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Titel der Diplomarbeit

Characterization of a novel porin from *Protochlamydia amoebophila*

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1. Introduction

1.1 Symbiosis

Microorganisms arose long before the appearance of the first multicellular organisms. When latter came into existence, their bodies provided new, persistent, stable and nutrient rich habitats for the microbes. As a result, large organisms have been inhabited by microorganisms at all times during their evolution (79). The most important of these interactions is probably the evolution of the eukaryotic organelles. More than 1.5 billion years ago, the cyanobacterial and alpha – proteobacterial ancestors of today's plastids and mitochondria established a stable symbiosis with their host cell, thereby giving rise to modern eukaryotes (72).

The term „symbiosis" was defined by de Bary in 1879 (24) as „the living together of two differently named organisms", independent of effects on the organisms involved and thereby including mutualism, parasitism and commensalism. A microbial symbiont can evolve to benefit its host, because by survival of its host it ensures survival of its current habitat. On the other hand symbiosis might also result in damage to the host (101). Today, a huge number of symbioses between eukaryotes of different hierarchical levels and bacteria is known, indicating the tremendous importance of symbiotic interactions for life on Earth.

1.2 The discovery of novel chlamydia-like bacteria as symbionts of amoebae

Free-living amoebae are ubiquitous protozoa that feed on a wide variety of microorganisms, including bacteria. However, some bacteria have developed strategies to survive uptake by amoebae and to multiply within them. Amoebae are therefore considered as "Trojan horses of the microbial world", acting as vectors and reservoirs for several bacteria, including numerous human pathogens, such as *Legionella pneumophila* and *Listeria monocytogenes* (52). In a study by Fritsche *et al*. (34), about 25% of studied *Acanthamoeba* isolates were shown to harbour intracellular bacteria. As most of these bacteria could not be isolated and cultivated in cell-free media, they were considered as obligate intracellular endosymbionts. In 1997, chlamydia-like bacteria were found as

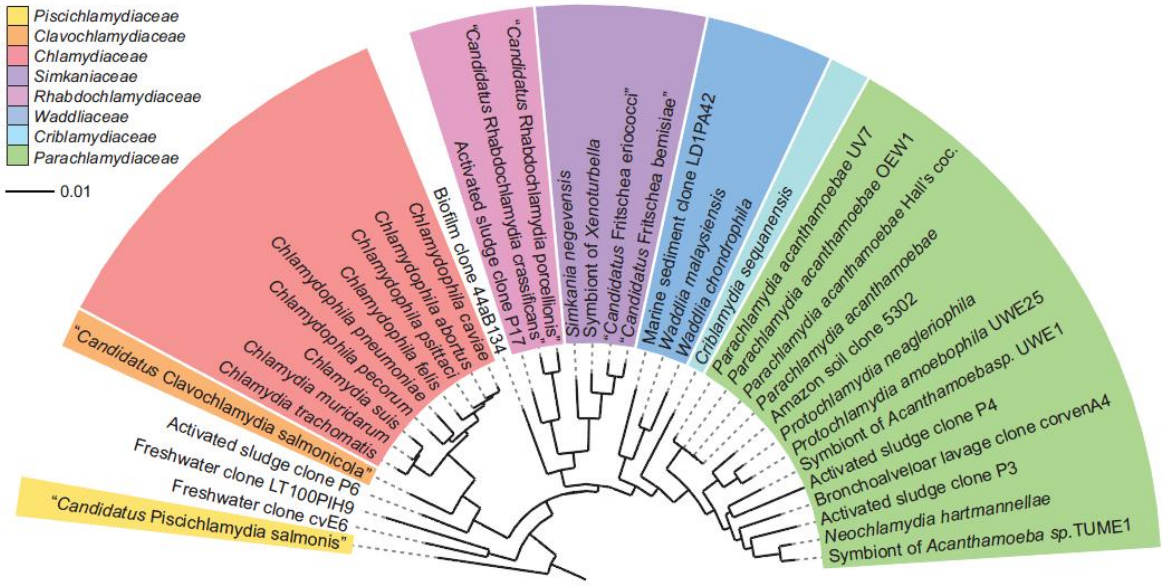

Fig. 1: Diversity in the phylum *Chlamydiae*. Phylogenetic tree of known chlamydiae based on comparative 16S rRNA analysis. From (50)

endosymbionts in amoebae (3, 12) and a later study identified 5% of amoeba endosymbionts as chlamydia-like bacteria (35). When additionally new chlamydia-like bacteria were found as contaminants in a laboratory cell culture (62) and in an aborted bovine foetus (95), this changed the picture of chlamydial diversity dramatically.

Before the discovery of these chlamydia-like bacteria, chlamydiae were believed to be a small group of closely related pathogens that formed a phylogenetically well separated phylum in the domain bacteria that contained a single family, the *Chlamydiaceae*, within a single order, the *Chlamydiales*. This family harbours important pathogens like *Chlamydia trachomatis*, the most frequently sexually transmitted pathogen (118) and the world's leading cause of preventable blindness (119), or *Chlamydophila pneumoniae*, causative agent of human respiratory diseases, which is suggested to play also a role in artheriosclerosis and coronary heart disease (8, 66).

The discovery of novel chlamydia-like bacteria, also termed 'environmental chlamydiae' in order to distinguish them from the *Chlamydiaceae* or 'clinical chlamydiae', lead to a significant expansion of the phylum *Chlamydiae*. Based on 16S rRNA sequence analysis, it currently contains eight families. Additionally to the *Chlamydiaceae*, the order *Chlamydiales* now consists of the families *Parachlamydiaceae*, *Waddliaceae*, *Simkaniaceae*, *Rhabdochlamydiaceae*, *Piscichlamydiaceae*, *Clavochlamydiaceae* and

Criblamydiaceae (Fig. 1). As the diversity is thought to be even larger based on detection of phylogenetically diverse rRNA sequences most similar to known chlamydiae in various habitats, the number of families might even increase in the next years (50, 53)

The pathogenic potential of environmental chlamydiae is so far not clear. Although there is some evidence that environmental chlamydiae might be involved in human diseases (5, 21) (42), more research is needed to show a causal relationship between chlamydia-like bacteria and human disease. However, there is considerable evidence for environmental chlamydiae infecting animals and thereby causing abortion in cattle (94) and epitheliocystis in fish (28, 63).

When the genome of *Protochlamydia amoebophila*, an amoeba endosymbiont, was sequenced in 2004, it was found to be nearly twice as large as that of clinical chlamydiae. The genome of *P. amoebophila* was found to harbour reduced central metabolic and biosynthetic pathways, comparable to genomes of clinical chlamydiae and other obligate intracellular bacteria, but to encode a complete tricarboxylic acid cycle in contrast to chlinical chlamydiae. Comparative genome analysis of members of the clinical chlamydiae and this member of the environmental chlamydiae showed that their last common ancestor lived about 700 million years ago and was already adapted to intracellular survival in early eukaryotes (51). Recent studies indicate that this ancestor might also have played a fundamental role in the evolution of plastids. The discovery of genes of chlamydial origin in plant genomes and a high number of plant- and cyanobacteria-like genes in chlamydial genomes lead to the suggestion that chlamydiae were involved in establishing a stable interaction between the ancestor of plants and its cyanobacterial symbiont, thereby giving rise to modern plants (56, 82).

The chlamydial developmental cycle

All known members of the phylum *Chlamydiae* share a characteristical biphasic lifecycle that alternates between an infectious extracellular form termed elementary body (EB) and an intracellular replicative form, the reticulate body (RB) (reviewed in 1). EBs are metabolically inactive and have a very rigid, osmotically stable cell wall that allows survival in harsh environments. In contrast, RBs are metabolically active and more fragile than EBs (43). In addition to these two developmental forms, the existence of crescent bodies (CB) has been suggested for *Parachlamydia acanthamoeba*. This form is regarded

as an additional infective developmental stage, whose presence has been associated with prolonged incubation time (40)

At the beginning of the developmental cycle an infectious EB attaches to a host cell and is taken up by the host. Upon entry, chlamydiae reside within a host-derived vacuole termed inclusion. In this inclusion, the EBs differentiate into RBs, which divide by binary fission. After several rounds of replication, RBs re-differentiate into EBs and leave the host cell by either lysis of the host or exocytosis (59) in order to infect new host cells (Fig. 2).

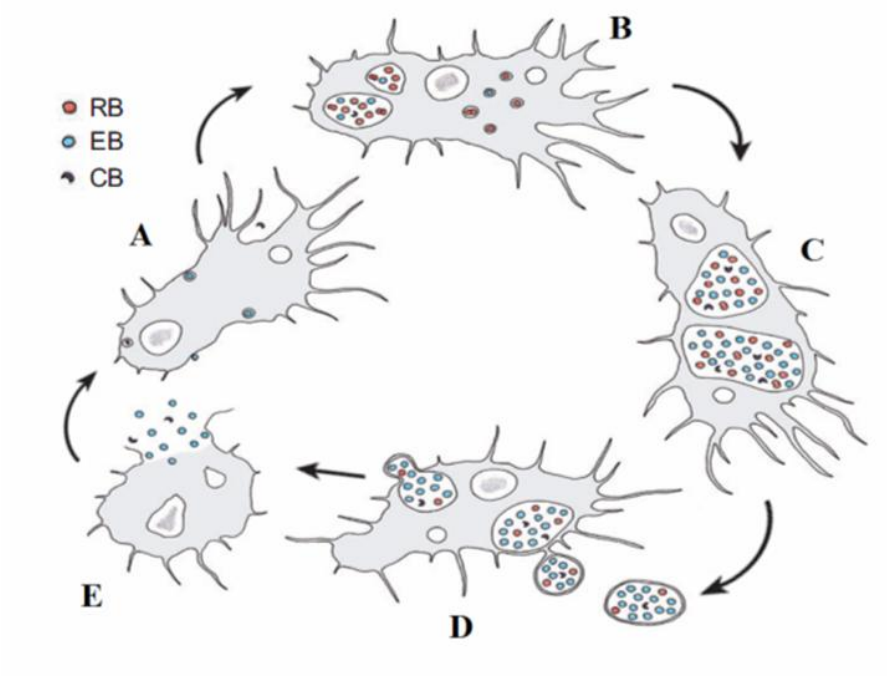

Fig. 2: The developmental cycle of chlamydia–like bacteria. Infectious EBs attach to their host cell and are internalized (A). Inside the host cell, EBs differentiate to RBs and multiply within large or single cell inclusions (B). After re-differentiation to EBs (C), chlamydiae leave their host by exocytosis or lysis of the cell (D and E). Modified from (50).

1.4 The chlamydial inclusion

The vacuole in which chlamydiae reside within their host cell is unique among intracellular bacteria and has been studied intensively for members of the *Chlamydiaceae*, but not for other members of the *Chlamydiales*.

After uptake of the bacteria, the chlamydial inclusion does not enter the endocytic pathway, but separates itself from this pathway at an early stage of development (98). Rab GTPases, known regulators of intracellular trafficking, were shown to be recruited to the chlamydial inclusion and might be involved in the escape from the endocytic pathway (23, 96). Expansion of the inclusion occurs by hi-jacking host-vesicles containing

sphingomyelin (41) and recently also transport of lipid droplets to the inclusion membrane has been shown (17). Active participation of chlamydiae is needed during these events, as the uptake of sphingomyelin and the escape from the endocytic pathway require chlamydial gene expression (99).

As chlamydiae live intracellularly, they depend on their host for the acquisition of nutrients. Fusion of the inclusion with vesicles of the phagolysosomal pathway is thought to be the main source for nutrients. All these interactions of chlamydiae with their host cell highlight the necessity of communication and transport mechanisms between bacteria and host cell across the barrier of the inclusion membrane.

Rockey *et al.* (93) identified the first chlamydial protein in the membrane of the host-derived inclusion, IncA, in 1995. Besides IncA, several other inclusio nmembrane proteins (Incs) have been identified in the last years by *in silico* approaches and for a subset of these proteins, localization in the inclusion membrane has been demonstrated (e.g. 68, 69). All Inc-proteins known so far share no discernable homology on the amino-acid level, but contain a characteristic bilobed hydrophobic domain of 50-80 amino acids. They are thought to be injected in the chlamydial inclusion membrane by the type-three-secretion system that is found in all chlamydiae (108).

A function is only known for few of these proteins. IncA plays a role in the fusion of chlamydial inclusions (26), probably by means of a SNARE-like-motif that was identified in this protein (26, 27). This case describes the first SNARE mimicry identified in a bacterium and supports the role of IncA for the fusion of inclusions, as SNARE signatures are known to play an essential role in targeting and fusion of vesicles in eukaryotes. Two Inc-proteins were reported to interact with host-cell rab GTPases, suggesting a role of these proteins in intracellular trafficking (23, 96). IncG is known to bind to the host-protein 14-3-3-beta, an interaction that is speculated to inhibit apoptosis of cells infected with *C. trachomatis* (100, 112).

Recently, putative Inc-proteins have also been identified in the chlamydia-like bacterium *P. amoebophila* by *in silico* analysis (47).

Although the function of most Inc-proteins is not known, these proteins seem to be central regulators of bacteria-host interactions and play an important role in communication between chlamydiae and their host cells and thereby in survival of chlamydiae within their host.

1.5 The chlamydial cell envelope

Chlamydiae have a cell envelope similar to that of other Gram-negative bacteria, consisting of an inner membrane, a periplasmic space and an outer membrane.

In contrast to other Gram-negative bacteria, chlamydiae seem to lack peptidoglycan, although the presence or absence of peptidoglycan is controversially discussed (75). Genome analysis showed the presence of a truncated pathway for the synthesis of peptidoglycan. Transcription of the genes of this pathway was shown by microarray analysis (73). Additionally, chlamydiae are susceptible to treatment with antibiotics that inhibit the peptidoglycan synthesis (81). But until now, detection of N-acetylmuramic acid, a key constituent of peptidoglycan, has not been possible in chlamydiae (75). Stability is thought to be conferred to chlamydial cell walls by cysteine-rich proteins of the outer membrane instead. These proteins are regarded as functional equivalent of peptidoglycan in chlamydiae (43) and are discussed below in further detail.

1.5.1 Outer membrane proteins of *Chlamydiaceae*

Proteins in the outer membrane are of great importance as they are involved in attachment to the host cell, uptake of nutrients, removal of waste and in case of the chlamydiae also in the differentness of EBs and RBs.

In the *Chlamydiaceae*, the two cysteine-rich proteins OmcB (60 kDa) and OmcA (12 kDa) are thought to provide rigidity to the cell wall of the EB and to render it osmotically stable. These two proteins harbour a high number of cysteines that are extensively cross linked by disulfide bridges in the EB cell envelope. Upon entry of the EB into the host cell, these disulfide bridges are reduced, resulting in osmotically fragile RBs (44). This mechanism is of great importance to chlamydiae as it allows them to divide intracellularly, when cross links are reduced, and to survive extracellularly when proteins are cross linked (43). Cross linking during conversion from RBs to EBs is thought to occur either spontaneously when the host cell is lysed (45) or late in the developmental cycle by an enzymatic reaction before host cell lysis (88), but the exact mechanism is not known.

The small cysteine-rich protein OmcA is a lipoprotein and thought to be anchored in the inner leaflet of the outer membrane (43). The large cysteine-rich protein, OmcB, was long thought to be localized to the periplasm, where it was proposed to form a stabilizing lattice by inter- and intramolecular disulfide bridges (83, 88). Therefore, it was not

regarded as a true member of the outer membrane, an assumption encouraged by its solubility in sarcosyl in contrast to other outer membrane proteins of *Chlamydiaceae* (30). However, there is strong evidence now for a localization of OmcB to the outer membrane of *C. trachomatis* and *C. pneumoniae* and involvement of this protein in heparin-binding on the surface of eukaryotic cells (31, 78, 103).

Other well characterized outer membrane proteins are the major outer membrane protein (MOMP) that forms the chlamydial outer membrane complex (COMC) together with OmcA and OmcB and is described in more detail below, and the members of the polymorphic outer membrane protein (POMP) family.

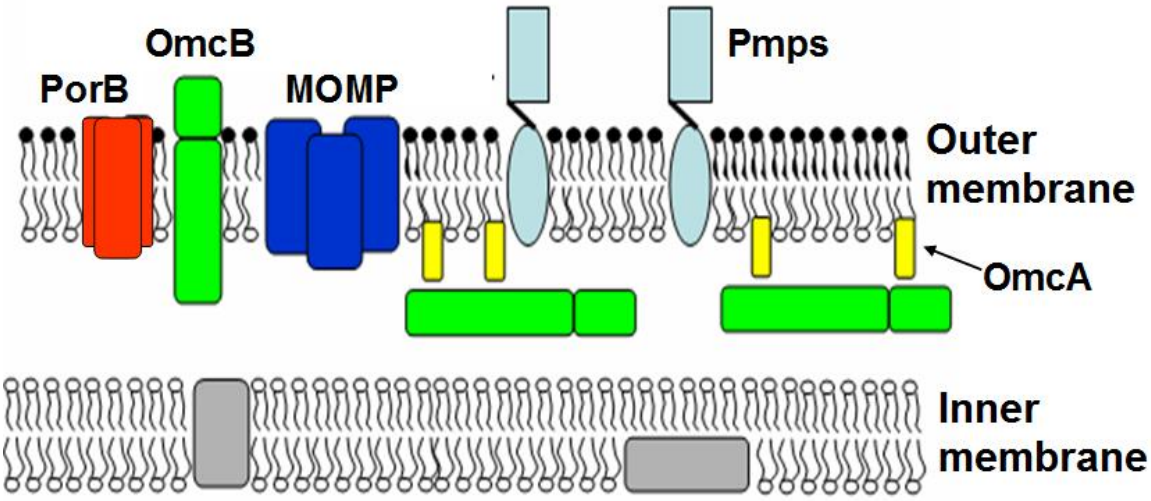

Fig. 3: Model of the cell envelope of *Chlamydiaceae*. OmcB is shown to be localized in the periplasmic space and the outer membrane. Modified from (47).

The chlamydial POMPs are a family of autotransporters (48) with heterologous primary amino acid sequence that can be recognized by two repeated sequence motifs. The unusually high polymorphism of these proteins is suggested to be due to selective pressure from the immune system (37) and might contribute to tissue specificity of different *C. trachomatis* disease groups (38, 105). Members of this family were shown to play a role in attachment to host cells (115) and in expansion of the chlamydial inclusion (61).

1.5.2 The major outer membrane protein

The outer membrane provides a barrier against harmful environmental factors, but also a barrier to the uptake of nutrients into the cell and the removal of waste from the cell. Permeability of the membrane is conferred by specific and unspecific transporters.

Unspecific transporters are termed porins. Porins are water-filled channels that allow the passage of small molecules with an exclusion limit of typically 600 Da and extremes of 5,000 Da, showing a transport rate proportional to the concentration gradient (117).

The major component of the chlamydial outer membrane is the major outer membrane protein (MOMP), a porin that is thought to make up about 60% of the chlamydial outer membrane (15). This 40 kDa protein has been intensively studied as it was shown to be an important chlamydial antigen that elicits a strong immune response (15). In EBs, MOMP is highly cross linked and mostly present as a trimer, whereas it is found mostly in its monomeric form in RBs (44). It has been suggest for a long time, that trimers of MOMP are held together by disulfide bonds (87). More recent studies suggest that although disulfide bonds are present between peptides of the trimers, they are not required for its folding, its outer membrane insertion or to stabilize it (32, 110).

The porin function of MOMP in different strains has been demonstrated by different approaches. Bavoil *et al.* (6) were the first to show that preparations of the COMC have porin activity by liposome swelling assays. Opening of the pore appeared to be regulated by disulfide bonds, because treatment of the isolated COMC with reducing agents resulted in a strong increase in channel activity, a mechanism also thought to occur after entry of chlamydiae into the host cell. The function of MOMP as porin was confirmed by lipid bilayer measurements using purified native and recombinant MOMP (32, 110, 121), but opening of the pore by reducing agents could not be shown for these preparations. This suggests that the effect observed by Bavoil was either due to other porins present in the purified COMC or to disulfide bonds formed between MOMP and other members of the outer membrane that are not present in preparations of purified MOMP.

MOMP forms a pore consisting of 16 antiparallel beta-sheets that form a barrel-like structure typical of porins of Gram-negative bacteria (110). MOMP is found in trimeric conformation in the outer membrane (74, 121). The barrel consists of five well conserved transmembrane domains that are connected to each other by four variable, surface exposed loops (4, 126). Removal of these loops has no influence on folding or incorporation of MOMP into the outer membrane of *Escherichia coli*, in contrast to removal of the transmembrane domains (32, 57). The four variable domains that are exposed to the surface show great variations in sequence (102) and reaction of MOMP with different antibodies targeting these variable regions is used for serovar typing in *C. trachomatis* (113, 126).

Antibodies against the major outer membrane protein were reported to neutralize infectivity of chlamydiae (16, 92, 106) and to protect mice from abortion after infection with chlamydiae (25, 91). Although it seems to be a promising candidate, development of a vaccine based on MOMP has not been successful yet. Vaccines based on recombinant and therefore non-native MOMP result in only partial protection (91).Vaccines containing native MOMP have been shown to be protective against chlamydial disease (91, 111), but high variation in parts of MOMP-sequence leads to difficulties in preparing a general vaccine and additionally, isolation of high quantities of the native form of MOMP is not practicable at the moment.

1.5.3 PorB

In 2000, a protein with weak sequence similarity to MOMP was identified in the genome of *C. trachomatis*. Localization of this protein to the chlamydial outer membrane complex and pore-forming activity of this protein, termed PorB, was demonstrated. Diffusion of sugars and amino acids was not as efficient for PorB as for MOMP. As PorB was only observed in small amounts in the COMC, the authors of the study suggested a more specific function of this porin compared to MOMP. Although PorB is present in much smaller amounts than MOMP, antibodies targeting this protein neutralized infectivity of *C. trachomatis* in vitro (65).

1.5.4 Outer membrane proteins of *P. amoebophila*

In contrast to *Chlamydiaceae*, little is known about the composition of the outer membrane of chlamydia-like bacteria. After sequencing the genome of the first member of the environmental chlamydiae in 2004 (51), only a homologue of the large cysteine-rich protein OmcB was found. An OmcA homologue was detected due to its position in the genome and the high amount of cysteines in this gene. No homologue of MOMP, PorB or members of the POMPs were found in *P. amoebophila*.

A recent study identified 38 outer membrane proteins of *P. amoebophila* by combining 1D and 2D gel electrophoresis of outer membrane fractions with mass spectrometry (47). The identified proteins included the *P. amoebophila* OmcB, pc0616, and OmcA, pc0617. In this study, a putative porin family was identified consisting of four proteins. Proteins of this family share an amino acid sequence identity of 22-28 % and have no homologues in other organisms except for *P. acanthamoeba*, a close relative of *P. amoebophila*. Two of

these proteins, pc1489 and pc1077, were present in high amounts in outer membrane fractions. Both were predicted to form a beta-barrel by *in silico* analysis and found to encode predicted signal peptides. Based on their high abundance and their putative function as porins, these two proteins were suggested to act as a functional replacement for MOMP in *P. amoebophila*, and form the COMC of *P. amoebophila* by interactions with pc0616 and pc0617. Pc0978 could be an additional part of the COMC, as it was found as frequently as pc0616 in outer membrane preparations and has a high number of cysteines. Therefore, it might be involved in disulfide-cross linking with other proteins to provide stability to the outer membrane of *P. amoebophila*.

