Under conditions of increased type III secretion (Demers et al., 1998), transcription of the gene coding for the $IpaH_{9.8}$-protein was found to be up-regulated. Secretion of this protein occurred after invasion of the organism into epithelial cells. Immunofluorescence analyses showed the localization of this protein in the nucleus of the host cell, while only a small amount of the protein could be found in the cytoplasm by Toyotome *et al.* Recently, Ashida and colleagues could confirm the secretion of $IpaH_{9.8}$ into the nucleus and show its role as an effector protein to modulate the host inflammatory response. They were also able to show an increased transcription when the bacterial cells were residing intracellularly, confirming the role of the protein as an effector influencing the properties of the host cell. Their results also provide in vivo and in vitro evidence that IpaH proteins contribute to attenuating the inflammatory response of the host (Ashida et al., 2007).

Another TTSS substrate also shown to be secreted into the nucleus of its eukaryotic host cell is the *Yersinia pestis* protein YopM. YopM is a TTSS substrate secreted into the nucleus of mammalian cells and secretion is microtubule-dependent. Secretion of this protein into the nucleus could be shown for epithelial cells as well as for macrophages. Secretion of YopM could only be observed when the bacteria were present at the cell's surface, indicating a contact-dependent activation of secretion. Further, it could be shown that drugs preventing acidification of the vacuole harbouring the bacterial cells greatly reduced YopM's entry into the nucleus (Skrzypek et al., 1998). Pathogenic and environmental chlamydiae are known to reside within a non-acidified vacuole that is disconnected from the phagosome-lysosome pathway and drugs preventing vacuole acidification could be shown to have no effect on *Chlamydia trachomatis* cells (Heinzen et al., 1996). This indicates that although YopM has a similar intracellular destination as Pc0373, both proteins seem to have significantly different effects on their host. Apart from that, like the Shigella protein $IpaH_{9.8}$, YopM contains a LRR in the N-terminal part. This is not the case for Pc0373, which is an indication that a LRR is probably not a determinant of TTSS substrates or the intracellular localization of the secreted protein. More probably, as

proteins involved in protein-protein interactions are known to contain a LRR (Kobe and Deisenhofer, 1995), this motif is not essential as a secretion recognition signal or for the localization of the secreted protein but rather determines the further fate of the protein and its involvement in protein interactions. Nevertheless, absence of a LRR in Pc0373 does not rule out its possible involvement in protein-protein interactions.

Finally, this study could show the localization of the *P. amoebophila*-protein Pc0373 in the nucleus of the amoebal host. The fact that it is predicted to be a soluble protein strengthens the hypothesis that Pc0373 is secreted via the TTSS into the host's nucleus where it can have different effects. Within the nucleus, Pc0373 could have different interaction partners: it could interfere with certain amoebal genes and so inhibit or enhance their transcription, or interact with mRNA by binding to it and so interfere with gene transcripts. The protein could also interact with amoebal proteins like structural or regulatory ones, thus influencing the host's regulatory pathways and its environment. *Ehrlichia chaffeensis*, an obligate intracellular bacterium, is known to be able to alter the transcriptional level of host genes, including those coding for apoptosis inhibitors, proteins regulation, cell differentiation, signal transduction and membrane trafficking proteins (Zhang et al., 2004). *Yersinia pseudotuberculosis* utilizes the TTSS to enhance its chances of survival and to overcome the host immune system but also to maintain the bacterial translational machinery in a fully operational state (Okan et al., 2006).

Since the genetic modification of chlamydiae has not been possible to date, the study of single genes with knockout-mutants is not possible. Proteins and their function must therefore be analysed on molecular level. Information on the TTSS of other organisms can provide useful information about possible TTSS substrates and effectors in chlamydiae.
One approach to determine a possible interaction of Pc0373 with host molecules would be its immobilisation via a His-tag and the addition of either amoebal DNA-, mRNA- or protein-preparations. The respective interaction partner could then bind to the immobilized protein followed by an elution step and characterization of the eluate. Another interesting assay would be the use of small-molecule-inhibitors of the TTSS and its effects on Pc0373 secretion and localization. The effect of these inhibitors on the TTSS could be demonstrated in several independent studies (Nordfelth et al., 2005; Muschiol et al., 2006; Wolf et al., 2006) and inhibition of Pc0373-secretion by this molecules would further confirm its role as a TTSS substrate.

Use of immunogold labeling in combination with electron microscopy would allow to further resolve the localization of Pc0373 in the amoebal nucleus (Herrera, 1992).