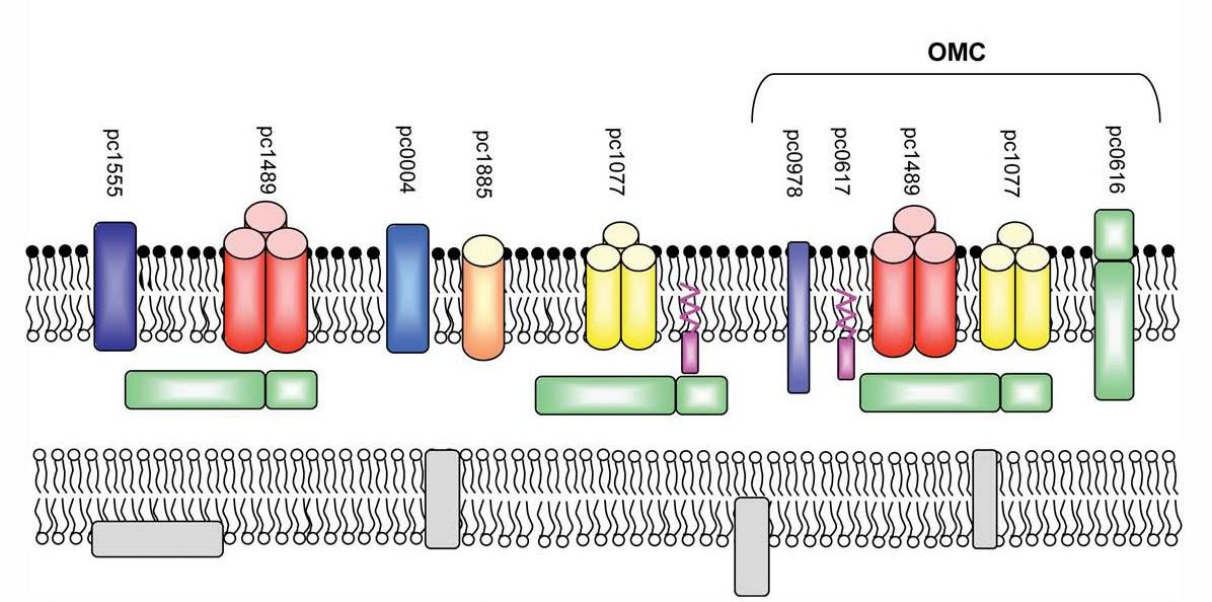

Fig. 4: Model of the outer membrane of *P. amoebophila* as proposed by Eva Heinz (47).

1. 6 Aims of this study

The outer membrane of bacteria plays an important role in the attachment to host cells, uptake of substances from the host cell and in the case of chlamydiae also in the differentness between EBs and RBs.

The major outer membrane protein is the most abundant component in the cell envelope of members of the *Chlamydiaceae*. The outer membrane of *P. amoebophila*, a member of the recently discovered environmental chlamydiae, seems to differ substantially from that of *Chlamydiaceae*. The most interesting feature is the absence of almost all main components of the chlamydial outer membrane, including MOMP and POMPs. MOMP was suggested to be replaced by the two putative porins pc1077 and pc1489. *In silico*

analysis predicted the presence of a signal peptide and localization to the outer membrane for these two proteins. Structure prediction indicated formation of a beta-barrel structure, a typical feature of porins of Gram-negative bacteria (47).
The aim of this study was to characterize the putative porin pc1489 concerning its localization, its expression during the developmental cycle and its putative function as a porin in *P. amoebophila*.

In the second part of this study, the localization of putative Inc-proteins of *P. amoebophila* was investigated. Proteins in the inclusion membrane of *P. amoebophila* are of exceptionally great interest, as this organism forms single cell inclusions in contrast to large inclusions containing many bacteria observed in members of the *Chlamydiaceae*. It is quite likely that differences in Inc proteins account for this. Additionally, Inc proteins might play an important role in survival of *P. amoebophila* in amoebae after internalization.

2. Material and Methods

2.1 Technical equipment

Table 1: Technical equipment used in this study

Equipment	Company
Agarose gel electrophoresis apparature Sub–Cell GT	Bio-Rad Laboratories GmbH, Munich, Germany
CCD camera AxioCam HRc	Carl Zeiss MicroImaging GmbH, Jena, Germany
Centrifuges: Centrifuge 5840 R OptimaTM L-100 XP ultracentrifuge	 Eppendorf AG, Hamburg, Germany Beckman Coulter, Inc., Palo Alto, CA, USA
Concentrator 5301	Eppendorf AG, Hamburg, Germany
Electroporator Micro PulserTM	Bio-Rad Laboratories GmbH, Munich, Germany
Devices for gelelectrophoresis: Electrophoresis cell (Sub-Cell GT) Electrophoresis power supply (PowerPac Basic)	 Bio-Rad Laboratories GmbH, Munich, Germany Bio-Rad Laboratories GmbH, Munich, Germany
icycler Thermal cycler	Bio-Rad Laboratories GmbH, Munich, Germany
Microbiological incubator KB 115	Binder GmbH, Tuttlingen, Germany
Laminar flow hood, model 1.8	Holten, Jouan Nordic, Allerod, Denmark
Magnetic stirrer RCT basic	IKA® Werke GmbH & Co.KG, Staufen, Germany
Microscopes: Inverse microscope Axiovert 25 Epifluorescence microscope Axioplan 2 imaging Confocal laser scanning microscope LSM 510 Meta	 Carl Zeiss MicroImaging GmbH, Jena, Germany Carl Zeiss MicroImaging GmbH, Jena, Germany Carl Zeiss MicroImaging GmbH, Jena, Germany
Mixing Block MB-102	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
NanoDrop® ND-1000 UV/Vis spectrophotometer	NanoDrop Technologies Inc., Wilmington, DE, USA
Neubauer counting chamber	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
pH meter inoLab pH Level 1	Wissenschaftlich technische Werkstätten (WTW) GmbH & Co.KG, Weilheim, Germany
Scales: OHAUS® Analytical Plus balance Sartorius BL 3100	 Ohaus Corporation, Pine Brook, NJ, USA Sartorius AG, Göttingen, Germany
ScannerEpson Expression 1680 Pro	Epson Deutschland GmbH, Meerbusch, Germany
Devices for SDS-PAGE analysis:	

Mini-PROTEAN Casting Stand Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories GmbH, Munich, Germany Bio-Rad Laboratories GmbH, Munich, Germany
Sonicator Bandelin Sonoplus HD2070	Bandelin electronic GmbH & Co.KG, Berlin, Germany
Sonotrode Bandelin Sonoplus UW 2070	Bandelin electronic GmbH & Co.KG, Berlin, Germany
Spectral photometer SmartSpec™ 3000	Bio-Rad Laboratories GmbH, Munich, Germany
TE77 semi-dry transfer unit	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Thermostatic circulator MultiTemp™ III	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Transilluminator	Biostep GmbH, Jahnsdorf, Germany
Ultrasonic Cleaner SC100T	VWR International bvba/sprl, Leuven, Belgium
UV sterilizing PCR workstation	PeqLab Biotechnologie GmbH, Erlangen, Germany
Water baths: DC10 GFL® type 1004	 Thermo Haake GmbH, Karlsruhe, Germany Gesellschaft für Labortechnik GmbH, Burgwedel, Germany
Water purification system MILLI-Q® biocel	Millipore GmbH, Vienna, Austria
Watervapour high pressure autoclaves: Varioclav® 135 S h+P Varioclav® 25 T H+P	 H+P Labortechnik GmbH, Oberschleißheim, Germany H+P Labortechnik GmbH, Oberschleißheim, Germany

2.2 Expendable items and kits

Table 2: Expandable items used in this study

Expandable item	Company
25 cm^2 Tissue culture flasks	Asahi Techno Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
500 cm^2 Tissue culture flasks	Nunc, Roskilde, Denmark
Cellulose acetate membrane filters (0.45 µm pore size, 25 mm diameter)	Sartorius Stedim Biotech GmbH, Göttingen, Germany
Cover glasses 24 x 60 mm	Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany
Electroporation cuvette (0.2 cm)	Bio-Rad Laboratories GmbH, Munich, Germany
Glass beads (0,75-1.0 mm)	Macherey-Nagel GmbH&Co.KG, Karlsruhe, Germany
Greiner tubes (15 ml, 50 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Isopore™ polycarbonate membrane filters (0.22 µm pore size, 25 mm diameter, black)	Millipore GmbH, Vienna, Austria

Microscope slides (76 x 26 mm)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Microscope slides, 10 well	Paul Marienfeld GmbH & Co KG, Lauda-Königshofen, Germany
Multiwell dishes, polystyrene (24 wells)	Nunc, Roskilde, Denmark

Needles Sterican® (ø 0,45 x 25 mm, ø 0,90 x 40 mm), single use, sterile	B.Braun Melsungen AG, Melsungen, Germany
PCR tubes (0.2 ml)	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Pipette Tipps (various sizes)	Carl Roth GMbH Co.KG, Karlsruhe, Germany
Plastic cuvettes	Greiner Bio-One GmbH, Frickenhausen, Germany
Plastic pipettes (10 ml, 2 ml), single use, sterile	Barloworld Scientific Ltd., Staffordshire, UK
Polyvinylidene fluoride membrane (Hydrobond P)	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Reaction tube (1.5 ml)	GenXpress Service & Vertriebs GmbH, Wiener Neudorf, Austria
Reaction tube (2 ml)	Greiner Bio-One GmbH, Frickenhausen, Germany
Round coverslips (12 mm diameter)	Carl Roth GmbH & Co., Karlsruhe, Germany
Syringe (1 ml) Inject® - F 1ml, single use, sterile	B.Braun Melsungen AG, Melsungen, Germany
Syringe (5 ml) Omnifix® single use, sterile	B.Braun Melsungen AG, Melsungen, Germany
Syringe filter, single use, sterile, 0.20 µm pore size	Asahi Techni Glass Corporation, Iwaki Glass Co., Ltd., Funabashi-City, Japan
Syringe filter, single use, sterile, 1.20 µm pore size	Sartorius AG, Goettingen, Germany
Ultracentrifuge tubes	Beckham Coulter, Inc. Paolo Alto, CA, USA
Whatman® Chromatography Paper 3MM Chr	Whatman Internation Ltd., Maidstone, UK

Table 3:Kits used in this study

Kit	Company
DNeasy Blood & Tissue Kit	QIAgen, Hilden, Germany
BCA Protein Assay Kit	Thermo Scientific Pierce Protein Research Products, Waltham, Massachusetts, USA
Western Lightning Chemiluminesence Plus Kit	Perkin Elmer, Waltham, Massachusetts, USA
HiTrap™ IgY Purification HP Column	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Qiaquick PCR purification Kit	QIAgen, Hilden, Germany
Vivapure Q Mini H Spin column	Sartorius AG, Göttingen, Germany

2.3 Chemicals and enzymes

Table 4: Chemicals used in this study

Chemical	Company
4´,6-diamidino-2-phenylindole (DAPI)	Laktan Chemikalien und Laborgeräte GmbH, Graz, Austria
Aceotnitrile	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Acetic acid	Carl Roth GmbH & Co.KG, Karlsruhe, Germany
LE Agarose	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Ammonium bicarbonate	Fluka Chemie AG, Buchs, Switzerland
Ammonium persulfate (APS)	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Ampicillin	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Boric acid	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Bovine serum albumin (BSA)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Brilliant Blue G-250	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Brilliant Blue R-250	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Bromphenol blue	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Calcium chloride dihydrate	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Citifluor AF1	Agar Scientific Ltd., Stansted, UK
Coomassie brillant blue G-250	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Disodiumhydrogenphosphate dihydrate (Na_2HPO_4 x $2H_20$)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Dithiothreitol (DTT)	Fluka Chemie AG, Buchs, Switzerland
Ethanol absolute	AustrAlco Österreichische Alkoholhandels GmbH, Spillern, Austria
Ethidium bromide (EtBr)	Fluka Chemie AG, Buchs, Switzerland
Ethylenediamine-tetraaceticacid (EDTA)	Carl Roth GmbH & Co KG, Karlsruhe, Germany
Ferrous ammonium sulfate hexahydrate	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Ficoll® 400	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Formaldehyde (37% (w/w))	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Gastrografin®	Schering Austria GmbH, Vienna, Austria
Glucose	Carl Roth GmbH Co.KG, Karlsruhe, Germany

Glycerol (87% (w/v))	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Glycine	Carl Roth GmbH Co.KG, Karlsruhe, Germany
HEPES	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Hydrochloric acid (HCl) (37% (w/w))	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Iodacetamide	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Isopropanol	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Magnesium sulfate heptahydrate	Merck GmbH, Vienna, Austria
Methanol	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Moviol 4-88	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
N,N,N´,N´-tetramethylethane-1,2-diamine (TEMED)	Fluka Chemie AG, Buchs, Switzerland
N-octyl polyoxyethylene (n-octyl POE)	BACHEM BIOCHEMICA GmbH, Heidelberg, Germany
Paraformaldehyde (PFA)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Proteose peptone	Oxoid Ltd., Hampshire, England
Rotiphorese® NF-Acrylamide/ Bisacrylamide-solution 30 % (29:1)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Sodium dihydrogen phosphate	Mallinckrodt Baker B.V., Deventer, Holland
Sodium dodecly sulfate (SDS)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Sodium hydroxide (NaOH)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Sucrose	Merck KGaA, Darmstadt, Germany
Trifluoretic acid	Fluka Chemie AG, Buchs, Switzerland
Trishydroxymethlaminomethane (Tris-HCl)	Carl Roth GmbH Co.KG, Karlsruhe, Germany
Trypticase Soy Broth	Oxoid Ltd., Hampshire, England
Trypsin gold powder	Promega Corporation, Madison, USA
Tween™ 20	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Xylene cyanole FF	Sigma-Aldrich Chemie GmbH, Steinheim, Germany
Yeast extract	Oxoid Ltd., Hampshire, England

Table 5: Enzymes and and corresponding enzyme buffers used in this study

Enzyme	Company
Benzonase® Nuclease	Novagen, Darmstatd, Germany
Buffer O	Fermentas Inc., Hanover, MD, USA
Lysozyme human (≥ 100 U/µg)	Sigma-Aldrich Chemie GmbH, Steinheim, Germany

Taq DNA Polymerase (5U/µl)	Fermentas Inc. Hanover, MD, USA
10 x Taq buffer	Fermentas Inc. Hanover, MD, USA
VspI (10 U/µl)	Fermentas Inc., Hanover, MD, USA

2.4 Organisms

Table 6: Amoeba strains and endosymbionts used in this study

Host	Endoymbiont	Source	Reference
Acanthamoeba castellanii Neff	none	American Type Culture Collection (ATCC), Manassas, VA, USA	(86)
Acanthamoeba castellanii Neff	*Protochlamydia amoebophila* UWE25	University of Washington, Seattle, USA	(19, 36)
Acanthamoeba sp. UWC1	*Parachlamydia acanthamoeba* UV-7	Wastewater treatment plant, Plattling, Germany	(18, 35)
Acanthamoeba sp. EI2	*Protochlamydia amoebophila* sp. EI2	Soil, Lower Austria, Austria	(97)

Table 7: *Escherichia coli* strains used in this study

Strain	Source	Genotype	Reference
BL21 (DE3)	Stratagene	F^-, *omp*T, *hsdS*(rB^-mB^-), *gal*, *dem*, λ(DE3)	(116)

2.5 Plasmids

For expression of proteins in *Escherichia coli* BL21 (DE3) the vector pet16b was used (Novagen, Darmstadt, Germany). *E. coli* cells harbouring the plasmids listed in table 8 were obtained from Eva Heinz (47).

Table 8: Plasmids used in this study

Plasmid	Insert	Weight of full length protein	Weight of protein fragment	Reference
pet16b – pc03991	Fragment of pc0399 (aminoacids 120 – 402)	95.9 kDa	35 kDa	(47)
pet16b – pc 0156	Fragment of pc0156 (aminoacids 1 – 180)	35.1 kDa	23.5 kDa	(47)

pet16b - pc1111	Fragment of pc1111 (aminoacids 1 – 199)	35.6 kDa	25.1 kDa	(47)
pet16b – pc0530	Fragment of pc0530 (aminoacids 78 - 252)	28.2 kDa	22.1 kDa	(47)
pet16b – pc0577	Fragment of pc0577 (aminoacids 2 - 201)	22.8 kDa	25.3 kDa*	(47)

* The higher weight of the protein fragment compared to the full length protein results from a His-Tag added to the fragment by cloning in the vector pet16b.

2.6 Antibodies

2.6.1 Primary antibodies

Table 9: Primary antibodies used in this study

Antibody	Antigen	Source of antibody	Dilution	Reference
anti-Pam	*P. amoebophila* elementary bodies	rabbit	1 : 2,000	(47)
anti-Pam	*P. amoebophila* elementary bodies	chicken	1 : 2,000	(47)
anti-pc0399-1	Fragment of pc0399 (aminoacids 120 – 402)	rabbit	1 : 1,000	(47)
anti-pc0156	Fragment of pc0156 (aminoacids 1 – 180)	rabbit	1 : 500	(47)
anti-pc1111	Fragment of pc1111 (aminoacids 1 – 199)	rabbit	1 : 100	(47)
anti-pc0530	Fragment of pc0530 (aminoacids 78 - 252)	rabbit	1 : 20	(47)
anti-pc0577	Fragment of pc0577 (aminoacids 2 - 201)	rabbit	1 : 20	(47)
preI pc0399-1 (pre-immune serum)	none	rabbit	1 : 1,000	(47)
preI pc0156 (pre-immune serum)	none	rabbit	1 : 500	(47)
preI pc1111 (pre-immune serum)	none	rabbit	1 : 100	(47)
preI pc0530 (pre-immune serum)	none	rabbit	1 : 20	(47))
preI pc0577 (pre-immune serum)	none	rabbit	1 : 20	(47)
anti-Hsp60	Hsp60 of *Chlamydophila caviae*	guinea-pig	1 : 2,000	(125)

	GPIC			
anti-pc1489	pc1489 purified from EBs	chicken	1 : 2,000	
preI pc1489 (pre-immune serum)	none	chicken	1 : 2,000	
anti-Cld	Chloride dismutase purified from "*Candidatus Nitrospira defluvii*"	rabbit	as indicated	(71)

2.6.2 Secondary antibodies

Secondary antibodies were purchased from Dianova (Hamburg, Germany) and GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Secondary antibodies used carried one of the following modifications:

- The fluorophor Indocarbocyanine (Cy3)
- The fluorophor Carbocyanine (Cy2)
- The fluorophor Indodicarbocyanine (Cy5)
- The fluorophor Fluorescein (FITC)
- The enzyme Horseraddish-Peroxidase (HRP)

Table 10: Secondary antibodies used in this study

Antibody	Antigen	Modification	Source of antibody	Dilution	Manufacturer
anti-chicken	chicken IgY	Cy3	donkey	1:1,000	Dianova
anti-chicken	chicken IgY	Cy2	donkey	1:1,000	Dianova
anti-chicken	chicken IgY	Cy5	donkey	1:1,000	Dianova
anti-chicken	chicken IgY	HRP	goat	1:10,000	Dianova
anti-guinea pig	guinea pig IgG	Cy3	goat	1:1,000	Dianova
anti-guinea pig	guinea pig IgG	Cy2	goat	1:1,000	Dianova
anti-guinea pig	guinea pig IgG	Cy5	goat	1:1,000	Dianova
anti-guinea pig	guinea pig IgG	HRP	goat	1:10,000	Dianova
anti-rabbit	rabbit IgG	Cy3	goat	1:1,000	Dianova
anti-rabbit	rabbit IgG	Cy2	goat	1:1,000	GE Healthcare Bio-Sciences AB
anti-rabbit	rabbit IgG	FITC	goat	1:1,000	Dianova
anti-rabbit	rabbit IgG	Cy5	goat	1:1000	Dianova
anti-rabbit	rabbit IgG	HRP	goat	1:10,000	Dianova

2.7 Primers

Primers were produced by Thermo Fisher Scientific GmbH (Ulm, Germany).

Table 11: Primers used for the amplification of 16SrRNA gene fragments

Primer name	Primer sequence* (5´-3´)	Size of amplified fragment	Specificity	Annealing temperature	Reference
PcR	GTC ATC RGC CYY ACC TTV SRC RYY TCT	1,260 bp	*Parachlamydiaceae, Waddliaceae*	58°C	(53)
PcF	TCA GAT TGA ATG CTG AC				
PanF	CGTGGATGAGGC ATGCRAGTCG	1,445 bp	*Chlamydiales*	65°C	(22)
PanR	GTC ATC RGC CYY ACC TTV SRC RYY TCT				
1492R	GGY TAC CTT GTT ACG ACT T	1,480 bp	*Bacteria*, but not *Chlamydiales*	52°C	(67)
616 V	AGA GTT TGA TYM TGGC				

* According to the NC-IUB the unspecified bases are represented as follows: R = G or A, Y = T or C, M = A or C, V = G or C or A, S = G or C, N = G or C or A or T;

2.8 Media and buffers

Media and buffers described here were sterilized for 20 min at 121°C and 1,013 x 10^5 Pa pressure in a watervapour-high pressure autoclave if not stated otherwise. Buffers containing ethanol or isopropanol were not autoclaved or were autoclaved before the addition of these components. If not indicated otherwise, media and buffers were stored at room temperature (RT) until further use.

If indicated in the text, buffers were filter-sterilized by passing them through a 0.2 µm filter.

2.8.1 Media for cultivation of organisms

2.8.1.1 Medium for axenic cultivation of amoebae

Trypticase Soy Broth with Yeast Extract (TSY)

Trypticase Soy Broth	30 g
Yeast extract	10 g
H_2O_{dd}	ad 1000 ml
pH 7.3	

PYG

Peptone	20 g
Glucose	18 g
Yeast extract	2 g
Sodiumcitrate	1 g
$MgSO_4$ x 7 H_2O	980 mg
Na_2HPO_4 x 7 H_2O	355 mg
KH_2PO_4	340 mg
$Fe(NH_4)_2(SO_4)2$ x 6 H_2O	20 mg
H_2O_{dd}	ad 1000 ml
pH 6.5	

2.8.1.2 Medium for cultivation of *E. coli*

Luria Bertani medium (LB medium)

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
H_2O_{dd}	ad1000 ml

pH 7.0 - 7.5

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

Antibiotics

Ampicillin stock solution (Amp)	100 mg/ml

Amp was dissolved in 50 % $EtOH_{abs}$ and stored at -20°C until further use.

Amp was added to medium to reach a final concentration of 100 µg/ml.

2.8.2 General buffers

10 x Page's Amoebic Saline (PAS)

NaCl	1.20 g
$MgSO_4$ x $7H_20$	0.04 g
$CaCl_2$ x H_20	0.04 g
NaH_2PO_4 x $2H_20$	1.78 g
KH_2PO_4	1.36 g
H_2O_{dd}	ad 1000 ml

1 x PAS

10 x PAS	100 ml
H_2O_{dd}	ad 1000 ml

PBS stock solution

Solution 1:	35.6 g/l Na_2HPO_4
Solution 2:	27.6 g/l NaH_2PO_4

pH of solution 1 was adjusted to 7.2-7.4 by adding solution 2

1 x PBS

PBS stock solution	50 ml
NaCl	7.6 g
H_2O_{dd}	ad 1000 ml

SPG-buffer

Sucrose	75 g
KH_2PO_4	0.52 g
Na_2HPO_4 x 2 H_20	2.30 g
Glutamc acid	0.75 g
H_2O_{dd}	ad l000 ml

2.8.3 Buffers, solutions and standards for gel electrophoresis

Buffers and solutions for gel electrophoresis were not autoclaved.

10 x TBE

Tris-HCl	162.0 g
Boric acid	27.5 g
EDTA	9.3 g
H_2O_{dd}	ad 1000 ml

pH 8.3 – 8.7

1 x TBE

10 x TBE	100 ml
H_2O_{dd}	ad 1000 ml

Loading buffer

Ficoll	25 % (w/v)
Bromphenol blue	0.5 % (w/v)
Xylencyanol	0.5 % (w/v)
EDTA	50 mM
H_2O_{dd}	

Ethidium bromide solution

Ethidium bromide stock solution:

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

Ethidium bromide staining solution:

Ethidium bromide stock solution diluted 1: 10,000 in H_2O_{dd}

DNA ladders

GeneRuler^{TM} 1kb (Fermentas Inc., Hanover, MD, USA)

GeneRuler™ 100 bp (Fermentas Inc., Hanover, MD, USA)

2.8.4 Buffers, solutions and standards for Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE)

Buffers and solutions for SDS-PAGE were not autoclaved, except for lower and upper buffer.

Lower buffer

Tris–HCl	90.8 g
10% (w/v) Sodium dodecyl Sulfat (SDS)	20 ml
H_2O_{dd}	ad 500 ml
pH 8.8	

Upper buffer

Tris-HCl	30.3 g
10 (w/w)% SDS	20 ml
H_2O_{dd}	ad 500 ml
pH 6.8	

10 x SDS-PAGE running buffer

Tris-HCl	30.2 g
Glycine	144 g
SDS	10 g
H_2O_{dd}	ad 1000 ml

4 x SDS - PAGE loading buffer

Tris-HCl pH 6.8	2.42 g
SDS	8 g
Bromphenolblue	0.02 g
Glycerol	40 µl
Dithiothreitol (DTT)	6.2 g
H_2O_{dd}	ad 100 ml

Coomassie brilliant blue staining solution

Methanol	50 % (v/v)
Acetic acid	10 % (v/v)
Brilliant Blue G-250	2.76 g
H_2O_{dd}	ad 1000 ml

Destaining solution

Acetic acid	5 % (v/v)
Ethanol	20 % (v/v)
H_2O_{dd}	ad 1000 ml

Colloidal Coomassie solution

$(NH_4)_2SO_4$	100 g
Orthophosphoric acid	20 g
Methanol	25 % (v/v)
Coomassie Brilliant Blue G-250	0.625 g
H_2O_{dd}	ad 1000 ml

Fixing solution

Ethanol	40 % (v/v)
Acetic acid	10 % (v/v)
H_2O_{dd}	ad 2000 ml

Protein Ladders

Unstained Protein Molecular Weight Marker (Fermentas Inc., Hanover, MD, USA)

PageRuler™ Prestained Protein Ladder (Fermentas Inc., Hanover, MD, USA)

2.8.5 Buffers and solutions for Western Blot analysis

Buffers and solutions for Western Blot analysis were not autoclaved, except for 10 x TBS.

Transfer buffer

Glycine	14.4 g
Tris-HCl	3 g
Methanol	20 % (v/v)
H_2O_{dd}	ad 1000 ml

10 x TBS

Tris-HCl	12.1 g
NaCl	43.8 g
H_2O_{dd}	ad 500 ml

pH 7.5; store at 4°C

1 x TBS

10 x TBS	100 ml
H_2O_{dd}	ad 1000 ml

store at 4°C

TBS-T

10 x TBS	50 ml
Tween 20	0.1% (v/v)
H_2O_{dd}	ad 500 ml

store at 4°C

Developer and Fixer solution

SIGMA Kodak GBX Developer

SIGMA Kodak GBX Fixer

Developer and Fixer were diluted as recommended by the manufacturer

2.8.6 Buffers for purification of pc1489

Buffer A

HEPES pH 7.5	2.9 g
NaCl	0.292 g
n-octyl–Polyoxyethylen (n-octyl-POE)	0.25 g
H_2O_{dd}	ad 500 ml

Buffer B

HEPES pH 7.5	0.29 g
NaCl	5.84 g
n-octyl–POE	0.025 g
H_2O_{dd}	ad 50 ml

300 mM Na_xPO_4

Solution 1:	53.4 g/l Na_2HPO_4
Solution 2:	41.4 g/l NaH_2PO_4

pH of solution 1 was adjusted to 6.5 by adding solution 2

3 x PEN

EDTA	0.087 g
NaCl	5.84 g
300 mM Na_xPO_4	ad 1000 ml

POP 05 – buffer

n-octyl–POE	0.5% (w/v)
3 x PEN	ad 50 ml

2.8.7 Buffers and solutions for antibody preparation and immunofluorescence

Binding Buffer

NaH_2PO_4	3.12 g
K_2SO_4	87.13 g
H_2O_{dd}	ad 1000 ml
pH 7.5	

Elution Buffer

NaH_2PO_4	3.12 g
H_2O_{dd}	ad 1000 ml
pH 7.5	

Cleaning Buffer

NaH_2PO_4	3.12 g
Isopropanol	30% (v/v)
H_2O_{dd}	ad 1000 ml
pH 7.5	

10 x PBS

Na_2HPO_4	26.8 g
NaH_2PO_4	13.8 g
NaCl	81.6 g
H_2O_{dd}	ad 1000 ml

4 % PFA

1 x PBS	25 ml
Paraformaldehyde (37% w/v)	2.7 ml

FA-Block solution

Bovine Serum Albumine (BSA)	20 g
10 x PBS	100 ml
H_2O_{dd}	ad 1000 ml

50 ml aliquots of FA-Block solution were stored at -20°C until further use. Upon thawing, FA-Block solution was stored at 4°C up to 2 weeks.

Mowiol

4 g Mowiol were mixed with 6 g glycerine and 6 ml H_2O_{dd}. 12 ml 0.2 M Tris-HCl solution (pH 8.5) was added and the solution was stirred at 50°C overnight. When the Mowiol had dissolved completely, the solution was centrifuged at 7,700 x g for 15 minutes (min) to remove air bubbles. The supernatant was collected and stored in aliquots at -20°C until further use.

2.9 Cell culture

2.9.1 Cultivation of *Acanthamoeba castellanii* Neff

Solutions

TSY medium

PYG medium

Procedure

Acanthamoeba castellanii Neff and *Acanthamoeba* sp. UWC1 isolates containing either no endosymbiont or harbouring endosymbionts were grown axenically in small or large culture flasks in 10 ml or 150 ml TSY or PYG medium. Cultures were grown at a constant temperature of 20°C in an incubator and were checked for contaminations with extracellular bacteria or fungi using an inverse microscope at regular intervals.

Medium was exchanged every one or two weeks, depending on the growth of amoebae, by pouring out the old medium in a glass bottle and adding new, fresh medium to the flasks.

2.9.2 Harvesting of amoebae

Amoeba cultures were harvested by shaking the culture flasks vigorously to detach amoebae from the bottom of the flask. The supernatant of the culture containing detached amoebae was collected in 50 ml Greiner tubes. Cell pellets were obtained by centrifugation at 7,323 x g for 5 min. TSY or PYG medium was added to the culture flasks to enable growth of remaining amoebae.

2.9.3 Cultivation of *E. coli*

Solutions

LB medium

Ampicillin stock solution

Procedure

E. coli cells were grown on LB plates or in liquid LB medium at 37°C. For culturing cells in liquid media, 5 ml of LB medium were inoculated with a single colony from a plate or cells from a glycerol stock under sterile conditions and incubated on an orbital shaker at 200 rpm. Recombinant *E. coli* cells harbouring a plasmid were grown in the presence of 100 µg/ml ampicillin to avoid the growth of cells without plasmid.
LB plates with *E. coli* colonies were stored at 4° for several weeks.

2. 10 General methods

2.10.1 Amplification of DNA fragments via Polymerase Chain Reaction (PCR)

Solutions

$MgCl_2$ (25 mM) (Fermentas Inc. Hanover, MD, USA)
Nucleotide Mix (2.5 mM each dNTP)
10 x Ex Taq polymerase buffer
Taq DNA Polymerase (5U/µl)
Forward primer (50 pmol/µl)
Reverse primer (50 pmol/µl)
$Aqua_{bidest.}$ (Mayrhofer Pharmazeutika GmbH & Co KG, Leonding, Austria)

Procedure

To avoid contaminations by external DNA, $Aqua_{bidest.}$, $MgCl_2$, 10 x Ex Taq polymerase buffer and tubes used for PCR were exposed to ultra-violet (UV) light in a UV sterilizing PCR hood for 20 min before preparing the standard reaction mixture. All pipetting steps, except for the addition of the positive control, were carried out under the PCR hood.
For amplification of DNA fragments via PCR, the following standard reaction mixture was prepared:

Standard reaction mixture

$MgCl_2$	4 µl
10 x Taq buffer Buffer	5 µl
dNTP – Mix	5 µl
Forward primer (50 pmol/µl)	1 µl
Reverse primer (50 pmol/µl)	1 µl
Template	100 ng
$Aqua_{bidest.}$	ad 50 µl

For each PCR, a positive and a negative control were included.
For the amplification of 16S rRNA gene fragments, PCR was performed at the following conditions:

PCR step	Temp. [°C]	Time	Number of cycles
Denaturation	95	90 sec	1
Denaturation	95	30 sec	35
Annealing	*	30 sec	
Elongation	72	90 sec	
Final elongation	72	420 sec	1

* for annealing temperatures, see Table 11

Obtained PCR products were analyzed quantitatively by photometric analysis and qualitatively by agarose gel electrophoresis.

2.10.2 Quantitative and qualitative analysis of nucleic acids

2.10.2.1 Quantitative, photometric analysis of nucleic acids

Concentration of nucleic acids was measured with a NanoDrop® ND-1000 spectralphotometer. 1.5 µl of nucleic acid solution was pipetted onto the end of the fibre optic cable of the NanoDrop® device. Measurements were performed at λ = 260 nm according to the instructions of the manufacturer.

2.10.2.2 Qualitative analysis of nucleic acids by agarose gel electrophoresis

Solutions

1 x TBE

Loading buffer

DNA ladder

EtBr staining solution

Procedure

Nucleic acid samples were analysed on agarose gels with concentrations of 1-2.5 % agarose (1.5 g–3.75 g agarose in 150 ml 1 x TBE). Samples were mixed with 1 volume of loading buffer and pipetted into the pockets of the gel. Nucleic acids were separated by applying a voltage of 80 – 100 V for 60 - 90 min, depending on type of analysis.

Separated nucleic acids were stained with EtBr staining solution for 30 min and visualized with a transilluminator emitting UV- light (λ = 312 nm). Band patterns were recorded and digitalised using a gel–documentation system (Biostep, Jahnsdorf, Germany).

2.10.3 Sequencing of PCR products

Certain PCR products were sequenced by Mag. Christian Baranyi using an ABI 3130*xl* DNA sequencer and the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems), which is based on Sanger's chain-termination method for sequencing. Brieflly, incorporation of ddNTPs linked to different fluorescent dyes into a newly synthezised DNA-strand results in termination of elongation. DNA-fragments of different

sizes are separated electrophoretically and recognized by a laser and the resulting chromatograph representing the DNA sequence is recorded.

For sequencing, the same primers as for PCR analysis were used at a concentration of 10 pmol/µl. Sequences were proof-read manually using the FinchTV software (Geospiza) and subjected to BLAST search using the general nucleotide collection database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the most similar sequence (2).

2.10.4 Analysis of proteins by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

2.10.4.1 Sample preparation for SDS-PAGE analysis

2.10.4.1.1 Sample preparation from cell cultures

Cell pellets were obtained by centrifugation at 17,949 x g for 2 min for *E. coli* or harvesting of amoebae at 7,323 x g for 5 min. Cells were resuspended in 1 x PBS and centrifuged as before. The obtained pellet was resuspended in 50-60 µl 4 x SDS-PAGE loading buffer, depending on the size of the pellet. Samples were heated to 95°C for 5 min to break the cells and cooled down again.

Nucleic acids present in the samples were removed by digestion with the nuclease Benzonase. 1 µl of Benzonase was added to the samples and digestion was performed for 1 hour at 4 °C. Samples were stored at -20°C until further use. Before loading on the gel, samples were heated to 95°C for 5 min.

2.10.4.1.2 Sample preparation for purified proteins

4 x SDS - PAGE loading buffer without DTT was added to the samples and samples were applied to the gel without heating.

2.10.4.2 SDS-PAGE-analysis

Solutions

Lower buffer

Upper buffer

Rotiphorese®NF-30% (w/v) Acrylamide/Bisacrylamide-solution

10% (w/v) Ammonium persulfate (APS)
N,N,N´,N´-tetramethylethane-1,2-diamine (TEMED)
1 x SDS-PAGE running buffer
Protein Ladder

Procedure

Separating and stacking gel were prepared according to the following standard reaction mixtures**:**

Separating gel

Lower buffer	2 ml
H_2O_{dd}	2.66 ml
Acrylamide/Bisacrylamide solution	3.33 ml
APS	40 µl
TEMED	8 µl

Stacking gel

Upper buffer	0.63 ml
H_2O_{dd}	1.63 ml
Acrylamide/Bisacrylamide solution	0.38 ml
APS	17.5 µl
TEMED	10 µl

12.5% polyacrylamide gels were prepared by use of a Mini-PROTEAN casting stand. Lower buffer, H_2O_{dd} and acrylamide/bisacrylamide solution were mixed and APS and TEMED were added to initiate polymerization of the gel. The separating gel solution was poured between the two glass plates of one cassette and gels were overlaid with isopropanol to produce a smooth surface of the separating gel. After polymerization of the separating gel, the isopropanol was poured off. The stacking gel was prepared by mixing upper buffer, H_2O_{dd} and acrylamide/bisacrylamide solution and adding APS and TEMED. A layer of stacking gel was added on top of the separating gel and combs were applied. Polymerized gels were wrapped in wet paper towels and saran wrap and stored at 4°C for a few days until further use.

For separation of proteins, gels were inserted into a Mini PROTEAN gel electrophoresis chamber and the chamber was filled with 1 x SDS-PAGE running buffer. Samples and marker were pipetted into the pockets of the gel. Proteins were separated by applying a voltage of 60 – 110 V for 1.5 hours, depending on type of analysis. The run was stopped when the marker front reached the bottom of the gel. The gel apparature was dissembled and gels were stained as described below.

2.10.4.3 Staining and destaining of SDS-PAGE-gels using Coomassie brilliant blue staining solution

Solutions

Coomassie brilliant blue staining solution

Destaining solution

Procedure

After electrophoresis, gels were stained in Coomassie brilliant blue staining solution on a rocking platform for 45 min followed by destaining in Destaining solution overnight. Gels were rinsed with H_2O_{dd} and digitalised by scanning.

2.10.4.4 Fixation, staining and destaining of SDS-PAGE-gels using colloidal Coomassie staining solution

Solutions

Colloidal Coomassie solution

Fixing solution

Procedure

After electrophoresis, gels were rinsed for 5 min with H_2O_{dd}, followed by incubation for 20 min in fixing solution. Gels were rinsed 2 times for 10 minutes in H_2O_{dd} and stained with colloidal Coomassie overnight. On the following day, gels were destained by rinsing them 2 times for 30 min in H_2O_{dd}. All these steps were carried out on a rocking platform. After destaining, gels were digitalised by scanning.

2.10.5 Western blot analysis

Solutions

Transfer buffer

1 x TBS

TBS-T

Developer and Fixer solution

Procedure

Proteins were separated by SDS-PAGE as described in 2.10.4. The gel electrophoresis chamber was dissembled and the stacking gel was removed from the resolving gel. The resolving gel was equilibrated for 15 min in transfer buffer. A polyvinylidene fluoride (PVDF) membrane and 6 pieces of Whatman paper were cut to the size of the separating gel. The PVDF membrane was activated by shaking in methanol for 5 min, followed by rinsing with H_2O_{dd} for 5 min. Transfer onto the membrane was performed by semi-dry blotting in a TE77 semi-dry transfer unit filled with transfer buffer for 1.5 hours at settings as recommended by the manufacturer.

Non-specific binding of proteins was prevented by incubating the membrane in 5% non-fat dry milk in 1 x TBS on a rocking platform overnight. Blocking of the membrane was followed by incubation in 10 ml TBS-T with the primary antibody against the respective protein for 1 hour. The membrane was washed 3 times in TBS-T for 15 min to remove excess primary antibody, followed by incubation in TBS-T with the corresponding HRP-labelled secondary antibody for 1 hour. After 3 additional washing steps, the signal was detected by using the Western Lightning Chemiluminescence Plus Kit as recommended by the manufacturer. The membrane was exposed to a photographic film in the dark. The film was developed with Developer Solution, fixed with Fixer Solution and washed in H_2O. Films were digitalised by scanning.

2.11 Purification of *P. amoebophila* EBs

For purification of the putative porin pc1489 and for infection studies, EBs were purified from *Acanthamoeba castellanii* cultures infected with *P. amoebophila*. Amoebae were broken up in a procedure that also destroyed RBs present in the sample. The cell debris was removed by filtration and centrifugation, yielding purified EBs.

2.11.1 Partial purification of *P. amoebophila* EBs

Solutions

1 x PAS

SPG-buffer

Procedure

12 – 15 large cultures of *A. castellanii* cultures infected with *P. amoebophila* were harvested (sec. 2.9.2). The pellet was washed in 1 x PAS, centrifuged (7,323 x g, 5 min) and the resulting pellet was resuspended in 1 x PAS. 4 freeze-and-thaw steps (freezing at -20°C/ thawing at 42°C in a water bath) were performed to disrupt amoeba cell membranes, followed by shearing of the samples with an equal volume of glass beads for 3 minutes. To remove glass beads and cell debris, the lysate was centrifuged for 10 min at 300 x g and 4°C. The resulting supernatant was filtered through a 1.2 µm filter and transferred to an ultracentrifuge tube. The cell suspension was filled up to 10 ml with precooled SPG-buffer and centrifuged at 40,000 x g for 40 min a 4°C in an ultracentrifuge. The resulting pellet was resuspended in 10 ml precooled SPG-buffer and centrifuged as before. The pellet was resuspended in 6 ml SPG–buffer and passed through a 0.45 µm syringe to singularize EBs. The resulting suspension was aliquoted to screw caps. To ensure that purified EBs were not contaminated with other bacteria or fungi, 1 ml of TSY was inoculated with 10 µl of each screw cap and incubated for 5 days at 20°C. Samples were checked for growth of bacteria or fungi with an inverse microscope. The remaining EB-suspension was stored at -80°C until further use.

2.11.2 High purification of *P. amoebophila* EBs

High purification of *P. amoebophila* involved two additional gradient centrifugation steps compared to partial purification. During gradient centrifugation, EBs formed a pellet at the bottom of the tube, whereas amoeba cell debris accumulated at the interface between gastrografin and sucrose.

Solutions

1 x PAS

SPG-buffer

30 mM Tris-HCl buffer (pH 7.3)
30% (v/v) Gastrografin in H_2O_{dd}
50% (w/v) sucrose in H_2O_{dd}

Procedure

12 – 15 large cultures of *A. castellanii* infected with *P. amoebophila* were harvested, washed in 1 x PAS and the resulting pellet was resuspended in 3 ml 30 mM Tris-HCl buffer. Amoeba cells were disrupted as described above (2.11.1). To remove glass beads and amoeba cell debris, the lysate was centrifuged for 10 min at 4°C and 300 x g. The resulting supernatant was filtered through a 1.2 µm filter and transferred to a clean ultracentrifuge tube. The cell suspension was filled up to 10 ml with 30 mM Tris-HCl buffer and centrifuged at 40,000 x g for 1 h at 4°C in an ultracentrifuge. The resulting pellet was resuspended in 1 ml precooled SPG-buffer. For further purification, a sucrose-gastrografin-gradient was prepared by overlaying 3 ml 50 % sucrose with 3 ml 30 % gastrografin and overlaying the gastrografin with 3 ml sample. Gradient centrifugation was performed at 40,000 x g for 2 h at 4°C two times. Between centrifugation steps, the cell suspension was passed through a 0.45 µm syringe in order to singularize bacteria. The pellet of the last centrifugation step was resuspended in 6 ml SPG-buffer and passed again through a 0.45 µm syringe. The resulting suspension was aliquoted to screw caps and screened for the presence of other bacteria or fungi as described in 2.11.1. Aliquots were stored at -80°C until further use.

2.12 Screening of amoeba cultures

To ensure that *Acanthamoeba castellanii* cultures were not contaminated with extracellular bacteria or other chlamydiae, cultures were screened at regular intervals and each time before purification of EBs was performed.

2.12.1 DAPI staining of amoebae

Every 2-3 weeks and before purification of EBs was started, cultures were screened by staining with 4',6-Diamidino-2-phenylindol (DAPI).

Solutions

DAPI

1 x PAS

4% PFA in 1 x PBS

Procedure

DAPI was prepared at a dilution of 1:10,000 and stored at 4°C until further use.

1 ml of an amoeba culture was harvested by centrifugation at 5,974 x g for 5 min. The resulting pellet was resuspended in 1 x PAS and centrifuged as before. The pellet was resuspended in 200 µl 1 x PAS. 20 µl of this suspension were applied to the well of a microscope slide. Amoebae were allowed to attach for 30 min. Then, the liquid was removed carefully and 10 µl of 4 % PFA were added instead to fix cells. After incubation for 10 min at RT, the fixative was removed and wells were washed with 10 µl H_20_{dd}. 10 µl of the DAPI solution were added onto each well and slides were incubated for 7 min in the dark. The DAPI-solution was removed and wells were washed with 20 µl H_20_{dd}. Slides were dried at RT in the dark, followed by embedding in Citifluor. Fluorescently labelled cells were analyzed by epifluorescence microscopy.

2.12.2 Screening of cultures via PCR

If investigation by light microscopy or staining with DAPI indicated the presence of contaminations with extracellular bacteria or other endosymbionts in amoeba cultures, DNA was isolated from these cultures and 16S rRNA gene-fragments were amplified by PCR followed by sequencing and BLAST search.

Procedure

10 ml of an amoeba culture were harvested and DNA was isolated from the resulting pellet using the DNeasy Blood & Tissue Kit according to instructions of the manufacturer and using the protocol for Gram-negative bacteria. DNA-concentrations were measured with a NanoDrop® ND-1000 spectralphotometer and 100 ng of DNAwere used as template for PCR.

To amplify 16S rRNA genes of bacteria, the primer pair 616V and 1492R was used. Primers PcF and PcR were used to amplify chlamydial 16S rRNA genes as described above (sec. 2.10.1). Resulting PCR products were checked quantitatively and

qualitatively (sec. 2.10.2), purified using the Qiaquick PCR purification Kit according to the manufacturer's instructions and subjected to sequencing (sec. 2.10.3).

2.12.3 Screening of cultures for bacteria present in low amounts

The primers 616V and 1492R do not only target 16S rRNA genes of bacteria, but also 16S rRNA genes of mitochondria. Mitochondria are present in high amounts in amoebae. Thus, bacteria that are present in low amounts were not detected by the approach described in 2.12.2. To detect these bacteria, samples were enriched before PCR analysis.

Solutions

1 x PAS (filter-sterilized)

TSY

Procedure

To detect extracellular bacteria present in low amounts, amoeba cultures were harvested and resuspended in 3 ml 1 x PAS (filter-sterilized). Amoebae were removed by passing samples through a 5 µm filter. The resulting suspension was used to inoculate 10 ml of TSY medium. TSY tubes were incubated for 7 days at RT to allow growth of bacteria. Samples were centrifuged at 5,974 x g for 5 min and resuspended in 50 µl 1 x PAS. 5 µl of this solution were subjected to DAPI-staining (2.12.1). The remaining solution was used to isolate DNA for PCR analysis using the DNeasy Blood & Tissue Kit according to instructions of the manufacturer and using the protocol for Gram-negative bacteria. 100 ng of DNA were used as template for PCR. Bacterial 16S rRNA gene-fragments were amplified using the primer pair 616V and 1492R as described in 2.10.1.

Resulting PCR products were checked quantitatively and qualitatively (2.10.2). PCR products were purified using the Qiaquick PCR purification Kit according to the manufacturer's instructions and subjected to sequencing (2.10.3).

2.12.4 Screening of purified *P. amoebophila* EBs by PCR and RFLP analysis

After partial and high purification, 16S rRNA genes of the resulting EBs were analysed by Restriction Fragment Length Polymorphism (RFLP) analysis with the restriction enzyme VspI. This restriction enzyme recognizes the sequence 5'-ATTAAT-3'. This sequence is present once in the 16S rRNA sequence of *P. amoebophila*, but twice in

Parachlamydia sp., thereby allowing differentiation of different environmental chlamydiae by RFLP analysis.