D.5. Potential virulence of environmental chlamydiae

In this study, sera from different patients of the Universitätsklinikum Ulm, Germany and of members of the Department of Microbial Ecology (DOME), University of Vienna, Austria were screened for antibodies against elementary bodies (EBs) of *Protochlamydia amoebophila* UWE25, *Parachlamydia acanthamoebae* UV-7 and *Simkania negevensis* Z. Antibody titres of positive sera were determined in order to get further insight into the infectivity and pathogenicity of these organisms.

D.5.1. Evidence for the pathogenicity of environmental chlamydiae

The natural host of most environmental chlamydiae found to date are amoebae, which are ubiquitous protozoa having been isolated from different habitats (as reviewed in (Rodriguez-Zaragoza, 1994)). Fritsche *et al.* found that approximately 25% of the *Acanthamoeba* spp. isolates contained uncultured bacterial endosymbionts (Fritsche et al., 1993). It could also be shown that intra-amoebal growth may increase the virulence of some intracellular bacteria (Cirillo et al., 1999), prompting the concern that other intracellular bacteria recovered from amoebae, such as the *Parachlamydiaceae*, could be pathogenic (Greub and Raoult, 2002). Growing indication for a pathogenic role of *Parachlamydiaceae* is present and antisera against environmental chlamydiae have been found in patients suffering from different diseases. For instance, Hall's coccus, a member of the *Parachlamydiaceae* was found in amoebae isolated from the source of an outbreak of humidifier-associated fever in the United States, as well as related serologic studies (Birtles et al., 1997). Increased antibody titres against *Parachlamydia* were found in patients with community-acquired pneumonia (Marrie et al., 2001). The identification in respiratory tract specimens of three new *Chlamydia*-like strains, which were found to be phylogenetically closer to the *Parachlamydiaceae* and *Simkaniaceae* than to the *Chlamydiaceae* known to be pathogens of different mammals (Ossewaarde and Meijer, 1999), is an additional argument in favour of a role of the *Parachlamydiaceae* in the pathogenesis of respiratory diseases.

Fritsche et al. reported that the cytopathic effect on human fibroblasts was enhanced by 66 to 70% when *Acanthamoeba* contained the *P. amoebophila* endosymbiont compared to cells devoid of endosymbionts (Fritsche et al., 1998b).

Simkania negevensis, the third chlamydia-like organism studied here is known to be able to infect human cell cultures of various tissue origins and to induce a host cell inflammatory response. Previous studies demonstrated its association with respiratory tract infections in infants and adults (Kahane et al., 1998; Lieberman et al., 2002).

As demonstrated above, there is a growing evidence for the infectivity and pathogenicity of environmental chlamydiae. Amoebae as their natural hosts, which are able to survive under adverse conditions and to form cysts as a survival strategy, are thought to act as Trojan horses for the infection of humans (Barker and Brown, 1994). These Trojan horses are not only able to transport the bacterial particles but also allow them to multiply within the host and to survive adverse conditions. Apart from that, environmental chlamydiae were shown to tap the amoebal nucleotide pool (Schmitz-Esser et al., 2004) and to influence the growth rate of its ameobal host (Collingro et al., 2004).

D.5.2. Cross-reactivity of environmental chlamydiae and screening of human sera from patients at the Universitätsklinikum Ulm, Germany

In this study, 15 randomly chosen patients from the Universitätsklinikum Ulm were screened for elevated antibody titres against the environmental chlamydiae *P. amoebophila*, *Parachlamydia* sp. and *Simkania negevensis*. Cross-reactivity of the sera was tested previous to titre determination. It could be shown that there was a strong cross-reactivity of *P. amoebophila* and *Parachlamydia* sera, while no cross-reactivity occurred for *Simkania* sera. This can be explained by the strong phylogenetic relationship of *P. amoebophila* and *Parachlamydia* as both belong to the family of *Parachlamydiaceae*, while *Simkania negevensis* falls within the more distantly related family *Simkaniaceae* (Fig. A.2.). These observation are in accordance with the results obtained by Casson *et al.* who could show that *P. amoebophila* and *Parachlamydia* showed cross-reaction events, while more distantly related *Chlamydia*-like organisms did not. No cross-reactivity against *C. pneumoniae*, *C. trachomatis* and *C. psittaci* was detected when sera elicited with any *Chlamydia*-like organisms were tested, except *Parachlamydia acanthamoebae* strain Hall's coccus that reacted with *C. pneumoniae* up to a titre level of 128 (Casson et al., 2007). As *Parachlamydia* sp. strains are reported to be involved in respiratory tract infections and *C. pneumoniae* is a causative agent of community-acquired pneumonia (Grayston et al., 1990), it is possible that cells of both organisms show similar epitopes, thus resulting in a cross-reaction of antisera against these organisms.