Procedure

Purified EBs of *P. amoebophila* were thawed at 37°C and centrifuged at 17,949 x g for 30 min at 4°C. From the resulting pellet, DNA was purified using the DNeasy Blood & Tissue Kit according to instructions of the manufacturer using the protocol for Gram-negative bacteria. The DNA-concentration was measured using a NanoDrop® ND-1000 spectralphotometer and 100 ng of DNA were used as a template for the following PCR. PCR was performed as described in 2.10.1 using the primer pair PanF and PanR.
Resulting PCR products were digested using the restriction enzyme VspI according to the following standard reaction mixture:

Standard reaction mixture

PCR-product	5 µl
Buffer O	1 µl
VspI	0.2 µl
H_20_{dd}	3.8 µl

The reaction mixture was incubated for 3 hours at 37°C. Digestion was stopped by addition of 5 µl loading dye. Samples were analysed by agarose gel electrophoresis as described in 2.10.2.2.

2.13 Purification of the putative porin pc1489 from highly-purified elementary bodies

The putative porin pc1489 was purified from elementary bodies to get amounts sufficient for immunization of one chicken and for performing lipid bilayer measurements.
EBs were incubated with detergent and the reducing agent DTT to solubilize membrane proteins. Obtained proteins were resuspended in a buffer with pH 7.5. Proteins were loaded on an anionic-exchange column and eluted from this column by applying a NaCl-concentration-gradient to the column. Pc1489 is predicted to have an isoelectric point of 6.75 in the environmental chlamydia genome database

(http://mips.gsf.de/genre/proj/uwe25/). At pH 7.5, pc1489 should be negatively charged and therefore be able to bind to the anionic exchange column.

Solutions

Buffer A

Buffer A without n-POE

Buffer B

3 x PEN

POP05–buffer with 100 mM DTT

4 x SDS - PAGE loading buffer without DTT

Procedure

Highly purified EBs were thawed, transferred to new 1.5 ml tubes and pelleted by centrifugation at 10,621 x g for 15 min at 4°C. The pellet was resuspended in 100 µl POP05-buffer with 100 mM DTT/3 mg EB (wet weight) and incubated for 1 hour at 37°C on a rocking platform. After this, cell debris was removed by centrifugation (10,621 x g, 10 min, 4°C) and the resulting supernatant containing solubilized membrane proteins was transferred into a 1.5 ml tube. A sample of 5 µl was withdrawn for SDS-PAGE analysis. An equal volume of ice-cold acetone was added to the supernatant and proteins were precipitated for 1 hour at -20°C. After 1 hour, the suspension was centrifuged as before and the resulting pellet was resuspended in 400 µl Buffer A. After incubation for 10 min at room temperature, the suspension was centrifuged at 10,621 x g for 10 min at 20°C to remove undissolved matter. 12 µl of the supernatant were withdrawn for SDS-PAGE analysis. A Vivapure Q-Mini-spin column was equilibrated by applying 400 µl Buffer A on top of the membrane and centrifugation at 2,000 x g for 5 min. The flow-through was discarded. The supernatant was loaded onto the Vivapure Q-Mini-spin column. Proteins were bound to the membrane of the column by centrifugation at 2,000 x g for 5 min and the flow-trough was collected. The column was washed 2 times with Buffer A and the flow through was collected. Elution of proteins was achieved by applying mixtures of Buffer A and Buffer B with increasing NaCl concentrations to the membrane, starting with the lowest concentration, and centrifugation at 2,000 x g for 5 min at 20°C. NaCl-concentrations used for protein elution are listed in Table 12. The flow-through of all elution-steps was collected. 12 µl of each fraction and 3 µl of the supernatant after incubation at 37°C were used for SDS-PAGE analysis. 5 µl of 4 x SDS - PAGE loading

buffer without DTT was added to each sample and samples were run on a SDS-PAGE-gel, followed by staining with colloidal Coomassie (sec. 2.10.4). Fractions that seemed to contain only a single band for pc1489 at the correct size after SDS-PAGE analysis were collected and pooled. Protein concentrations of these fractions were determined using the BCA™ Protein Assay Kit (2.14).

Table 12: Volumes of Buffer A and B mixed to obtain the required concentration of NaCl for elution of pc1489.

NaCl-concentration	Buffer A	Buffer B
100 mM NaCl	955 µl	45µl
250 mM NaCl	879 µl	121µl
300 mM NaCl	854µl	146µl
350 mM NaCl	829µl	171µl
400 mM NaCl	804µl	196µl
550 mM NaCl	729µl	271µl
700 mM NaCl	653µl	347µl
850 mM NaCl	578µl	422µl
1000 mM NaCl	503µl	497µl

To increase protein concentrations, another precipitation step was performed by adding 4 volumes of ice-cold acetone to 1 volume of sample. Proteins were precipitated overnight at -20°C. The sample was centrifuged at 10,621 x g for 30 min at 4°C and the pellet was resuspended in the amount of Buffer A without n-POE necessary to obtain a protein concentration of 1 mg/ml. The purity of the precipitated proteins was checked by SDS-PAGE analysis (2.10.4).

2.14 Determination of protein concentrations with the BCA™ Protein Assay Kit

Protein concentrations were measured using the BCA™ Protein Assay Kit.

All samples and standards were prepared and measured as suggested by the manufacturer for the Enhanced Protocol.

All measurements were performed in duplicates and the mean absorbance of these duplicates was used for the determination of protein concentrations.

2.15 Mass spectrometry analysis

To identify proteins that were present in gel bands after purification of pc1489, bands of interest were excised from the gel. Proteins were digested with Trypsin and analysed by linear trap quadruple – mass spectrometry.

2.15.1 Sample preparation for mass spectrometry

Solutions

10% (v/v) and 0.1% (v/v) trifluoroacetic acid (TFA)

50 mM ammonium bicarbonate (ABC-buffer)

Acetonitrile (ACN)

Alkylation buffer (10 mg/ml iodacetamide in 50 mM ABC)

Destaining solution (100 µl 50 mM ABC + 80 µl ACN (44% (v/v))

Reduction buffer (10 mM DTT in 50 mM ABC)

Trypsin gold working solution:

Trypsin gold was added to 50 mM acetic acid to yield a concentration of 100 ng/µl and aliquots were stored at -80°C. Immediately before use, 10 µl were thawed and diluted by addition of 70 µl ABC-buffer, resulting in a final concentration of 12.5 ng/µl

Procedure

Band of interest stained with colloidal Coomassie were excised from gels after SDS-PAGE and were transferred to screw caps and stored at -80°C for further use or immediately processed for analysis by mass spectrometry. Gel bands were washed three times in 200 µl H_20_{dd}, followed by destaining of spots by adding 100 µl destaining solution. The solution was replaced every 15 min, until the gel bands were completely destained. This was followed by incubation for 5 min in acetonitrile. All these steps were carried out on a rocking platform at 20°C. ACN was removed and bands were vacuum-dried for 5 minutes at RT.

Samples were reduced in 200 µl reduction buffer for 30 min at 56°C, followed by incubation in 160 µl ACN for 5 min. ACN was removed and proteins were alkylated with 100 µl iodacetamide for 20 min in the dark. Samples were washed 3 times with 200 µl ABC-buffer. Gel bands were dehydrated with 200 µl ACN and vacuum-dried.

20 µl of trypsin gold working solution was added to the samples and samples were re-swollen for 10 min at 4°C. Afterwards, trypsin solution was removed, samples were covered with ABC-buffer and tryptic digest was performed overnight at 37°C. On the next day, digestion was stopped by addition of 2 µl 10 % TFA. Peptides were extracted by sonication for 10 min in a sonication water bath. The peptide solution was transferred to a clean tube and the sonication step was repeated by addition of 10 µl 0.1 % TFA to the sample. The peptide extracts were pooled and stored at -80°C until they were subjected to nano-liquid chromatography matrix-assisted laser desorption-ionization (MALDI) - time of flight (TOF)/TOF (nanoLC-MS/MS) analysis.

2.15.2 Identification of proteins by nanoLC-MS/MS

Mass spectrometry analyses were performed at the Institut für Biochemie und molekulare Zellbiologie der Universität Wien at the Vienna Biocenter by Dr. Pichler.

MS/MS spectra were matched against a custom generated database with Mascot version 2.2 (Matrix Science). This database contained the protein sequences of *P. amoebophila* UWE25 and protein sequences availabe for other environmental chlamydiae. Additionally, it included protein sequences availabe for *Acanthamoeba* sp. and the two relatives *Dictyostelium discoideum* and *Entamoebae histolytica*, whose genomes have been sequenced, as well as a list of frequently found contaminants in MS samples, like trypsin, keratins and BSA.

2.16 Lipid bilayer measurements

Lipid-bilayer measurments were performed at the laboratory of Prof. Roland Benz (Germany) as described before (58). Before addition to the aqueous phase, the samples containing pc1489 were diluted 1:100 in 1 % Genapol.

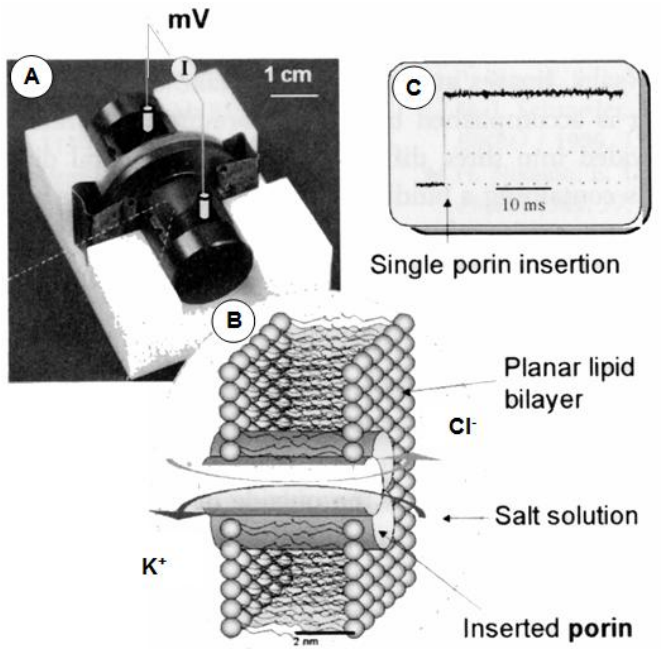

Fig. 5: Principles of lipid bilayer measurments. This technique is based on the increase in conductance measured if a channel-forming protein is incorporated in an aritificial lipid-bilayer (Fig.5, B and C). The apparature used consists of two chambers (cis- and trans-chamber) that are connected by a small circular hole (Fig.5, A). An artificial lipid-bilayer is formed across this hole, the protein to be investigated is added and pore-formation in the membrane is measured. Modified from (9).

2.17 Fixation of cells

Samples were fixed with PFA and methanol and the obtained results were compared.

2.17.1 Fixation of cells with 4 % PFA

Solutions

4 % PFA in 1 x PBS

1 x PBS

Procedure

Round cover slips were sterilized in ethanol, flame-sterilized and placed into each well of a multiwell-dish. Amoeba cultures were harvested and 1 ml of medium with uninfected amoebae or amoebae harbouring chlamydial endosymbionts was added to each well. Cells were allowed to attach overnight.

The medium was removed and wells were washed with 500 µl 1 x PBS. 500 µl 4 % PFA in 1 x PBS were added to each well and cells were fixed for 1 hour at RT. The fixative

was removed and cells were covered with 1 x PBS. Fixed cells were stored in 1 x PBS up to 2 weeks at 4°C.

2.17.2 Fixation of cells with Methanol

Solutions

Methanol

1 x PBS

Procedure

Fixation procedure was the same as for fixation with 4% Paraformaldehyde (see 2.17.1), except that cells were fixed with methanol for only 10 min at RT.

2.18 Immunofluorescence analysis and antibody preparation

2.18.1 Preparation of antibodies targeting the putative porin pc1489

Solutions

Binding Buffer

Elution Buffer

Cleaning Buffer

Procedure

400 µg of pc1489 in Buffer A without n-POE were used for immunization of one chicken. Antibodies were produced by Eurogentec (Seraing, Belgium). Pre-immune egg yolks were collected before the first immunization. After the first immunization, 3 boosts followed. Egg yolks of the immunized chicken were collected, starting 8 days after the first immunization.

IgY antibodies from different egg yolk collections and also from the pre-immune yolks were purified using a HiTrap™ IgY Purification HP column according to the manufacturer's instructions.

2.18.2 Preparation of amoeba lysate

Solutions

FA-Block solution

Procedure

An amoeba culture was harvested by centrifugation (5 min, 7,323 x g). The pellet was resuspended in 500 µl – 1 ml FA-Block solution, depending on the size of the pellet. Amoeba cells were broken by either 4 sonication steps for 30 seconds each or by two freeze-and-thaw steps (freezing at -20°C/ thawing at 42°C in a water bath). The resulting lysate was stored at -20°C until further use.

2.18.3 Removal of antibodies targeting amoeba proteins

To remove antibodies that would result in an unspecific background in immunofluorescence analysis, all sera used were inactivated against amoeba proteins.

Procedure

Antibody sera were mixed 1:1 with amoeba cell lysates, vortexed briefly and incubated overnight at 4°C. On the following day the solution was mixed and incubated again for 30 min at 4°C. Amoeba cell debris that had bound the host-reactive antibodies was removed by centrifugation for 2 min at 6,797 x g. The supernatant was transferred to a new tube and used for further analysis.

2.18.4 Immunofluorescence analysis

Solutions

1 x PBS

10 mM Tris-HCl pH 6.5

0.05% Tween^{TM} 20 in 1 x PBS

FA-Block solution

Mowiol

Procedure

For samples fixed with 4% PFA, a permeabilisation step was performed prior to immunofluorescence analysis in order to permeabilize cells.

1 x PBS was removed and cells were incubated with 300 µl 0.05% Tween^{TM} 20 in 1 x PBS for 25 min at room temperature. After removal of Tween^{TM} 20, cells were covered with 300 µl 10 mM Tris-HCl (pH 6.5) containing 5 µg/ml Lysozyme and incubated for 1 hour at room temperature. Permeabilized cells or cells fixed with methanol were incubated with FA-Block solution for 10 min. After this blocking step, the blocking solution was replaced with the primary antibody diluted in FA-Block solution and cells were incubated for 1 hour at RT. Cells were washed 3 times with 1 x PBS, followed by incubation with secondary antibody diluted in FA-Block solution for 1 hour in the dark. Cells were washed 3 times with 1 x PBS and cover slips were embedded in Mowiol. Embedded samples were analyzed by epifluorescence microscopy and confocal laser scanning microscopy.

2.18.6 Immunofluorescence on thin sections

Solutions

1 x PBS

FA-Block solution

100 mM HEPES buffer, pH 7.2, with 2 % PFA and 0.1 % glutaraldehyde

12 % gelatin

Cryoprotective solution (2.3 M sucrose in 100 mM phosphate buffer)

Procedure

Thin sections (75 nm) of *A. castellanii* infected with *P. amoebophila* were provided by Jacqueline Monatanaro.

Cells were fixed with 2% PFA, 0.1 % glutaraldehyde in 100 mM HEPES buffer (pH 7.2) for 20 mins at 20°C and then transfered to 2 % PFA in 100 mM HEPES buffer for a further 2 hours. Fixed cells were pelleted at 3000 x g and washed in 100 mM HEPES buffer. The cells were encapsulated in 12 % gelatin at 37°C. After 10 min infiltration, the cells were pelleted in an eppendorf tube and cooled on ice. The gelatin-cell pellet was cut into 0.1 mm^2 cubes and infiltrated in Cryoprotective solution overnight at 4°C. Cell cubes

were mounted onto aluminium stubs and frozen in liquid nitrogen. Thin sections were cut using a Leica Ultracut UC6-FC6 ultramicrotome (Leica Microsystems, Austria) and mounted onto carbon-coated formvar-pilioform films supported on hexagonal 200 mesh nickle EM grids (Gilder, Netherlands). Sections were preserved prior to use under Cryo-EM pick-up solution containing 1% methyl cellulose (Electron Microscopy Sciences, Germany) and 1.3 M sucrose in 100 mM phosphate buffer at 4°C. 10 µl 1 x PBS were added to each well to solubilize methyl cellulose on top of the thin-sections. After 10 minutes, 1 x PBS was replaced with 10 µl of the primary antibody diluted in FA-Block solution and cells were incubated for 1 hour at RT. Cells were washed 3 times with 10 µl 1 x PBS, followed by incubation with 10 µl of the secondary antibody diluted in FA-block solution for 1 hour in the dark. Cells were washed again and slides were embedded in Citifluor. Embedded samples were analyzed by epifluorescence microscopy and confocal laser scanning microscopy.

2.19 Infection studies

2.19.1 Counting of DAPI-stained cells

The number of *P. amoebophila* cells in suspensions was determined by filtering cells onto a membrane, followed by DAPI-staining to visualize cells.

Solutions

1 x PBS (filter-sterilized)

DAPI

Procedure

DAPI was prepared at a dilution of 1:10,000 and stored at 4°C until further use.

Partially purified EBs were thawed at 37°C. 1 µl of the EB–suspension was added to 5 ml of filter-sterilized 1 x PBS and stored on ice. The filter device was flame-sterilized and assembled. 5 ml 1 x PBS were filtered to rinse the device by applying a vacuum pump running at 250 mmHg (millimetre of mercury). The bacterial suspension was filtered onto a 0.22 µm polycarbonate membrane filter and a 0.45 µm cellulose acetate membrane support underneath the filter. The device was rinsed with 5 ml 1 x PBS to wash down

bacteria bound to the glass funnel. 150 µl of DAPI solution were added on top of the filter and the filter was incubated for 5 min in the dark. The filter was washed with 1 x PBS, placed on a microscope slide and embedded in Citifluor. Samples were analyzed with an epifluorescence microscope. The number of cells on 10 x 10 fields on a grid was counted. The cell number/ml was calculated using the following formula:

$$\frac{\text{Cells}}{\text{ml}} = \frac{\text{Number of all cells counted x M x DF}}{\text{Number of fields counted}}$$

M …Microscope factor = 11,264

DF … Dilution factor = 1,000

2.19.2 Infection cycle

Solutions:

TSY

1 x PAS (filter-sterilized)

1 x PBS (filter-sterilized)

4% PFA in 1 x PBS

Methanol

Procedure

A large flask (150 ml) of *A. castellanii* Neff was harvested (sec. 2.9.2) and amoebae were counted using a Neubauer counting chamber. The number of amoebae/ml was calculated using the following formula:

$$\frac{\text{Cells}}{\text{ml}} = \frac{\text{Number of all cells counted x D}}{\text{Number of fields counted x Depth of chamber}}$$

DF … Dilution factor = 100

Depth of chamber = 0.1 mm

After counting, amoebae were pelleted by centrifugation and resuspended in the volume of TSY needed to obtain the required number of cells/ml. Round cover slips were

sterilized in ethanol, flame-sterilizd and placed into each well of a multiwell-dish. 1 ml of TSY containing a defined number of amoebae was pipetted in the wells of the multiwell-dish. Samples were incubated for 30 min – 60 min at 20°C to allow attachment of amoebae. Proper attachment of amoebae was controlled by light microscopy. Partially purified EBs were thawed at 37°C and placed on ice. Amoebae were infected with EBs at a multiplicity of infection (MOI) of 5 or a MOI of 10 by adding a defined amount of EB suspension to each well. Multiwell-dishes were centrifuged at 600 x g for 15 min at 20°C to enhance infection. The end of centrifugation was regarded as time point 0 hours post infection (h p.i.). After centrifugation, the medium was replaced with fresh TSY to remove excess EBs that were not attached to amoebae. Infected cultures were grown at 20°C. At indicated time points, cells were fixed. For fixation, the medium was removed and cells were washed with 1 x PBS. Cells were fixed with 4% PFA or with methanol (2.18.1 and 2.18.2). The fixative was replaced by 1 x PBS and cells were stored at 4°C until immunofluorescence analysis was performed.

2.19. 3 Heat inactivation of EBs

Conditions for heat inactivation were chosen similar to those already published for heat inactivation of clinical chlamydiae (16).

Procedure

Partially purified EBs were thawed at 37°C and 1 volume of 1 x PBS was added. For heat inactivation, cells were incubated at 56°C for 30 min.

2.19.4 Infection - inhibition assay using anti-Pam and anti-pc1489-antibody

Infectious EBs were incubated with antibodies targeting the protein of interest and these EBs were then used for infection of host cells. The ability to attach to host cells and the number of bacteria/host cell were compared to non-treated EBs at certain time points.

Anti-pc1489-antibody and anti-Pam-antibody, an antibody targeting the immunodominant proteins of the outer membrane of *P. amoebophila*, were used.

Solutions

TSY

1 x PAS (filter-sterilized)

FA-Block solution (filter-sterilized)
1 x PBS (filter-sterilized)
4% PFA in 1 x PBS
Methanol

Procedure

Anti-pc1489-antibody and anti-Pam-antibody were diluted in FA-Block solution to reach concentrations of 200 µg/ml and 20 µg/ml. Additionally, a solution containing 200 µg/ml anti-Cld-antibody, targeting the Chloride dismutase of "*Candidatus Nitrospira defluvii*" (generously provided by Frank Maixner) was prepared.
EBs and heat-inactivated EBs were incubated with 20 µg/ml or 200 µg/ml of anti-pc1489-antibody or anti-Pam–antibody for 30 min at 37°C. As controls, EBs and heat – inactivated EBs were incubated with FA-Block solution only and additionally, EBs were incubated with 200 µg/ml anti-Cld-antibody under the same conditions. Amoebae were infected to obtain a final MOI of 5 as described above (sec. 2.19.2). Infections were done in duplicates. One of these duplicates was fixed with methanol, the other with 4% PFA (sec. 2.18.1 and sec. 2.18.2). The following time points were investigated: 0, 6, 12, 24, 48, 72 and 96 hours post infection. After fixation of cells, multiwell-dishes were stored at 4°C until immunofluorescence analysis was performed. Samples were examined by fluorescence microscopy and the number of infected amoebae at 72 and 96 h p.i. was counted. The ratio of infected amoebae to all amoebae counted was calculated to allow a comparison of infectivity of treated and non-treated EBs.

2.15 Heterologous expression of proteins in *Escherichia coli*

2.15. 1 Overexpression of proteins

Vector-constructs used in this study are listed in table 8 and were provided in the *E. coli* expression strain BL21 (DE3) by Eva Heinz.

Solutions

LB medium
Ampicillin
1 M Isopropyl-β-D-thiogalactopyranosid (IPTG) in H_2O_{dd}, filter-sterilized

4 x SDS - PAGE loading buffer

Procedure

5 ml of LB medium were inoculated with *E. coli* cells from a glycerol stock and grown overnight at 37°C. Recombinant *E. coli* cells harbouring a plasmid were grown in the presence of ampicillin to avoid the growth of cells without a plasmid. 250 µl of the overnight-culture were used to inoculate 5 ml of fresh LB medium and cells were grown for 2 hours at 37°C. Expression of proteins was induced by addition of IPTG to obtain an end concentration of 1 mM to the culture. After 6 hours, cells were harvested by centrifugating 300 µl of the culture at 17,949 x g for 2 min. The pellet was resuspended in 60 µl of 4 x SDS - PAGE loading buffer and analysed by SDS-PAGE analysis (sec. 2.10.4).