Out of 15 sera of randomly chosen patients at the Universitätsklinikum Ulm for whom no chlamydial infection was reported, only 2 were seropositive for the *Parachlamydiaceae P. amoebophila* and *Parachlamydia* reaching a titre level of 32, and one serum was seropositive for *Simkania*, also up to a level of 32. These results show rather low titre levels against the analysed organisms due to the fact that the patients were randomly chosen and not suspected to be suffering from an infection with these organisms or showing symptoms assigned to an infection with chlamydia-like organisms. Therefore, these patients had probably not come in contact with higher loads of these organisms or suffered from an infection. Or, if an infection had taken place, either it was some time ago and the antibody titres had decreased over time, or the infection had happened shortly before the serum was obtained and production of IgG had not started yet. In this case, microimmunofluorescence (MIF) with secondary antibody against IgM which is produced rapidly after infection – in contrast to the delayed production of IgG – would show whether a recent infection had taken place.

D.5.3. Screening and titre determination of sera obtained from members of the DOME

Another aim of this study was to show, whether persons who are regularly in contact with infectious particles of these three organisms would have significantly elevated antibody titres or not. Therefore, volunteers from the environmental chlamydiae group and other groups at the DOME were tested. No significantly high antibody titres against *Simkania*-EBs could be observed. Indeed, little work with infectious particles of this organism is performed in the environmental-chlamydiae group. Most of the work is performed with *P. amoebophila*-EBs and especially the purification of EBs leads to an increased exposure of the respective researcher to the infectious particles, even if efforts for its prevention are taken. On the other hand, due to the cross-reactivity of *P. amoebophila*- with *Parachlamydia*-antibodies and vice versa, the distinction of antibody titres against each of these organisms was not possible.

The obtained results clearly show that persons who are not members of the chlamydiae group did not have elevated antibody titres against any of the three organisms. On the other hand, people working extensively with EBs, especially those of *P. amoebophila*, showed highly elevated antibody titres reaching up to 1024 (Fig. C.16.). These two persons had recently extensively worked with *P. amoebophila* and performed several EB-purifications, thus resulting in a close and extensive contact with the infectious elementary bodies.

The person who only occasionally worked with environmental chlamydiae showed titres of 256, while the three persons who regularly worked with these organisms had elevated titres of 512. One person, who had worked with EBs of environmental chlamydiae some time ago, was not expected to show elevated titres. Indeed, antibody titres were zero. Compared to the strong increase in antibody titres of persons who had recently extensively worked with EBs of *P. amoebophila*, this indicates that antibody titres are degraded over time after encounter. Although the interpretation of results obtained with the MIF assay underlie the subjective judgement of the researcher (Wong et al., 1999), congruent results were obtained from two independent screenings in a blind experiment. This shows that results are reliable and deviations among the results were low.

D.5.4. Concluding remarks

The occurrence of elevated antibody titres in persons extensively working with EBs of environmental chlamydiae shows that these organisms are capable of entering the human body and triggering an immune response. Assumptions whether an infection takes place and the organism is able to multiply cannot be made, although for *Parachlamydia* sp. it could be shown that EBs can enter human macrophages and multiply therein (Greub et al., 2003). This is not the case for *P. amoebophila*-EBs, which can enter macrophages but fail to multiply (Collingro, unpublished data).
On the other hand, clear indication is given in this study that the human body reacts to the entry of infectious particles by producing large amounts of specific antibodies. What the further implications are is difficult to speculate. It should be noted that persons from the environmental chlamydiae-group have been working with environmental chlamydiae and have not suffered from any symptoms or diseases in which environmental or pathogenic chlamydiae are known to be involved. Nevertheless, the fact that the human body reacts with increased antibody production after encountering infectious particles of the here studied organisms is a clear indication that these particles are recognized as invasive and perhaps even pathogens.

A long-term screening of the titre levels of the DOME volunteers and the evaluation of titre levels taking into account the time and degree of exposure of the persons to the EBs of environmental chlamydiae would help to better understand the parameters influencing antibody generation and amount of antibodies generated by the human organism. Apart from that, comparison of IgG and IgM titre levels would allow conclusions about the point

of infection and the time span necessary for the generation of higher antibody levels by the human body.