3. Results

3. 1 Purification of the putative porin pc1489

The putative porin pc1489 was purified from elementary bodies of *P. amoebophila*. The different fractions obtained after applying a NaCl-gradient to the anion-exchange column were controlled on a SDS-PAGE gel. In the fractions after solubilisation and precipitation of proteins (Fig.6, lanes 1 and 2), a high number of different proteins was present. In lane 3, the flow-through fraction, one band at ~ 50 kDa can be observed. This band represents the OmcB homologue pc0616 that was identified in a previous study by mass spectrometry analysis of gel bands (47). A prominent band corresponding to pc1489 is present in lanes 1 and 2 at ~30 kDa and also in lanes 5-9, corresponding to the eluates of the 250 mM, 300 mM, 350 mM and 400 mM NaCl- fractions. The molecular weight of the observed band is not in accordance with the predicted weight of pc1489 (36 kDa), but outer membrane proteins have been reported to migrate abnormally in SDS-PAGE gels (see sec. 3.3). Only very weak additional bands are present in lanes 6, 7 and 8, showing that pc1489 is highly enriched in these fractions. For some purifications, up to 7 bands could be observed in the eluate after applying 350mM NaCl, suggesting the presence of

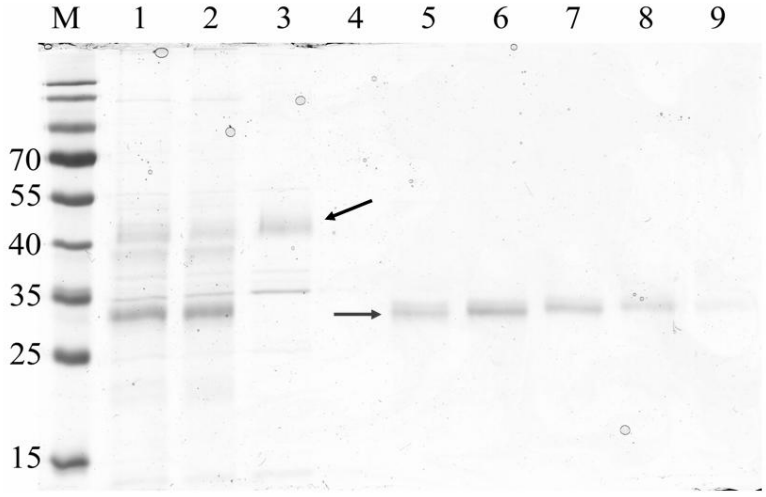

Fig. 6: Typical gel to check the purity of pc1489 after purification. Bands for OmcB (upper arrow) and pc1489 (lower arrow) are indicated. M:Marker; Lane 1: Proteins after incubation at 37°C; Lane 2: Proteins after precipitation with acetone; Lane 3: Flow-through after applying the sample to the column; Lane 4: Eluate after applying 100 mM NaCl; Lane 5: Eluate after applying 250 mM NaCl; Lane 6: Eluate after applying 300 mM NaCl; Lane 7: Eluate after applying 350 mM NaCl; Lane 8: Eluate after applying 400 mM NaCl; Lane 9: Eluate after applying 550 mM NaCl; Weight of marker proteins in kDa is indicated on the left.

various other proteins than pc1489 in these fractions. Fractions where strong additional bands to that at 30 kDa were observed, were not used for production of antibodies or lipid bilayer measurements. After the second precipitation step, samples were highly enriched in pc1489 as can be seen by the presence of a strong band at 30 kDa in Fig.7 (left and central picture). In the enriched fractions, bands were visible at 55 kDa, 60 kDa and 70 kDa. To see whether these bands represent pc1489 and to identify other proteins present in these bands, they were subjected to analysis by mass spectrometry.

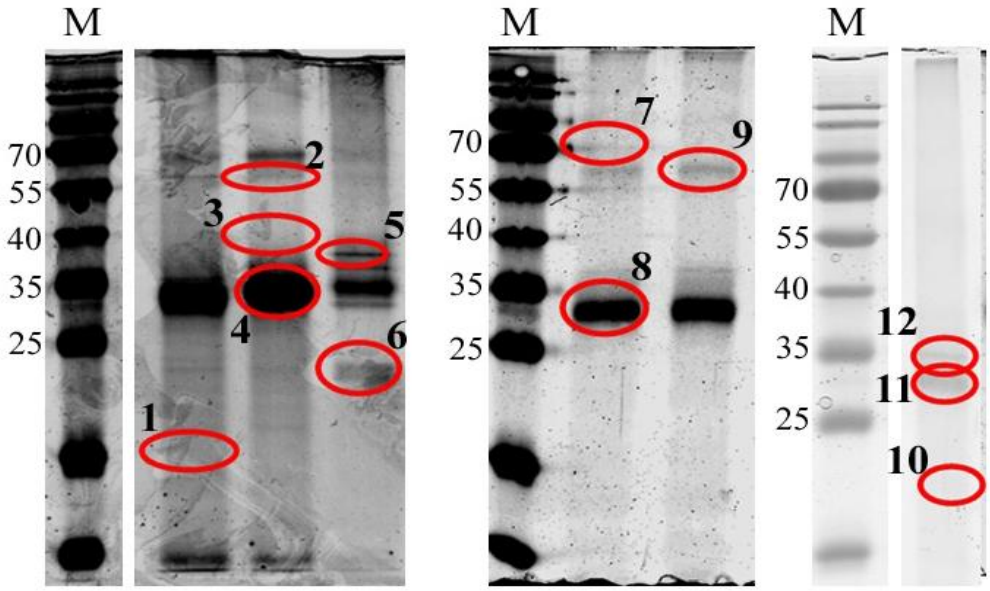

Fig. 7: SDS-PAGE gels of pc1489 after the second precipitation step (left and central picture) and of a second, independant purification of pc1489 (right picture). Bands analyzed by mass spectrometry are indicated by red circles and numbers correspond to table 13. For these scanned images, the contrast was enhanced. Weight of marker proteins in kDa.

3.2 Analysis of bands of interest by mass spectrometry

In total, 12 bands of two independent purifications of pc1489 were analysed.

Pc1489 was the most abundant protein in most bands in Fig.7. It was present in high amounts in the band at 30 kDa and also in the bands at 55 kDa, 60 kDa and 70 kDa (table 13). The presence of pc1489 in various bands suggests formation of multimers by pc1489 as found in porins of Gram-negative bacteria or interaction with other proteins of the outer membrane. The bands at higher molecular weights could represent the dimeric and trimeric form of pc1489 comparable to those observed for MOMP of *Chlamydiaceae* (74). MOMP has been reported to run at a molecular weight of 90 kDa, representing the porin trimer, when loaded on gels without heating the sample (110). However, for

Band	Protein	Function/ Similarity to known proteins*	Signal peptide predicted**
1	pc1885	homologue of OmpW	yes
	pc1077	putative porin	yes
	pc1974	unkown protein	yes
2	pc1489	putative porin	yes
	pc0383	similar to macrophage infectivity potentiator (Mip)	yes
	pc1499	similar to DnaK (heat shock protein)	no
3	pc1489	putative porin	no
	pc1077	putative porin	yes
4	pc0383	similar to Mip	yes
	pc1077	putative porin	yes
	pc1885	homologue of OmpW	yes
5	pc1771	similar to citrate -synthase	no
	pc1105	conserved hypothetical protein	no
	pc1489	putative porin	yes
6	pc1489	putative porin	yes
	pc1180	strongly similar to 60 kDa chaperonin GroEL	no
	ACL00009089 ***	amoeba protein	no
7	pc1489	putative porin	yes
	pc0675	hypothetical protein	yes
	pc1070	unknown	yes
8	pc1489	putative porin	yes
	pc1077	putative porin	yes
	pc1885	homologue of OmpW	yes
9	pc1489	putative porin	yes
	pc1077	putative porin	yes
	pc0004	hypothetical protein	no
10	pc1885	homologue of OmpW	yes
	pc1489	putative porin	yes
	pc1077	putative porin	yes
11	pc1489	putative porin	yes
	pc1885	homologue of OmpW	yes
	pc1077	putative porin	yes
12	pc1489	putative porin	yes
	ACL00000461***	mitochondrial porin from *A. castellanii*	no
	pc1077	putative porin	yes

Table 13: Proteins identified by mass spectrometry in analyzed bands. For each band, only the 3 hits with the highest protein score are listed. Rows coloured grey were identified in a previous studies in outer membrane fractions (47).

*** according to http://mips.gsf.de/genre/proj/uwe25/**

**** according to TargetP (29)**

***** according to http://tbestdb.bcm.umontreal.ca**

MOMP, no band for the monomer can be observed under these conditions in contrast to pc1489, where the monomer is the most prominent band.In the fraction after elution with 350 mM NaCl, results indicated a considerable contamination with other proteins. Proteins that were identified by mass spectrometry are listed in Table 13. Most of the proteins identified by mass spectrometry were predicted to harbour a signal peptide according to TargetP, indicating a localization in the outer membrane, and were found in preparations of the outer membrane of *P. amoebophila* in a previous study (47).

3.3 Heat modifiability of pc1489

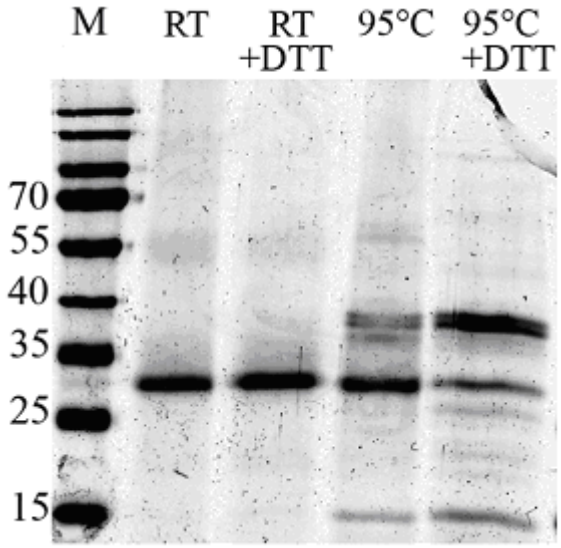

Fig. 8: Heat modifiability of pc1489 demonstrated by the appearance of a band at a higher molecular weight after heating of samples to 95°C. Contrast was enhanced. Molecular weight marker in kDa.

When pc1489 was applied to SDS-PAGE gels in loading buffer without DTT and without heating, the major band for the protein was found at a weight of ~30 kDa and additional weak bands were observable at ~55 kDa and ~ 70 kDa (Fig.8, lane 1). According to *in silico* predictions, pc1489 should have a molecular weight of 36 kDa. The migration behaviour didn't change in the presence of the reducing agent DTT (lane 2). However, when samples were heated to 95°C prior to loading, the band pattern changed considerably. In addition to the band at 30 kDa, bands appeared at ~ 36 kDa and ~15 kDa. The upper band at 36 kDa could represent pc1489 migrating at its correct size after boiling, whereas the lower band could be a degradation product or a cleaved form of pc1489. This change in band patterns was even more pronounced in the presence of DTT. As the band at 55 kDa visible in lanes 1 and 2 got weaker in lane 4, the higher amount of protein at 36 kDa could be related to breaking up of a multimer of pc1489 or breaking of crosslinks between proteins by heating in the presence of DTT.

3.4 Western blot analysis of pc1489

The specificity of purified anti-pc1489-antibody was tested by Western Blot analysis. Western Blot analysis showed specific detection of purified pc1489 by the antibody, indicated by the presence of one distinct band at ~ 30 kDa. Additional weak bands were present at ~ 55 kDa and ~ 70 kDa (Fig.9 A). As pc1489 has been identified in bands of this weight by mass spectrometry (see sec.3.2), these additional bands could result from detection of pc1489 by the antibody. Samples with equal amounts of proteins of uninfected *A. castellanii*, *A. castellanii* infected with *P. amoebophila* and purified EBs of *P. amoebophila* were loaded on gels and Western blot analysis was performed. As these samples had to be heated prior to loading on the gel in order to break up cells, the signal for pc1489 was expected at ~ 36 kDa.

In samples containing amoebae without symbionts, no band was observed by Western blot analysis, showing that the anti-pc1489-antibody does not cross-react with amoeba proteins (Fig.9 B, lane 2).

In samples of *A. castellanii* infected with *P. amoebophila*, several bands were detected (Fig.9 B, lane 1). One prominent band was found at ~ 60 kDa, and a weak signal was

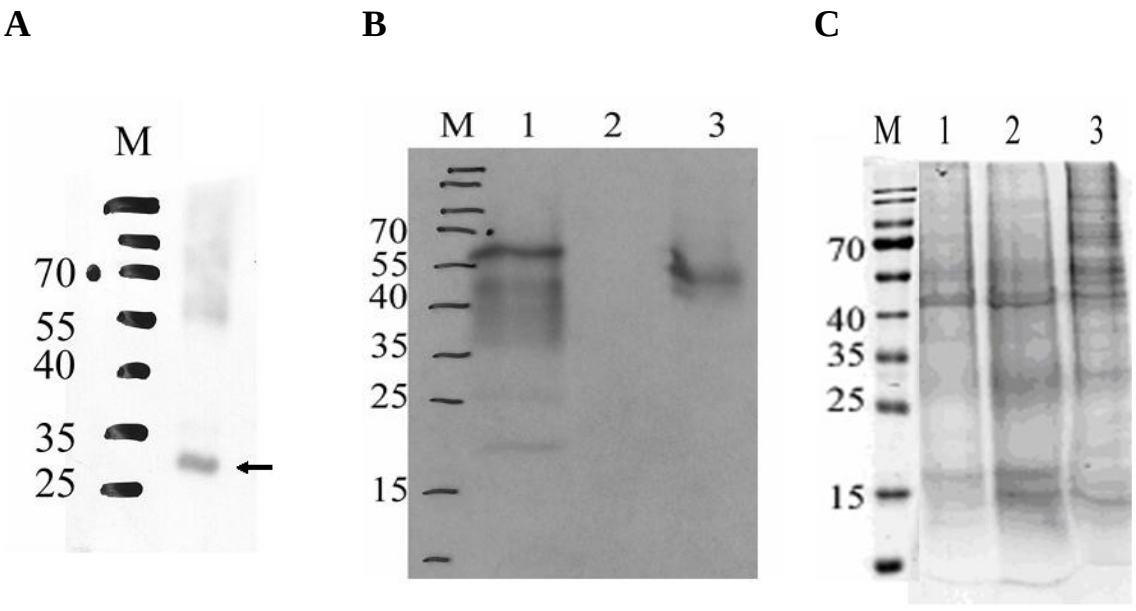

Fig. 9: Western blot analysis with anti-pc1489-antibody. A: Detection of a band at the correct size in SDS gels with purified pc1489 shows specificity of anti-pc1489-antibody. B: Detection of multiple bands in *A. castellanii* infected with *P. amoebophila*. Lane 1: *A. castellanii* infected with *P. amoebophila;* Lane 2: Uninfected *A. castellanii;* Lane 3: Purified EBs of *P. amoebophila*. For comparison, a SDS gel with samples applied like in B and stained with colloidal Coomassie is shown in C. Molecular weight marker in kDa.

present from 35 kDa to 50 kDa. The smear might represent the monomeric form of pc1489 after heating to 95°C which results in several bands at these molecular weights (see sec 3.3). The band at 55 kDa could represent a multimer of pc1489 or the protein pc1489 cross linked to other proteins in the outer membrane of EBs and RBs as both developmental forms were present in asynchronized cultures used for this experiment. The band at 20 kDa could result from degradation products of pc1489. In a study by Heinz *et al.* (47), pc1489 was detected in bands of various molecular weights in outer membrane fractions of *P. amoebophila*, supporting the detection of several bands by anti-pc1489-antibody in amoebae infected with *P. amoebophila*. In EBs, only one prominent band was observable at ~55 kDa (Fig.9 B, lane 3), possibly representing a tightly cross linked multimer of pc1489.

3.5 Immunofluorescence analysis of pc1489

The localization of pc1489 in *P. amoebophila* during co-existence with its host was studied by immunofluorescence analysis. Anti-pc1489-antibody was used simultaneously with anti-Pam and anti-Hsp60-antibodies. Anti-Pam-antibodies were raised against whole elementary bodies of *P. amoebophila* and therefore target the immunodominant components of the outer membrane. Anti- Hsp60-antibodies target the chlamydial heat-shock protein GroEL that is located in the cytoplasm and in the outer membrane of chlamydiae (120).

Signals for pc1489 showed a halo-like shape and overlapped with signals for the outer membrane of *P. amoebophila* by anti-Pam-antibody in methanol and PFA-fixed samples (Fig.10). Signals observed for the cytoplasmic protein Hsp60 were surrounded by pc1489 (Fig.11 B). The halo-shape of signals was even more evident when immunofluorescence was performed on thin-sections (Fig.11 A). The shape of the signal for pc1489 indicates a localization of this protein in either the inner or the outer membrane or in the inclusion membrane of *P. amoebophila*. As *P. amoebophila* thrives in single cell-inclusions in its host, the inclusion membrane is adjacent to the outer membrane. Differentiation between signals of the outer membrane and the inclusion membrane is not possible by immunofluorescence analysis. Hsp60 was detected mostly in the cytoplasm of bacteria, but also a few halo-like shaped signals could be observed, consistent with recent reports that Hsp60 is also located to the outer membrane (120).

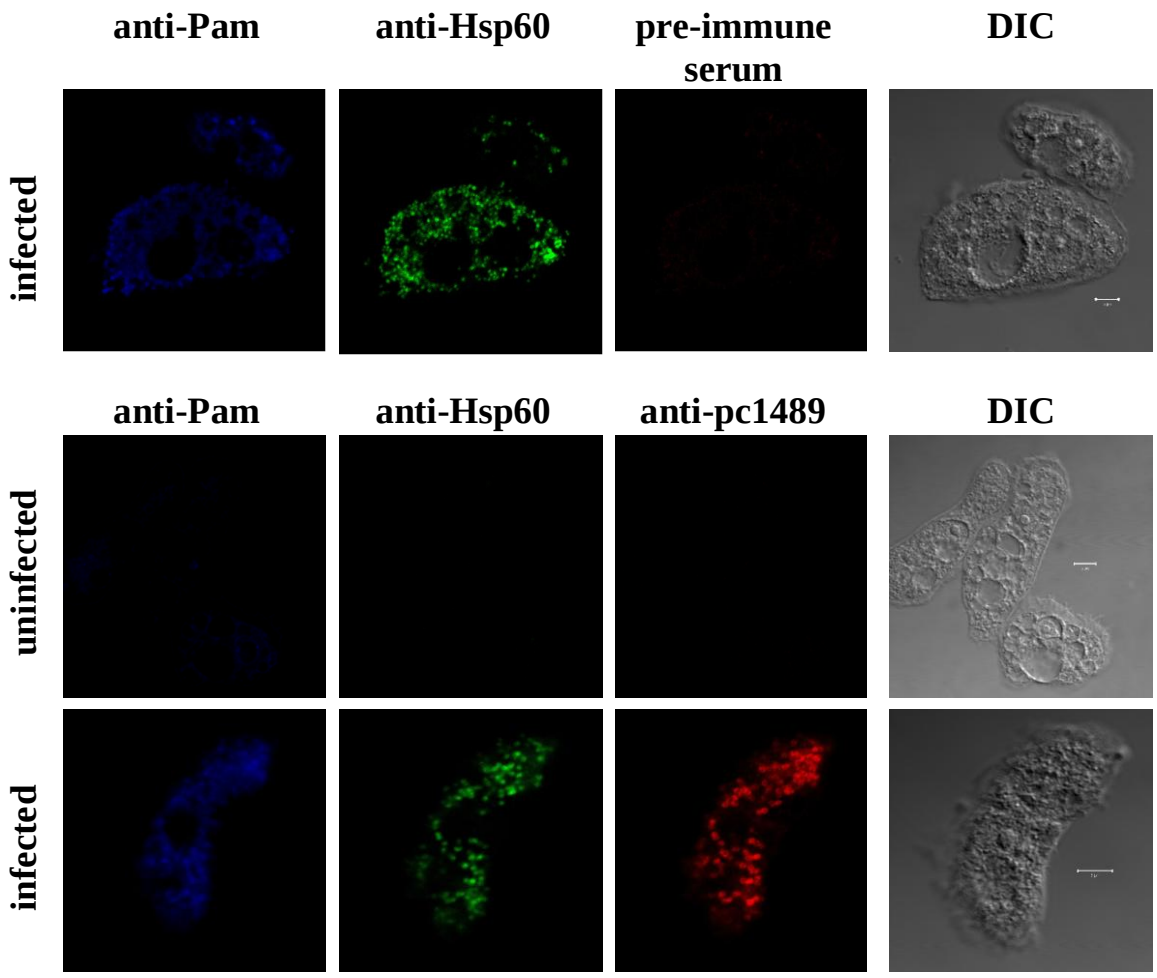

Fig. 10: Immunofluorescence analysis using preI-pc1489 and pc1489-antibody. No signal is observed for the pre-immune serum in infected amoebae or for pc1489 in uninfected amoebae. Differential interference contrast (DIC) channel is shown for better overview. Rows 1-4 represent identical microscopic fields. Bars 5 µm.

No fluorescent signal was observed in uninfected amoebae for any of the antibodies used, although an elevated level of background noise/signal could be observed for the anti-Pam-antibody (Fig.10).

Serum of pre-immune egg yolks gave no signal in uninfected and in amoebae infected with *P. amoebophila* (data no shown). No differences in the shape of the signal could be observed for methanol and PFA-fixed samples (data not shown).

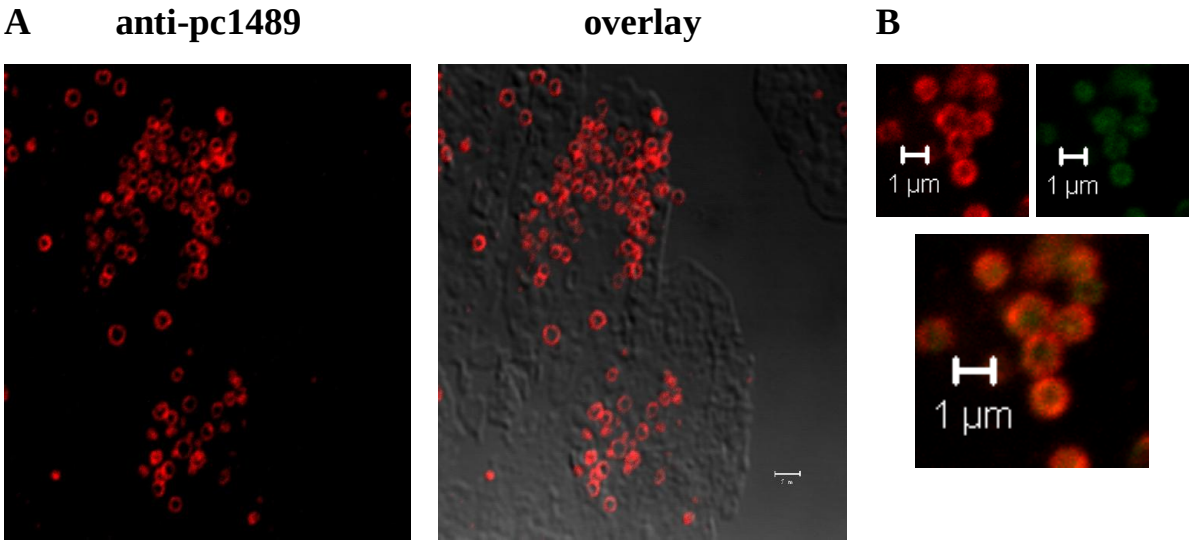

Fig. 11: A: Immunofluorescence analysis of thin-sections (75 nm). Detection of pc1489 (red) gives halo-shaped signals with anti-pc1489-antibody. Left: Detection of pc1489 by anti-pc1489-antibody. Right: Overlay of DIC and anti-pc1489-antibody; Bar 2 µm

B: Detection of *P. amoebophila* by anti-pc1489-antibody (red, upper left) and anti-Hsp60-antibody (green, upper right). Signals for pc1489 surround those for Hsp60 in an overlay (bottom). Bars 1 µm

3.6 Detection of proteins homologous to pc1489 in other chlamydia-like bacteria

Signals resembling those observed for *P. amoebophila* E25 were seen in immunofluorescence analysis of samples of *P. amoebophila* EI2. Pc1489 showed halo-shaped signals overlapping with signals of anti-Pam-antibody, indicating presence of a pc1489-like protein in this strain (Fig.12).