E. Summary

Environmental chlamydiae are a group among the order *Chlamydiales*, which is closely related to the pathogenic chlamydiae. One important member of this group is the amoebal endosymbiont *Protochlamydia amoebophila* UWE25.

In this study, two unknown proteins of *Protochlamydia amoebophila* were analysed - Pc0373 and Pc0374 - which were found to be homologous to Type III secretion system (TTSS) substrates of pathogenic chlamydiae.

Bioinformatic analyses showed that the proteins are soluble, which is in favour of the hypothesis that these proteins might be secreted by the TTSS and act as effectors within the amoebal host cell. Apart from that, several motifs were predicted for these proteins, indicating that they might be involved in protein-protein interactions.

It could be confirmed that the proteins were transcribed by the *Protochlamydia amoebophila*-endosymbiont residing within its amoebal host cells.

Cloning of the open reading frames encoding these proteins and heterologous protein overexpression were performed followed by analysis of the protein fraction. Heterologous protein expression could be shown for both proteins, which were further purified and used for antibody generation.

Immunofluorescence analyses with sera obtained after immunisation of guinea pigs and rabbits with the purified proteins were performed on amoebal cells harbouring *Protochlamydia amoebophila*. Pc0374 was shown to form ring-shaped fluorescence signals around the bacterial cells and the protein is most probably localized in the inclusion rather than in the bacterial or the inclusion membrane due to its predicted solubility. The other protein, Pc0373 was shown to be translocated into the nucleus of the amoebal host. This observation suggests that Pc0373 is indeed a TTSS substrate and is targeted to the host's nucleus where it could manipulate different cellular processes of the host, including gene expression.

Another part of this work was the optimization of a standard microimmunofluorescence protocol in order to detect antibodies against three different species of environmental chlamydiae.

As there are several indications that environmental chlamydiae have pathogenic potential and might be able to infect humans, blood sera from different persons were checked for the presence of antibodies against environmental chlamydiae. Microimmunofluorescence using elementary bodies of three different environmental chlamydiae with blood sera

obtained from randomly chosen patients, for whom no former chlamydial infection was reported, showed only marginally elevated antibody titres. On the other hand, screening of blood sera obtained from members of the environmental chlamydiae group of our laboratory showed a clear correlation between degree of exposure to the infectious elementary bodies of these organisms and titre levels of the respective person. This clearly shows that environmental chlamydiae are able to trigger an immune response in humans.

F. Zusammenfassung

Umweltchlamydien sind eine Gruppe innerhalb der Ordnung *Chlamydiales*, die nahe Verwandtschaftsverhältnisse zu den pathogenen Chlamydien zeigt. Ein wichtiger Vertreter dieser Gruppe ist *Protochlamydia amoebophila* UWE25, ein Endosymbiont in Amoeben.
In der vorliegenden Arbeit wurden zwei unbekannte Proteine vom *Protochlamydia amoebophila* untersucht, Pc0373 und Pc0374, von denen bekannt ist, dass sie Homologien zu Substraten des Typ III Sekretionssystems (TTSS) pathogener Chlamydien aufweisen.
Bioinformatische Analysen ergaben, dass diese Proteine löslich sind, was die Hypothese unterstützt, sie könnten über das TTSS sekretiert werden und als Effektorproteine in der Amoeben-Wirtszelle wirken. Darüber hinaus wurden mehrere Strukturmotive für diese Proteine errechnet, was ein Hinweis auf mögliche Protein-Protein-Interaktionen ist.
Es konnte zudem gezeigt werden, dass die Proteine vom Endosymbionten *Protochlamydia amoebophila* transkribiert werden, während sich dieser intrazellulär im Amoebenwirt befindet.
Die Gene, die für die zwei genannten Proteine codieren, wurden kloniert und heterolog überexprimiert, gefolgt von einer genaueren Analyse der Proteinfraktion. Die überexprimierten Proteine wurden anschließend zwecks Herstellung spezifischer Antikörper aufgereinigt.
Die Blutsera, die nach Immunisierung von Hasen und Meerschweinchen gewonnen wurden, dienten zur weiteren Analyse mittels Immunfluoreszenz-Verfahren. Im Amoebenwirt, der den Endosymbionten beherbergte, zeigte das Protein Pc0374 ringförmige Fluoreszenzsignale rund um die Bakterienzellen. Es ist anzunehmen, dass das Protein aufgrund der erwarteten Hydrophilie eher in der bakteriellen Inklusion lokalisiert ist, als in der Bakterien- oder Inklusionsmembran. Für das Protein Pc0373 konnte gezeigt werden, dass es in den Zellkern des Amoebenwirtes transloziert wird, was die Vermutung nahelegt, dieses Protein ist tatsächlich ein Substrat des TTSS. An seinem Bestimmungsort - den Wirtszellkern - angelangt, könnte das Chlamydien-Protein verschiedene Zellprozesse des Wirtes beeinflussen, darunter auch die Genexpression.