In immunofluorescence analysis of *P. acanthamoeba* UV7, only a weak signal could be detected in a subset of bacteria with anti-pc1489-antibody (Fig.13). In most cases, signals for Hsp60 did not overlap with signals for serum raised against EBs of *P. acanthamoeba*. Signals for pc1489 did not localize with signals for anti-UV7-antibody, but seemed to surround mainly signals for Hsp60. No signals were observed with the pre-immune serum of anti-pc1489-antibody (data not shown)

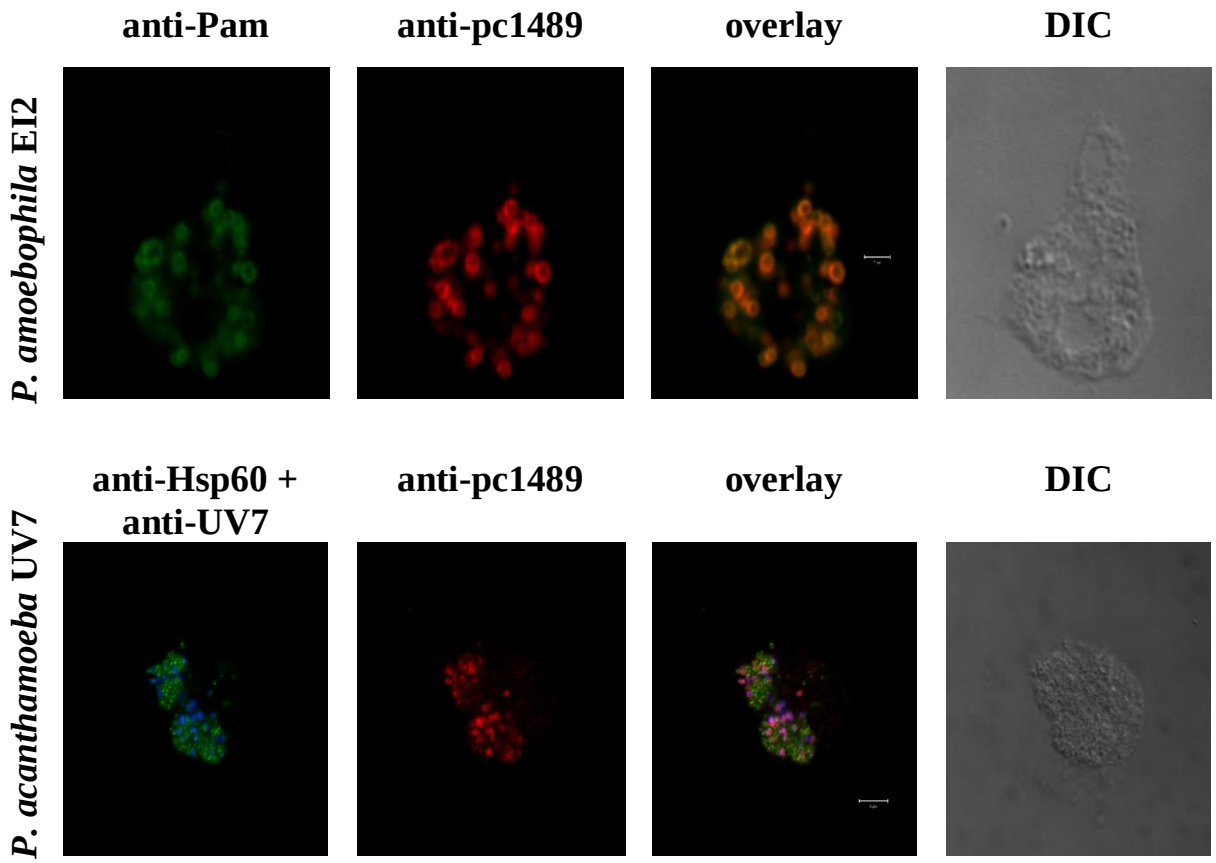

Fig. 12: Immunofluorescence analysis to detect pc1489 in other chlamydia-like bacteria. Upper panel: Detection of *P. amoebophila* EI2 by anti-Pam-antibody (green) and anti-pc1489-antibody (red). Lower panel: Detection of *P. acanthamoeba* UV7 by anti-pc1489-antibody. For *P. acanthamoeba* UV7, an overlay of signal by Hsp60 (blue) and signal given by anti-UV7-serum (green) is shown in the first column in order to show that these two antibodies detect different subsets of bacteria in amoebae. Differential interference contrast (DIC) channel is shown on the right. Rows 1-4 represent identical microscopic fields. Bars 5 µm

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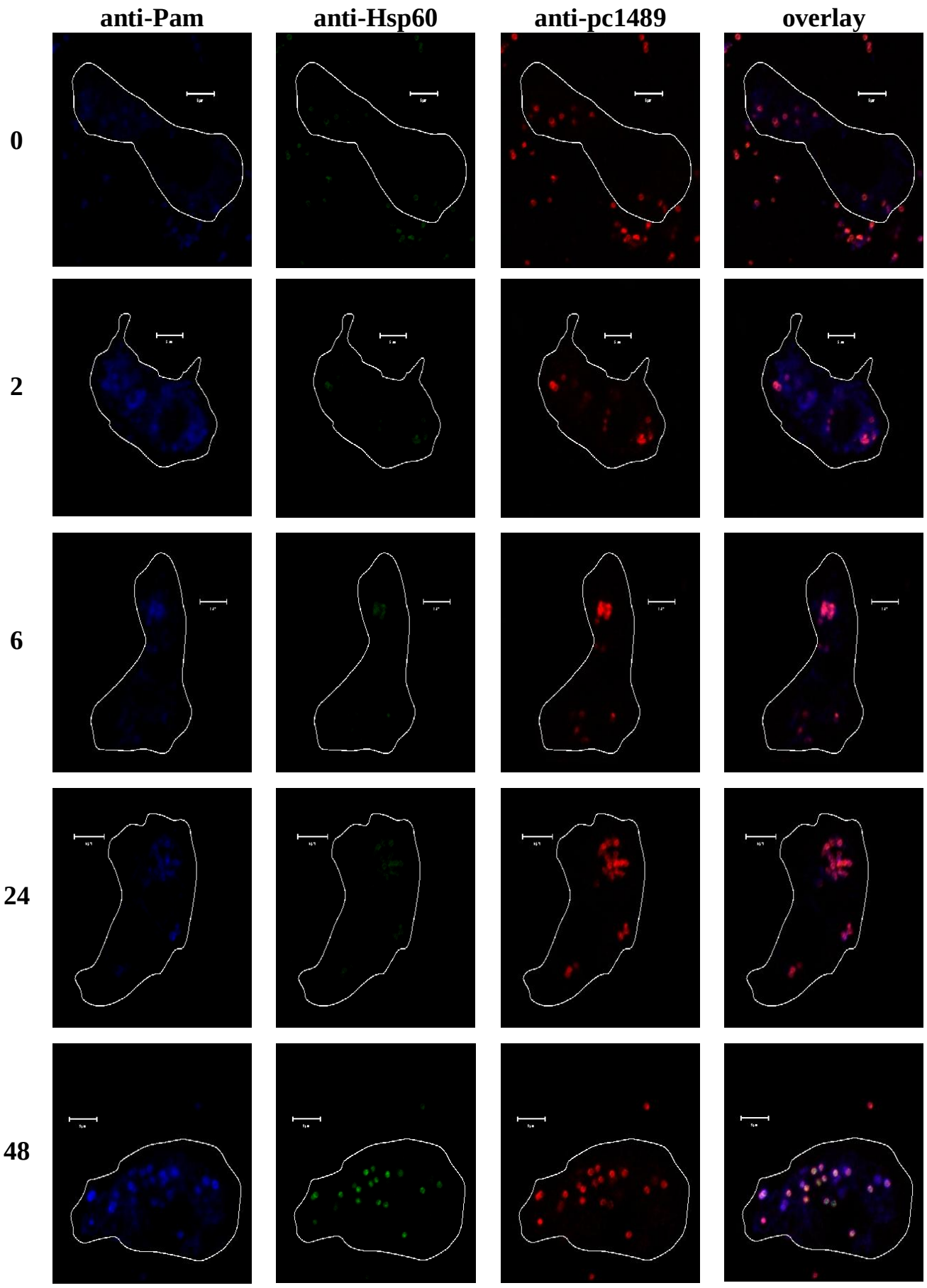
3.7 Expression of pc1489 during an infectious cycle

At 0 hours post infection (h p.i.), pc1489 could be detected by immunofluorescence analysis, giving a strong but not well-defined signal and indicating the presence of this protein in the infectious EBs (Fig.13). Signals for pc1489 were observed throughout the whole infection cycle for all chlamydiae present, with the halo-like shape getting better defined around 6 h p.i. Signal intensity increased slightly at later time points. Anti-Pam-antibody resulted in a high unspecific background, especially at early time points of the infection (0 h p.i. – 6 h p.i.). Therefore, this antibody seems to bind amoeba proteins despite pre-treatment with amoeba lysates. As this antibody was raised against EBs purified from amoebae, it seems possible that not all amoeba proteins were removed

during purification of EBs, thereby giving rise to antibodies targeting proteins of amoebae. The unspecific background got lower at later time points and halo-shaped signals of anti-Pam-antibody overlapped with signals for Hsp60 and pc1489. Only very weak signals could be observed for the heat shock protein Hsp60 at early time points. Signals for Hsp60 at 0 h p.i. showed a halo-like shape, pointing to the presence of a low amount of this protein in the outer membrane of EBs. Very weak signals could be seen for Hsp60 up to 24 h p.i., with a strong increase in signal intensity at 48 h p.i. From 48 h p.i. to 96 h p.i., nearly all chlamydiae labelled with anti-Pam-antibody and anti-pc1489-antibody were also detected by anti-Hsp60-antibody and gave a strong signal. Signals for Hsp60 were surrounded by signals for pc1489 at later time points. Expression of Hsp60 seems to be up-regulated significantly after conversion of EBs in metabolically active RBs as indicated by the rise of signal for this protein. At later time-points, sometimes enlarged chlamydiae were seen in amoebae (Fig.14), maybe indicating stress due to depletion of iron or other nutrients.


anti-Pam
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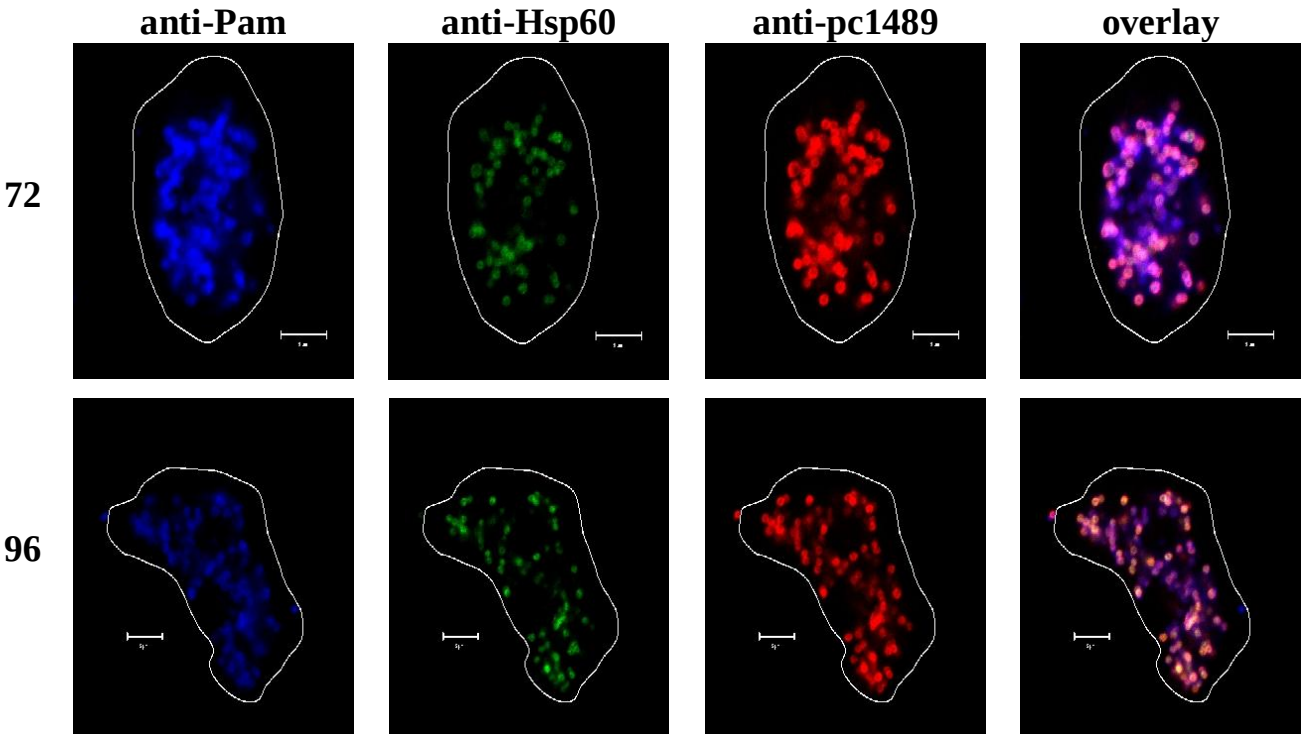

Fig. 13: Infection cycle of *P. amoebophila* in *A. castellanii*. P. amoebophila cells were detected with anti-Pam-antibody (blue), anti-Hsp60-antibody (green) and anti-pc1489-antibody (red). Amoeba cell outline are indicated in white. Numbers on the left indicate hours post infection. Rows 1-4 represent identical microscopic fields. Bars 5 µm

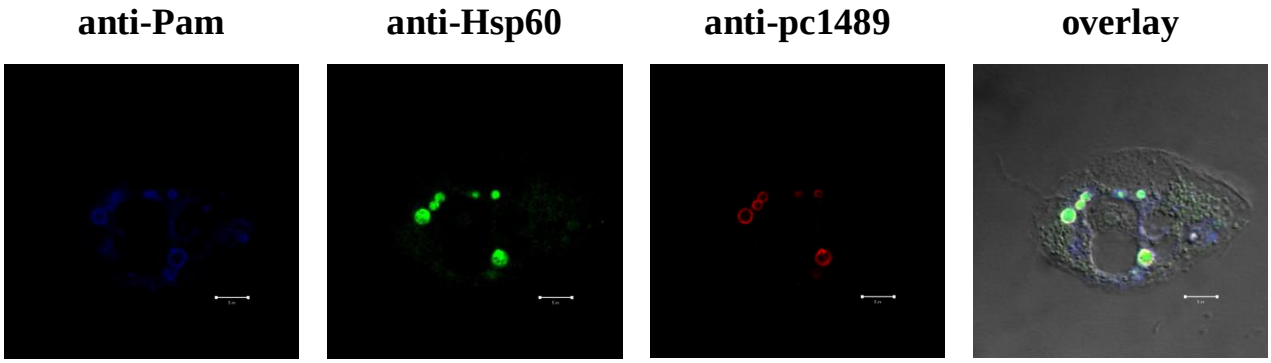

Fig. 14: Enlarged chlamydiae at 96 h p.i. Comparison with Fig.13 shows that chlamydiae are 2-4 times larger than normally. Bars 5 µm

3.8 Porin activity of pc1489

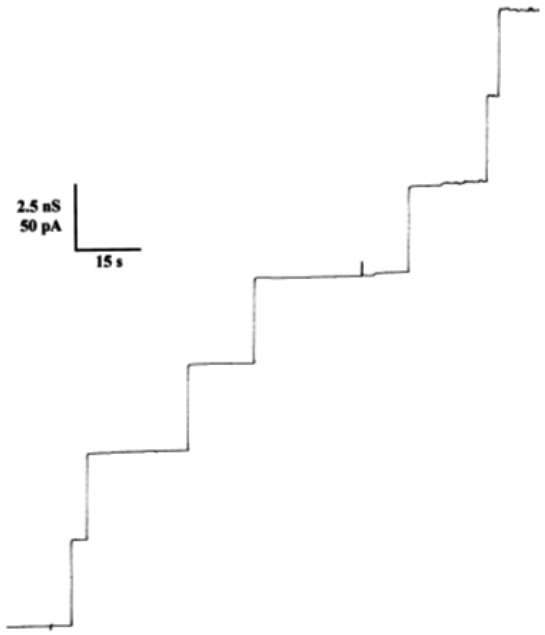

Fig. 15: Single-channel analysis of purified pc1489 in 1 M KCl. Each step indicates insertion of one pore in the lipid bilayer. Conductance in nS and time in seconds

Salt	Concentration (M)	Single-channel conductance (nS)
KCl	0.01	0.15
KCl	0.03	0.25
KCl	0.1	0.6
KCl	0.3	1.2
KCl	1	3.25
KCl	3	11
LiCl	1	2.25
KAc	1	1.5

Table 14: Single channel conductance of pc1489 in different salt solutions

Lipid-bilayer measurments were performed at the laboratory of Prof. Roland Benz (Würzburg, Germany). Single-channel analysis showed that pc1489 formed channels in a lipid bilayer, indicated by a step-wise increase in the measured conductance after addition of pc1489 to the lipid bilayer (Fig.15). The single channel conductance was 3.25 nS in 1 M KCl. The dependence of the single-channel conductance on the KCl-concentration (Table 14) suggested that ions can pass the formed channel easily, indicating the presence of a large water-filled pore in the membrane.

The single-channel conductance was shown to be more dependent on the aqueous mobility of anions than of cations. Replacement of the mobile Cl^- ion by the less mobile acetate-ion resulted in a 2.1 –fold decrease in conductance, whereas replacement of the mobile K^+ -ion by the less mobile Li^+ -ion resulted in a 1.4 decrease.

These results indicate selectivity of the pc1489-channel for anions. Anion selectivity of the porin was confirmed by additional measurements and calculation of the P_c/P_a- ratio using the

Goldman-Hodgkin-Katz equation (10). This equation shows the ratio of the permeability coefficient for a cation (P_c) in relation to the permeability coefficient for an anion (P_a) for a porin. A value for $P_c/P_a > 1$ indicates selectivity for cations of a channel, $P_c/P_a < 1$ indicates selectivity for anions and if $P_c/P_a = 1$, a channel is not selective regarding the chosen anion or cation (46). P_c/P_a – ratios were 0.48 for KCl, and 0.78 for LiCl. All calculated P_c/P_a – ratios are smaller than 1, confirming selectivity of pc1489 for anions.

A typical feature of porins is closure of the pore if high voltage is applied. Channels formed by pc1489 showed closure for both positive and negative potentials of 30 mV and -30 mV, shown by a decrease in conductance (Fig.16). Closure behaviour of pores did only slightly change if pc1489 was added only to the trans- or to the cis-chamber of the apparature in a way that closure was observed at a voltage of 40 mV and -40 mV. These results indicate a symmetric response of the pore to the voltage applied and thereby suggest the formation of a symmetrical pore by pc1489.

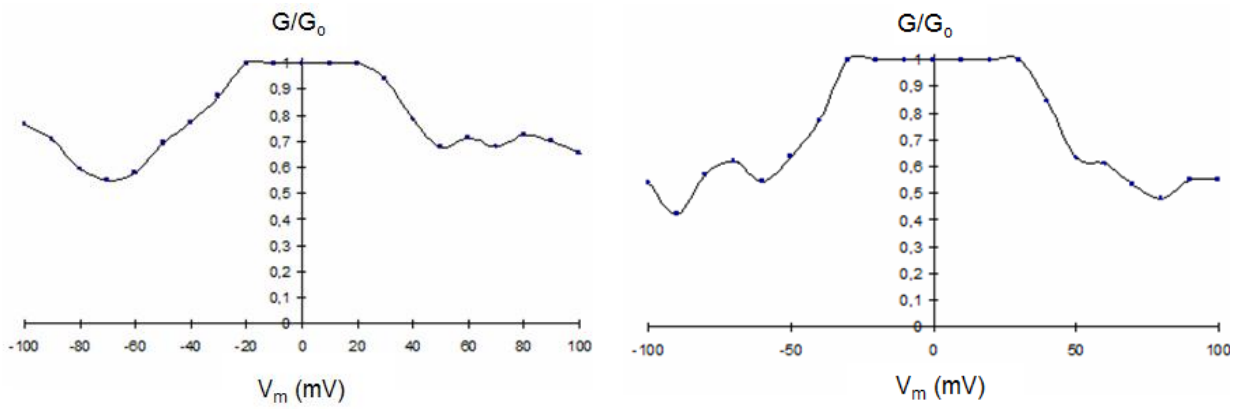

Fig. 16: Ratio of the conductance G at a given potential (V_m) divided by the conductance G_o at 10 mV as function of V_m. The aqueous phase contained 1 M KCl. The obtained graph shows voltage dependent closure of pores formed by pc1489 as seen by a decrease in the measured conductance. Channels closed if pc1498 was added on both sides (left) or only to the cis-chamber of the apparature.

3.9 Heat inactivation of EBs

It has been demonstrated in previous studies that incubation of infectious EBs at 60°C for a period of times as short as 3 min results in a dramatic decrease in attachment to and uptake by host cells for some members of the *Chlamydiaceae* (80).

The effect of incubation at 56°C for 30 min on *P. amoebophila* was tested.

Non-treated and heat treated EBs were compared concerning their uptake by amoebae as well as their ability to multiply within the amoeba host cells. No difference for uptake by amoebae and the following 6 h p.i. and 12 h p.i. time points could be noticed (Fig.17, 0 and 6 h p.i.). At 24 h p.i., dividing chlamydiae could be seen in non-treated samples, although differentiation between heat treated and non-treated bacteria was still difficult. The number of bacteria in infected host cells started to rise clearly at 48 h p.i. in non-treated samples, accompanied by an increase in signal for the heat-shock protein Hsp60. Heat treated *P. amoebophila* cells appeared unable to divide, as numbers of bacteria per host-cell dropped instead of increasing. Additionally, no gain in Hsp60 signal was seen for the heat treated sample. This got even more obvious for the following time points, where non-treated chlamydiae continued multiplying and filled the host cell, whereas signals for treated chlamydiae could only be observed for single cells in a few amoebae. The shape of signals for heat treated cells was well defined up to 72 h p.i., but got diffuse at 96 h p.i., suggesting slow digestion of heat-inactivated chlamydiae by amoebae.

The obtained results suggest that heat-inactivation of EBs does not inhibit uptake of *P. amoebophila* by amoebae, but renders chlamydiae unable to multiply within their host cells.

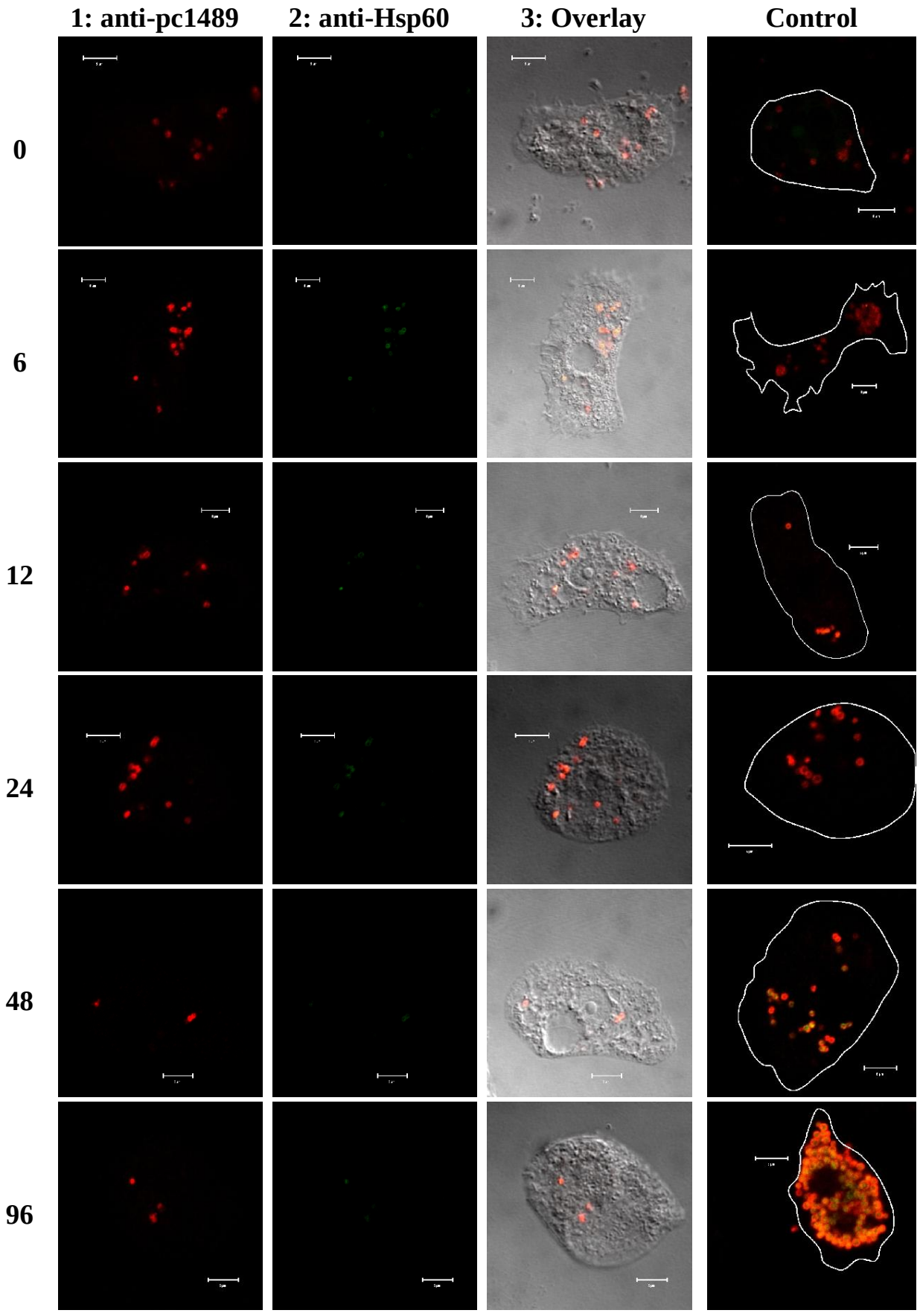

Fig. 17: Infection cycle with heat-inactivated EBs. Rows 1-3 represent identical microscopic fields for infection with heat treated EBs. On the far right, infection with non-treated EBs is shown for comparison (Hsp60 in green, pc1489 in red) and for this control, amoeba cell outline are indicated in white. Numbers on the left indicate hours post infection. Bars 5 µm

3.9 Infection inhibition assay

No difference in uptake or the ability to multiply within host cells could be observed between EBs incubated with antibody and the control (Fig.18). For samples incubated with 200 µg/ml anti-Pam-antibody, this high concentration of antibody lead to agglutination of EBs. This resulted in large aggregates of EBs that were taken up by amoebae in contrast to other samples, where EBs did not stick together. These aggregates of chlamydiae were observed up to 48 h p.i. in samples treated with 200 µg/ml anti-Pam-antibody. However, agglutination did not seem to influence multiplication of *P. amoebophila* within host cells as an increase of signal for Hsp60 could be observed for chlamydiae within these aggregates in contrast to heat-inactivated EBs (Fig. 20).

At 72 h p.i., no aggregates were observable any more. The number of amoebae infected after 72 h p.i. and 96 h p.i. showed no difference between EBs treated with the low concentration of anti-Pam-antibody and the controls (Fig.19). However, in samples of EBs treated with the higher anti-Pam-concentration, the percentage of infected amoebae was even higher than that of the control. However, these results have to be treated with caution, as no duplicates could be investigated for some time points for samples incubated with 200 µg/ml anti-Pam-antibody as no amoebae were found on the slides fixed with PFA. Pre-incubation with anti-pc1489 did not show any effect on infectivity of chlamydiae even at high concentrations. The number of infected amoebae was comparable to that observed for untreated EBs and the EBs incubated with anti-Cld-antibody. No effect of incubation with anti-Cld-antibody, targeting the chloride dismutase of "*Candidatus Nitrospira defluvii*" that is not be present in *P. amoebophila,* was observable. Heat-inactivated EBs were taken up by amoebae and did not multiply as expected. No differences in uptake after incubation with antibodies were observable. Heat-inactivated EBs incubated with high concentrations of anti-Pam-antibody did not agglutinate and no large aggregates of chlamydiae could be seen. It is possible that treatment of EBs at 56°C leads to denaturation of heat-sensitive target sites of this antibody, resulting in lower amounts of anti-Pam-antibody bound to EBs and thus EBs do not aggregate.

These results suggest that pre-treatment of EBs with anti-Pam-antibody or anti-pc1489-antibody does not inhibit infection of amoeba host cells. However, as not all samples from all time points contained amoebae, these results have to be validated by further experiments.

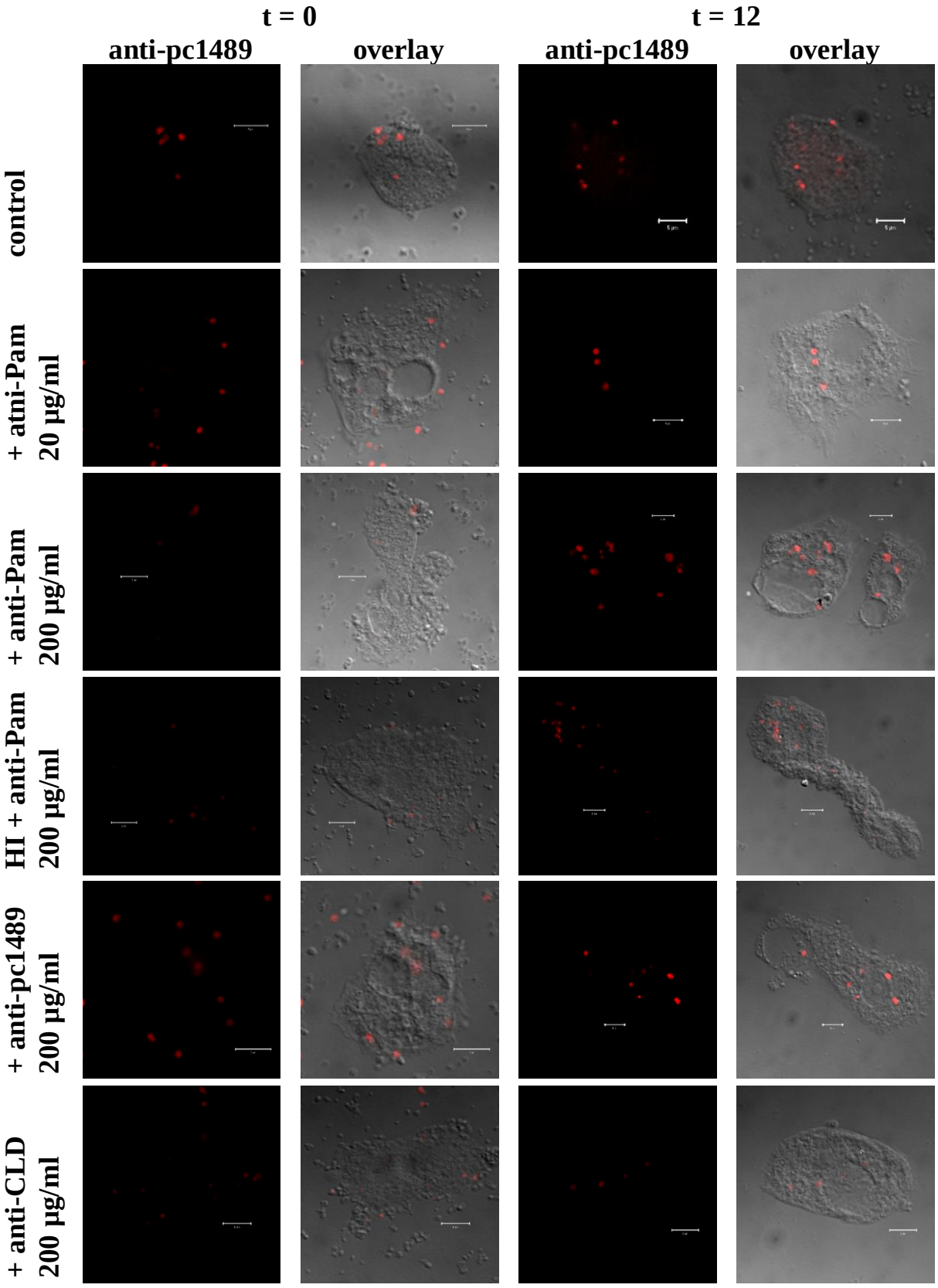

Fig. 18: Infection inhibition assay with anti-Pam-, anti-pc1489- and anti-Cld-antibody for 0 h p.i. and 12 h p.i. Signals for pc1489 and an overlay for pc1489 with the DIC-images is shown. Bars 5 µm

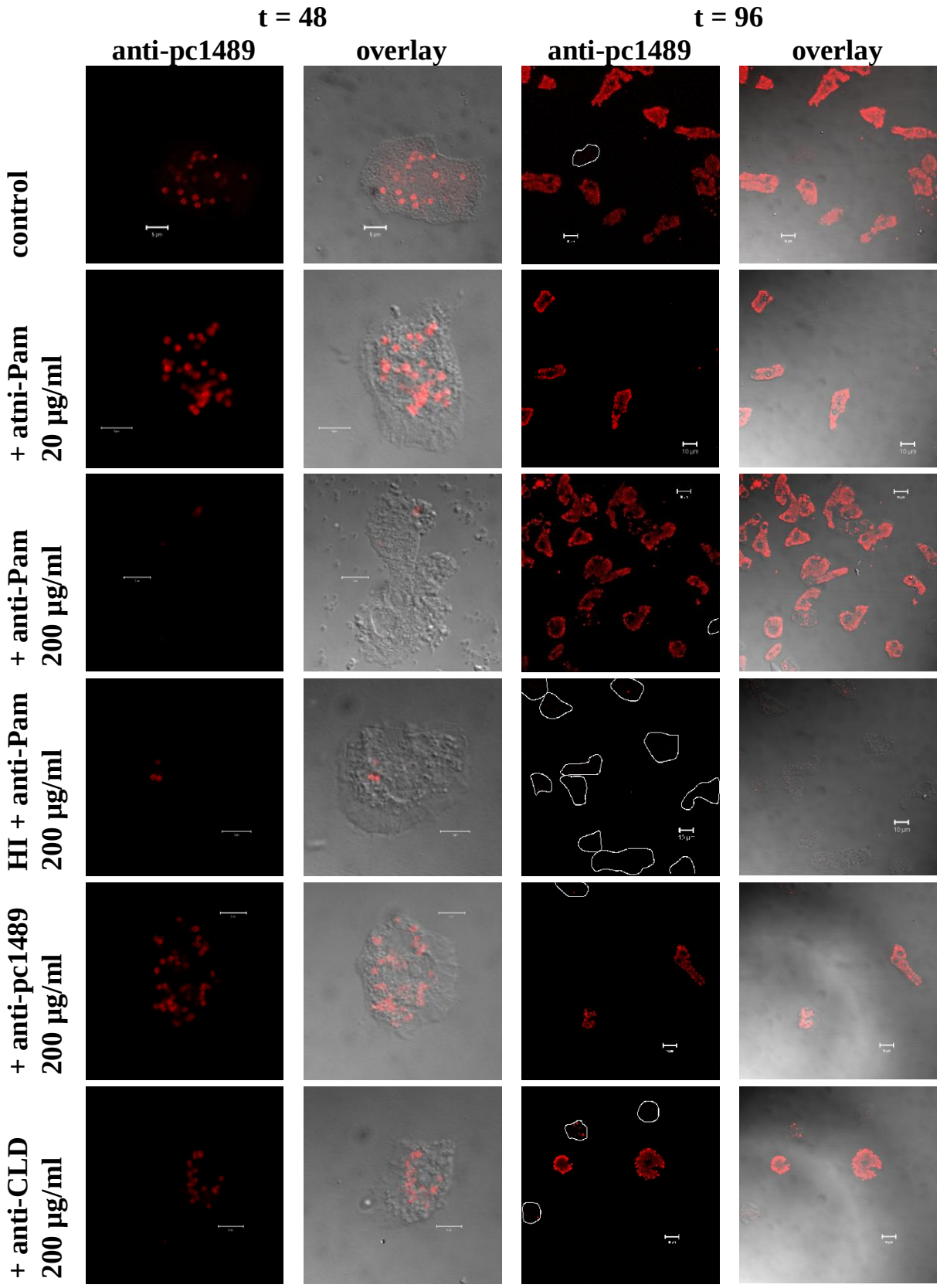

Fig. 19: Infection inhibition assay with anti-Pam-, anti-pc1489- and anti-Cld-antibody for 48 h p.i. and 96 h p.i. Signals for pc1489 and and an overlay for pc1489 with DIC-images is shown. Rows 1 and 2 as well as rows 3 and 4 represent identical microscopic fields. In row 3, the outlines of uninfected amoebae are indicated in white. Bars 5 µm for pictures on the left and 10 µm for pictures in the right panel.

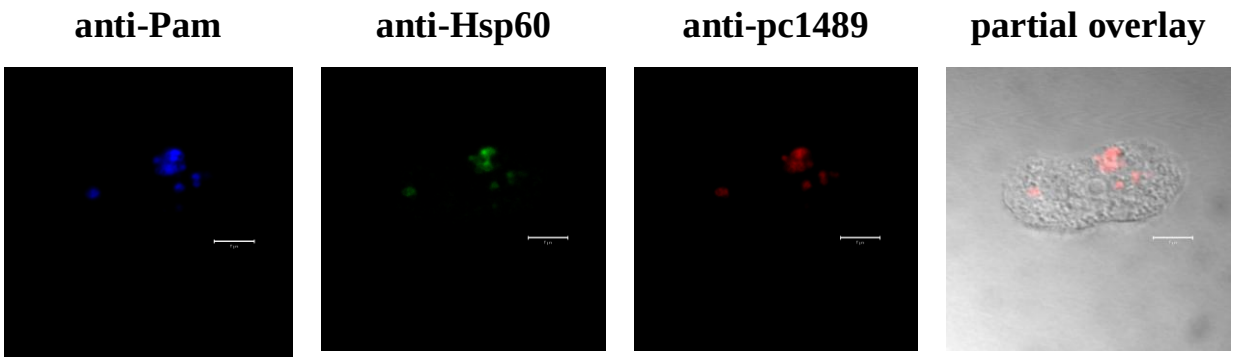

Fig. 20: Activity of huge lumps of chlamydiae taken up by amoebae after preincubation with 200 µg/ml anti-Pam-antibody is shown at 48 h p.i. by increase in Hsp60-signal for these bacteria. On the right, the DIC-channel and the signal of anti-pc1489 is shown in an partial overlay. Bars 5 µm

3.10 Western blot analysis of Incs

The specificity of antibodies against putative Inc - proteins of *P. amoebophila* was tested by the reaction to lysates of *E. coli* cells heterologously expressing the Inc-proteins. Lysates of *E. coli* without plasmid were used as negative control. All antibodies detected their target protein. Sera against pc0156, pc0399 and pc0577 showed strong and specific detection indicated by a strong band at the correct size and almost no background signals. Antibodies targeting pc0530 and pc1111 also showed strong signals for their target proteins, but bound also unspecifically to proteins of *E. coli*, resulting in several unspecific bands (Fig.21). As proteins for immunization of animals were expressed in *E. coli* and purified via a His-tag (47), histidine-rich proteins of *E. coli* might have bound to the column during purification of the overexpressed proteins and been eluted with the Inc-proteins. Upon immunization of animals, these contaminating proteins would give rise to antibodies targeting histidine-rich proteins of *E. coli*.

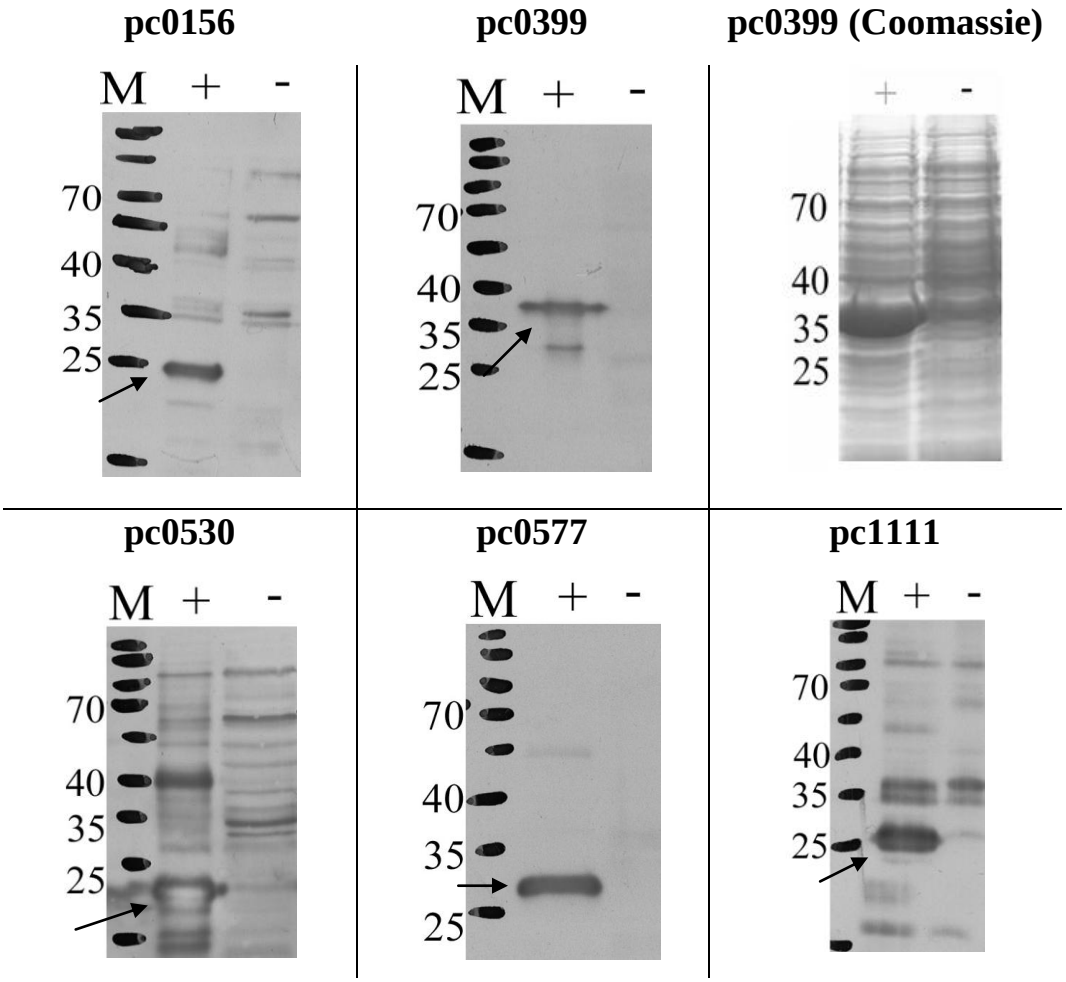

Fig. 21: Western blots showing the specificity of anti-Inc-antibodies to parts of putative Inc-proteins expressed in *E. coli*. Lysates of *E. coli* expressing the Inc-proteins are indicated with +, *E. coli* cells without expression vector with -. Bands for Inc-proteins are indicated by arrows. For pc0399, a SDS gel stained with Coomassie is shown for comparison. Molecular weight marker in kDa on the left of each picture.

3.11 Immunofluorescence analysis with anti-Inc-antibodies

Immunofluorescence analysis showed halo-shaped signals for pc0156, pc0399, pc0530 and pc1111 overlapping with signals of anti-Pam-antibody. Anti-pc0399-antibody and anti-pc0156-antibody bound to all bacteria detected with anti-Pam-antibody, whereas pc0530 and pc1111 were detected in only a subset of bacteria (Fig.22). All antibodies resulted in a high unspecific background even though sera were inactivated against amoeba proteins several times. Pre-immune sera also yielded high unspecific background, but no defined signals were observed. Signals for pc0577 were difficult to distinguish from the high background and were observed in the cytoplasm or the outer membrane.

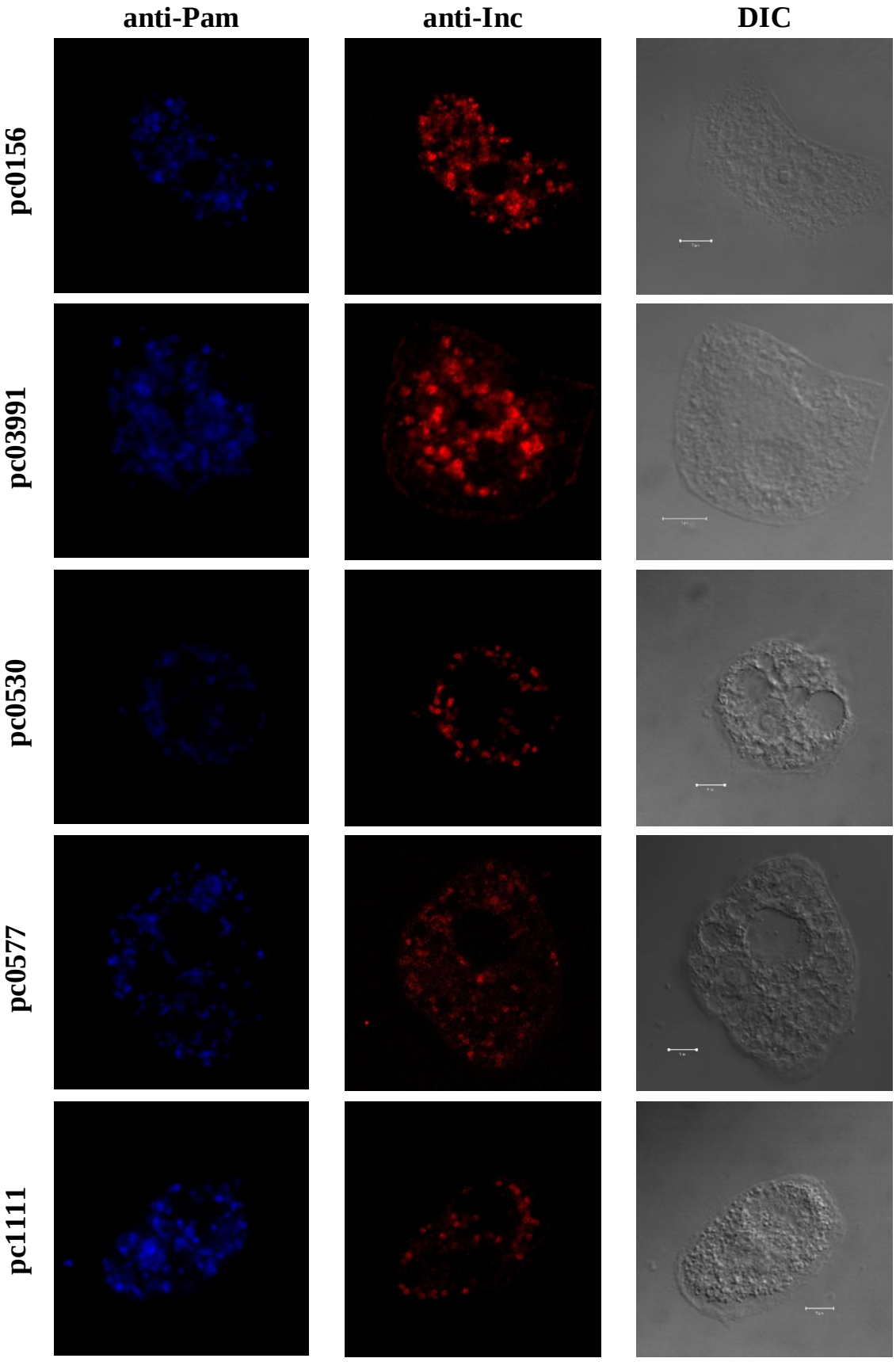

Fig. 22: Immunofluorescence analysis with antibodies targeting the putative Inc-proteins of *P. amoebophila*. Rows 1-3 indicate identical microscopic fields. Bars 5 µm

Differences compared to the pre-immune serum were hard to see for this protein (data not shown).As stated above, differentiation between signals for proteins in the inner membrane, the outer membrane and the inclusion membrane is not possible by immunofluorescence analysis in *P. amoebophila*. To get additional proof for the localization of the putative Inc-proteins in the inclusion membrane, highly-purified elementary bodies fixed with methanol were investigated by immunofluorescence analysis. During high-purification of EBs, the inclusion membrane should be removed as observed by transmission electron microscopy. Highly-purified EBs gave a strong signal with anti-Pam-antibody, but no signal could be observed for the putative Inc-proteins (Fig.23). These results support the localization of these proteins to the inclusion membrane of *P. amoebophila*.