Ein weiteres Ziel dieser Arbeit war die Optimierung eines Standard-Mikroimmunfluoreszenz-Verfahrens, mit dem Ziel, Antikörper gegen verschiedene Spezies von Umweltchlamydien zu detektieren.

Da mehrere Hinweise vorliegen, dass auch Umweltchlamydien pathogenes Potential besitzen und Humanzellen infizieren können, wurden Blutsera verschiedener Personen auf die Anwesenheit von Antiköpern getestet, die spezifisch für die untersuchten Umweltchlamydien sind. Hierfür wurde das optimierte Mikroimmunfluoreszenz-Verfahren für Elementarkörperchen (die infektiöse Form im Lebenszyklus der Chlamydien) dreier verschiedener Umweltchlamydien und Blutsera zufällig ausgewählter Patienten angewandt. Da für diese Patienten keine bestehende oder zurückliegende Chlamydien-Infektion bekannt war, zeigten sich erwartungsgemäß nur gering erhöhte Antikörper-Titer.
Im Gegensatz dazu zeigten die Blutsera von Personen aus der Umweltchlamydien-Gruppe unseres Departments eine klare Korrelation zwischen dem Expositionsgrad der einzelnen Labormitglieder zu den Umweltchlamydien und den Titern ihrer Blutsera.
Dies lässt den Schluss zu, dass Umweltchlamydien eine Immunantwort im menschlichen Organismus in Gang setzen können.

G. List of abbreviations

16S rRNA	small subunit of ribosomal ribonucleic acid
λ	wavelength
Ω	Ohm
µ	mikro (10^{-6})
°C	degree Celsius
%	percent
A	adenine
aa	amino acid
AA/BA	acryl amide/bisacryl amide
abs	absolute
Amp	ampicillin
APS	ammonium peroxy sulfate
bidist	double-distilled and filtered
BLAST	basic local alignment search tool
BCA	bicinchoninic acid
BSA	bovine serum albumin
bp	base pair(s)
C	cytosine
C1	clinical isolate 1
cDNA	complementary desoxyribonuleic acid
CPAF	chlamydial protease-like activity factor
Cy2	3-(6-carboxyhexyl)-2-{3-[3-(5-carboxypentyl)-6-sulfo-1,3-benzoxazol-3-ium-2-yl]prop-2-en-1-ylidene}-2,3-dihydro-1,3-benzoxazole-6-sulfonate
Cy3	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy3.18-derivative N-hydroxysuccimidester
Cy5	5,5'-di-sulfo-1,1'-di-(X-carbopentynyl)-3,3,3',3'-tetra-methylindol-Cy5.18-derivative N-hydroxysuccimidester
Da	Dalton ($1{,}66018\times10^{-24}$ g)
DABCO	1,4-Diazabicyclo(2,2,2)octane
DAPI	4'-6'-di-amidino-2-phenylindole
DEPC	di-ethyl-pyrocarbonate
$DiOC_6$	3,3'-dihexyloxacarbocyanine-iodide

dist	plainly distilled
DMSO	dimethyl sulfoxide
DNA	desoxyribonuleic acid
DNase	desoxy-ribonuclease
dNTP	desoxy-nucleotide-tri-phosphate
DOME	Department of Microbial Ecology, University of Vienna, Austria
EB	elementary body
EDTA	ethylene-di-amine-tetra-acetic acid
e.g.	*exempli gratia* (lat., “example given”)
ERT	Eppendorf reaction tube
et al.	*et alteri* (lat., “and others”)
EtBr	ethidium bromide
EtOH	ethanol
F	forward (used for labeling of primers)
fb	final bleeding
Fig.	Figure
FITC	fluorescein isothiocyanate
FLA	free-living amoebae
g	gram(s)
G	guanine
GC	mol % guanine and cytosine
gp	guinea pig
h	hour(s)
hyp.	hypothetical
H_2O	water
HCl	hydrochloric acid
His	histidine
HRP	horseradish peroxidase
i.e.	*id est* (lat., "that is")
IF	immunofluorescence
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
k	kilo (10^3)
KBL	kilobase-ladder (DNA length standard)

KCl	potassium acetate
l	liter(s)
lb	large bleeding
LB	Luria Bertani
LRR	leucine-rich repeat
m	milli (10^{-3})
M	molar
MHC	major histocompatibility complex
MIF	microimmunofluorescence
min	minute(s)
n	nano (10^{-9})
NaOH	sodium hydroxide
NC	negative control
NCBI	National Center for Biotechnology Information
o/n	overnight
OD_x	optical density, meassured at a wavelength of x nm
ORF	open reading frame
p.a.	*pro analyticum* (lat., “for analysis”), grade of purity
PAA	poly-acrylamide
PAGE	Polyacrylamide-gelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
pi	pre-immune
PI	pathogenicity island
pI	isoelectric point
R	reverse (used for labeling of primers)
rab	rabbit
RB	reticulate body
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute

rRNA	ribosomal RNA
RT	room temperature; reverse transcription
sb	small bleeding
SPG	sucrose-phosphate-glutamate
SDS	sodium dodecyl sulfate
sp.	species (singular)
spp.	species (plural)
Taq	thermostable DNA-polymerase from *Thermus aquaticus*
TBE	Tris-boric acid-EDTA
TEMED	N,N,N',N'-tetra-methyl-ethylene-di-amine
Temp.	temperature
tRNA	transfer ribonucleic acid
TSA	tyramide signal amplification
U	uracil
UV	ultraviolet
UV-7	University of Vienna isolate 7
UWE25	University of Washington environmental isolate 25
w/v	weight per volume

H. Literature

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I. Acknowledgements

I kindly want to thank the following people:

- Michael Wagner for giving me the opportunity to carry out my diploma thesis at his department and for the relaxed and amicable atmosphere at DOME
- Matthias Horn for the opportunity to plunge into the world of bacteria, his support and patience
- My supervisor Elena for the challenge, support and the great time
- Schmitzi, for knowing everything, his time-saving "home-made" protocols and for always having time
- Roland (who will hopefully not forget to thank me in his Nobel laureate speech for always believing in him) for his friendship, his humour and encouragement
- Dulli for all the fun time and for the unforgotten "Fürstenfeld"-duet with Roland
- Gerti for lending me her clever little index cards and for helping me find my way around the lab
- Christian, "the Baron", for sharing his computer and his great sense of humour
- All other members of the DOME: Alex, Holger, Susi, Eva, Lena, Barbara, Regina, Frank, Basti, Kilian, Ivan, Astrid, Silvia, Christian H., Christian K., Chrissi, Kristin, Stefan, Doris, Sergej, Steffi, Ingo, Manuel and Ingrid for a "working"-atmosphere which could hardly be better.

I would also like to thank my colleagues at the VDRC for the smooth entry into working life: Reini, Thomas, Michi, Lisa, Virginia, Judith, Andi, Joanna, Florence, Selen, Rainer, Svetlana, the Babsis, the Renés and the guys from KK and VT.

Big thanks to my friends for the serious and the fun times spent together and for their support

- the girls: Ursi, Jutta, Johanna, Liz, Sharis
- the boys: David R., Manuel, Markus

I would like to thank David for his support, patience and encouragement; Batman and Emma for running over the keyboard, so I can blame any spelling error on them ☺

Most of all I want to thank my father for his love and support, and my grandmother who will always be in my heart.

J. Curriculum vitae

Irina Kolarov

Personal data

Date of birth	August 6, 1979
Place of birth	Lom, Bulgaria
Nationality	Austrian

Education

Juni 1998	high-school diploma (Matura) at the Gymnasium der Kreuzschwestern Gmunden, Austria
Since October 1998	study of biology at the University Of Vienna, Austria, with focus on ecology
2007 – 2008	diploma work at the Department of Microbial Ecology Center, University of Vienna

Publications

Heinz, E., **Kolarov, I**., Kastner, C., Toenshoff, E.R., Wagner, M., and Horn, M. (2007) An Acanthamoeba sp. containing two phylogenetically different bacterial endosymbionts. *Environ Microbiol* **9**: 1604-1609.