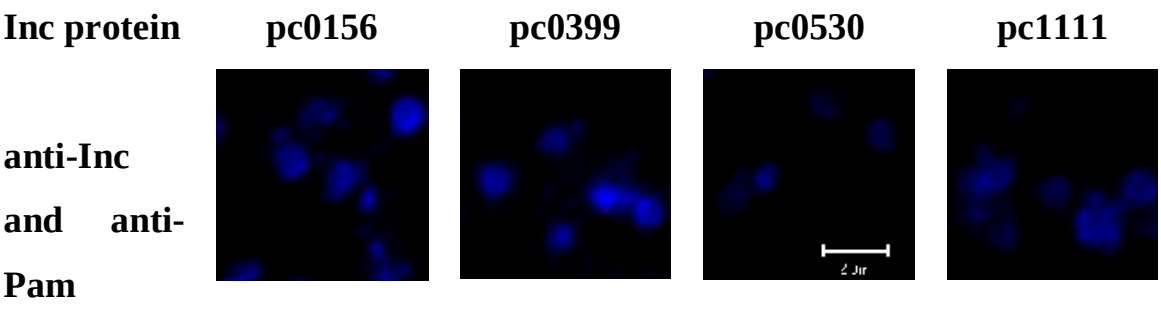

Fig. 23: Immunofluorescence analysis of highly-purified EBs of *P. amoebophila* with anti-Pam-antibody and antibodies targeting four of the putative Inc-proteins. Signals can be seen for anti-Pam-antibody (blue), but not for Inc-proteins (red), because the inclusion membrane is removed during purification. Bar 2 µm

4. Discussion

4.1 Characterization of pc1489

4.1.1 Localization of pc1489

MOMP, a porin with a mass of 40 kDa, is the most abundant component in the outer membrane of *Chlamydiaceae* (15), but interestingly there is no homologous gene encoded in the genome of *P. amoebophila* (19). In a recent study that combined 1D- and 2D- gel electrophoresis with mass spectrometry, a family of putative porins was identified in outer membrane preparations of the chlamydia-like bacterium *P. amoebophila*. The prediction of a beta-barrel structure and the high abundance in preparations of the outer membrane suggested that the two proteins pc1489 and pc1077 function as porins and act as functional replacement of the major outer membrane protein of *Chlamydiaceae* in *P. amoebophila* (47).

In this study, antibodies were raised against pc1489 purified from elementary bodies of *P. amoebophila*. Immunfluorescence demonstrated the localization of this protein in the inner, outer or inclusion membrane of *P. amoebophila* by forming a halo-shaped signal surrounding the bacteria. Localization of pc1489 to the outer membrane was supported by heat modifiability of this protein. Heat modifiability is considered to be a hallmark feature of outer membrane proteins and has been studied in detail for several outer membrane proteins like OmpA from *E. coli* (7) or outer membrane proteins of *Rhodopseudomonas sphaeroides* (64). Due to their high beta-sheet content, outer membrane proteins bind unusually high amounts of SDS. This results in faster migration through the gel compared to other proteins leading to an underestimation of the weight of the protein. However, excessive binding of SDS is reduced by boiling and therefore heating of the samples leads to migration of the protein at the correct size. This behaviour is suggested to result from the conversion of beta-sheets to alpha-helices by heating (85). Pc1489 was migrating at 30 kDa without heating, but an additional band appeared at 40 kDa after heating, showing that the migration behaviour of this protein changes upon heating. This heat modifiability of pc1489 suggests a high beta-sheet content of this protein as also has been proposed by in *silico analysis* (47) and points to a localization in the outer membrane of *P. amoebophila*.

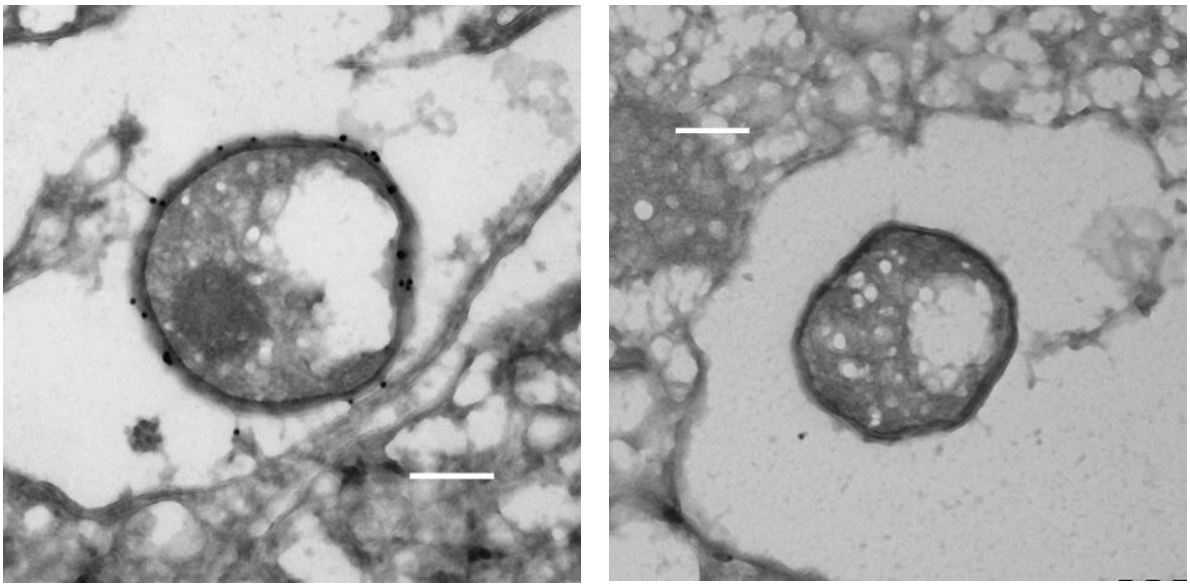

Fig. 24: Pictures taken by transmission electron microscopy using anti-pc1489-antibody (left) and pre-immune serum (right). Bars 200 nm. Pictures courtesy of Jacqueline Montanaro

The final proof of a localization of pc1489 to the outer membrane has recently been given by immuno-transmission electron microscopy studies by Jacqueline Montanaro. Gold particles are located at the membrane of *P. amoebophila*, but not the inclusion membrane or the

cytoplasm of the bacterium (Fig. 24). To investigate the presence of a homologue of pc1489 in a member of the same family but different genus than *P. amoebophila*, immunofluorescence was performed with *P. acanthamoeba*. The detection of a signal in a subset of bacteria is well-supported by the identification of a homologue of pc1489 by *in silico* analysis in *P. acanthamoeba*, suggesting the recognition of a protein similar to pc1489 in this bacterium by the anti-pc1489-antibody. This protein could be identical with the one identified by *in silico analysis* and assigned to the same Pfam domains as members of the putative porin family of *P. amoebophila* (pc0870, pc1077, pc1489 and pc1860) was identified (47). The detection of a protein in *P. acanthamoeba* by anti-pc1489-antibody is rather surprising, as antibodies against MOMP are often strain-specific in clinical chlamydiae and serovar-specific in *C. trachomatis*, where they are used for serotyping (102, 104).

The low number of detected cells could be the result of differences in the organisation of the outer membrane in EBs and RBs of *P. acanthamoeba*, for example due to extensive cross linking of proteins in EBs that would mask binding sites for the anti-pc1489-antibody in proteins of the outer membrane. This hypothesis is supported by the finding

that serum raised against whole EBs and therefore targeting proteins of the outer membrane of EBs only binds to a subset of bacteria, presumably EBs in immunofluorescence analysis (Fig.12). It is also possible that the antibody does not fit the protein detected in *P. acanthamoeba* perfectly and therefore less of the antibody is bound, leading to fewer cell being detected. To investigate this further, Western blot analysis with samples of *P. acanthamoeba* should be performed and differences in band patterns between purified EBs and bacteria isolated at a time point in the developmental cycle when mainly RBs are present, should be compared. It will be also interesting to contrast band patterns of *P. acanthamoeba* with *P. amoebophila*.

Pc1489 was shown to be present in *P. amoebophila* throughout the whole developmental cycle, demonstrating the presence of this protein in EBs and RBs comparabe to MOMP of *Chlamydiaceae*. The observed increase in signal intensity at later time points could be the result of incorporation of newly synthesized pc1489 into the outer membrane, thereby increasing the amount of pc1489 present. It could also result from changes in the structure of pc1489 which lead to better binding of anti-pc1489-antibody to its target protein or be due to different fixation behaviour of different samples.

4.1.2 Functional characterization of pc1489

In SDS-PAGE gels with purified pc1489 and also in Western blot analysis, weak bands at 55-60 kDa were observed in addition to bands at 30 kDa or 40 kDa. This raises the question about the organization of pc1489 in the outer membrane of *P. amoebophila*. "Classic porins" of other Gram-negative bacteria are usually found as trimers in the outer membrane, although monomeric forms of porins are known as well (109, 124). These trimers have been reported to be extremely stable even in the presence of SDS and to dissolve only by heating above 60°C in SDS (76, 84). This is the case for most known porins, though exceptions from this rule like the unusually labile PorB-trimers of *Neisseria gonorrhoeae* exist, that dissolve in the presence of SDS at room temperature (89). MOMP of *Chlamydiaceae* is also present in a trimeric conformation in the outer membrane and trimers of this proteins only dissociate upon heating to 95°C (74). The band found at 55 kDa in Western blots with purified pc1489 could represent the trimeric form of pc1489, because due to the unusual binding of SDS trimers of other porins of Gram-negative bacteria migrate with a molecular mass of 65 to 70 kDa on SDS-PAGE gels (13). If pc1489 is present as trimer in the outer membrane of *P. amoebophila*, it

could be unusually labile, because even for samples in buffer without DTT that were not heated, the band at 30 kDa, representing the monomer, was dominant. It is possible that multimers of pc1489 were destroyed during purification, although similar protocols are used for extraction of other porins and were not reported to destabilize these protein complexes. However, pc1489 trimers could be comparable to PorB of *Neisseria gonorrhoeae* and dissolve in the presence of SDS at room temperature. As in asynchronized cultures of amoebae infected with *P. amoebophila* the most prominent band is observed at around 55 kDa in Western blot analysis, this would support the presence of multimers of pc1489. However, these results suggest that multimers would then be quite stable as they were not broken by heating of samples to 95°C, but that the multimers would be somehow destroyed during purification of pc1489 from elementary bodies.

If pc1489 is present as a monomer in contrast to MOMP of *Chlamydiaceae*, bands at higher molecular weight could result from interactions with other proteins of the outer membrane, for example the cystein-rich proteins pc0616 and pc0617. This is supported by the finding that other outer membrane proteins were identified in the same bands as pc1489 by mass spectrometry (47). In order to further analyze the organization of pc1489 in the outer membrane, cross linking experiments of outer membrane fractions should be performed.

The putative function of pc1489 as a porin was confirmed by lipid bilayer measurements that showed a stepwise increase in conductance after addition of pc1489 to lipid bilayers, each step indicating incorporation of one pore into the membrane. The single-channel conductance for this porin was 3.25 nS in 1 M KCl and thereby considerably higher than those observed for the so-called “classic porin” OmpF of *E. coli* (0.7 nS) (89). It is also higher than single-channel conductance values observed for MOMPs of the *Chlamydiaceae*. MOMP of *C. trachomatis* and *Chlamydophila psittaci* have a single-channel conductance of 1 nS and 1.5

nS in 1 M KCl, respectively (57). This shows that major outer membrane proteins of members of the *Chlamydiaceae* differ from each other and porins from environmental chlamydiae in their porin activity. Values observed for porins of other Gram-negative bacteria range from 10 pS for Tsx of *E. coli* (11) up to extremes of 20 nS for the thermophilic bacterium *Thermus thermophilus* (70).

Porin	Organism	Configuration	Single channel conductance in 1 M KCl	Size of pore	Reference
MOMP	*C. trachomatis*	Trimer	1 nS	2 nm	(57, 110)
MOMP	*C. psittaci*	Trimer	1.5 nS	not known	(57)
OmpA	*E. coli*	Monomer	50-80 pS or 260-320 pS*	1 nm	(109)
OmpF	*E. coli*	Trimer	0.7 nS	1.2 nm	(89)
OmpG	*E. coli*	Monomer	0.81 nS**	1.2 nm	(20)
pc1489	*P. amoebophila*	Monomer or Trimer	3.25 nS	not known	
PorB	*N. gonorrhoeae*	Trimer	1 nS***	1.6 nm	(76)

Table 15: Characteristics of a few porins of gram-negative bacteria

***the two different values presumbably correspond to two different conformational states of this porin**

**** measured in 1 M NaCl**

***** measured in 200 mM NaCl**

Dependence of the single-channel conductance on the concentration of KCl was shown, indicating the presence of a wide water-filled pore formed by pc1489 in lipid bilayers, although the exact size of pore has not been determined. Formation of a wide pore has also been demonstrated for the major outer membrane proteins of *C. psittaci* and *C. trachomatis* (57). The pore formed by MOMP is proposed to have a diameter of 2 nm based on penetration rates of sugars of different sizes through MOMP in liposome swelling assays (110). With a proposed diameter of 2 nm, the pore formed by MOMP is considerably larger than channels formed by other Gram-negative bacteria. Diameters range from 0.3 nm for Tsx over 0.7 nm for OmpF and 1.2 nm for the large porin OmpG of *E. coli* (89, 107).

The porin pc1489 was shown to be selective for anions with a Pc/Pa-ratio of 0.48 for KCl, similar to that measured for native MOMP (0.5) by Wyllie *et al.* (121). The same authors reported slight cation selectivity for a truncated recombinant MOMP of *C. psittaci* and *C. pneumoniae* and for a full-length recombinant construct of *C. psittaci* and *C. trachomatis* in later studies (57, 122). They suggested that small proteins or molecules bound to the channel present in native preparations might have modified selectivity of MOMP in their

previous study (57). MOMPs of *C. psittaci* and *C. trachomatis* were shown to differ significantly in ion selectivity at certain KCl concentrations, again pointing out differences in this channel for different members of the *Chlamydiaceae*. Removal of the largest variable domain of MOMP, VS4, made the pore even more cation selective, suggesting that selectivity for cations or anions results from variable domains exposed to the surface (57). As these domains vary largely between different members of the *Chlamydiaceae*, this will result in different ion selectivities of MOMPs. Indeed, different selectivities were reported in other studies where cation selectivity (32) and no selectivity for anions or cations was reported (60). These variable results may not only result from different organisms used, but also from the use of native MOMP or different recombinant constructs in different studies. As it is quite difficult to express the full-length MOMP of some chlamydiae in *E. coli*, truncated constructs are often used, probably resulting in differences in folding of the protein and therefore other pore-forming characteristics. In this study, native pc1489 was used to determine selectivity for anions. Therefore, biased results due to recombinant expression can be excluded.

As a family of four putative porins with an amino acid sequence identity of 22-28 % was identified in *P. amoebophila*, characterization of the other members of this family will be interesting. The three major porins of *E. coli*, OmpC, OmpF and PhoE share ~60 % homoloy and differ slightly in their ion-selectivity and pore size (77). The expression of *ompC* and *ompF* was shown to be regulated by the omsotic strength of the medium, whereas *phoE* is expressed under phosphate limitation (reviewed in 90). In *C. trachomatis*, a protein with 20.4% sequence identity to MOMP was identified. This protein, termed PorB, was only observed in small amounts in the COMC and a more specific function of this porin compared to MOMP was suggested (65). Similiarly, different members of the putative porin family of *P. amoebophila* could be expressed at different time points in the developmental cycle or differ in ion selectivity. Further analysis should be performed to show whether the other members of this family do indeed function as porins and how they differ from each other.

To sum up, pc1489 was shown to be localized to the outer membrane of *P. amoebophila*, to be present during the whole developmental cycle of this organism and to function as porin in this study.

4.2 Influencing the infectivity of *P. amoebophila*

4.2.1 Inhibition of infection by pre-incubation with antibodies

It has been shown more than 40 years ago that incubation of chlamydiae with sera prepared against whole chlamydiae resulted in inhibition of infection of host cells by these organisms (e.g. 55). Caldwell and Perry (16) were the first to show that incubation of EBs with antibodies targeting MOMP of *C. trachomatis* resulted in neutralization of infection of host cells. They reported that incubation of EBs with antibody did not influence the attachment or entry, but inhibition of infection occurred at a later time point in the infection cycle. Later studies using antibodies targeting MOMP resulted in either inhibition of attachment (106, 114) or in neutralization of infection at steps after internalization of the chlamydiae (92). The results of these studies suggest that different antibodies against MOMP, targeting different regions of this protein, lead to neutralization of infection at different time points. Prevention of attachment can be easily explained by masking of domains important for binding to host cells, comparable to reports about neutralization of infection by antibodies against the outer membrane proteins OmcB or PmpD (78, 115). Neutralization at later time points is more difficult to explain. The study by Caldwell and Perry (16) also demonstrated that inhibition of infection after uptake of cells depended on the use of intact dimeric IgG molecules, because Fab fragments targeting MOMP did not neutralize infectivity. Based on this observation, they suggested that effects on infectivity of *C. trachomatis* could result from higher structural rigidity of the outer membrane due to cross linking by IgG, thereby preventing transformation of EBs to RBs or inhibiting transport of host-derived nutrients to the bacteria. Alternatively, they proposed that cross linking of MOMP by IgG molecules could restrict fluidity of the outer membrane and prevent other components of the outer membrane from interacting with host receptors (16).

In contrast to the pronounced effects of incubation with anti-MOMP-antibody on infectivity of members of the *Chlamydiaceae*, no effect was observed on the infectivity of *P. amoebophila* after incubation with two different antibodies. Anti-Pam-antibody was raised against whole EBs of *P. amoebophila*. EBs that were incubated with this antibody were still taken up by host cells and were able to multiply within amoebae comparable to non-treated controls. No effect was observed for pre-incubation with antibodies targeting pc1489, a protein thought to be the functional replacement of MOMP in *P. amoebophila*. These results suggest that neither pc1489 nor proteins targeted by anti-Pam-antibody play

a role in attachment to amoeba host cells. *P. amoebophila* might attach to amoebae by means of other proteins that are not targeted by these antibodies, it might attach to amoebae by unspecific interactions (e.g. electrostatic) or might just be taken up by amoebae like other bacteria on which these protozoa feed.

In addition, the obtained results show that multiplication is not blocked by treatment with antibodies in *P. amoebophila* in contrast to *C. trachomatis* (16) and therefore either no cross linking of the outer membrane occurs or cross linking does not influence outer membrane rigidity or fluidity. It is possible that cross linking is not as effective as in *C. trachomatis*, because pc1489 might not be as abundant in the outer membrane as MOMP that is thought to make up about 60% of the outer membrane of *Chlamydiaceae* (15). On the other hand, inhibition of infection has also been observed for pre-incbuation with antibodies targeting PorB, a porin of *C. trachomatis* that is present in the outer membrane in lower amounts than MOMP (65).

Although differences in the abundance of pc1489 and MOMP could account for differences in inhibition of infection by antibodies targeting these proteins, finding an explanation for no observed effect by treatment with serum against *P. amoebophila* is more difficult.

It was shown that EBs of *C. psitta*ci treated with serum against this bacterium were taken up by macrophages and destroyed rapidly, because they lost the ability to prevent phagolysosomal fusion (123). In contrast, *P. amoebophila* EBs treated with anti-Pam-antibody were not digested by amoebae, but multiplied within these cells. Therefore, *P. amoebophila* must be able to escape from the endocytic pathway even after blocking proteins of the outer membrane. These observations are similar to results obtained for the intracellular bacteria *Legionella pneumophila* and *Coxiella burnettii*. In both organisms, treatment with sera against these organisms does not inhibit intracellular multiplication (49, 54).

In *P. amoebophila*, proteins in the inclusion membrane could participate in escape of the bacterium from the endocytic pathway. Inc proteins identified in *P. amoebophila* differ substantially from those of clinical chlamydiae (47) and therefore could account for differences in the ability to survive phagocytosis even after preincubation with antibodies. However, it can not be excluded that antisera used in this study did not crosslink outer membrane proteins of *P. amoebophila* in a way comparable to sera against *C. psittaci* or other members of the *Chlamydiaceae*.

Additionally, it has to be considered that *A. castellanii* Neff is not the native host of *P. amoebophila*. Maybe effects of antibody-treatment on infectivity would be observable in the native host. If outer membrane proteins of *P. amoebophila* are adapted to attachment to only the native host, they would not be required for uptake by *A. castellanii*. In this case, *A. castellanii* that feed on bacteria would take up this bacterium unspecifically. However, as the native host of *P. amoebophila* is not known yet, it is not possible to investigate this issue further at the moment.

4.2.2 Inhibition of infection by heat-treatment

Members of the *Chlamydiaceae* lose their infectivity upon heat treatment and heat-labile components of the outer membrane were suggested to be the cause for this, although slightly contradictory results were reported, maybe generated by different incubation conditions and different host cell lines used (14, 16, 33, 123) .

Heat treatment resulted in loss of infectivity of *P. amoebophila*. Heat treated cells were taken up by amoebae comparable to non-treated control cells, but were not able to multiply. As no increase of Hsp60-signal, that is thought to be a sign of metabolically active cells, was observed for the treated cells, heat-inactivated EBs might be unable to convert to metabolically active RBs. Intriguingly, anti-Pam-signals for *P. amoebophila* cells were observed up to 96 h p.i. in amoebae and morphology of cells was preserved up to 72 hours. This stands in contrast to rapid destruction of heat treated *C. psittaci* in macrophages (123) and also to the fact that *E. coli* normally taken up by amoebae are digested within 72 hours (Illias Lagkouvardos, personal communication). The observation that heat inactivated chlamydiae are not immediately destroyed by amoebae is supported by a report by Greub (39) who found heat-inactivated *P. acanthamoeba* cells in macrophages as long as 48 hours post infection. Because this study also showed that the lysosomal hydrolase Cathepsin D, an enzyme involved in caspase activation, did not colocalize with heat-inactivated bacteria, Greub suggested a passive modulation of the intracellular fate of heat-inactivated *P. acanthamoeba*, maybe by a special composition of the bacterial outer membrane. As EBs were incubated for a longer period of time and at higher temperatures in the study by Greub in comparison to this study, this putative protective effect could be even more pronounced for treatment in this study and explain the observed results.

4.3 Inc-proteins of *P. amoebophila*

Modification of the host-derived membrane of the inclusion by incorporation of chlamydial Inc-proteins was discovered more than 10 years ago (93). The exact role of most of these proteins for interactions with the host is not known, but they are considered as important means for the communication between host cell and chlamydiae, to play a role in transport of nutrients to the chlamydiae and to ensure survival of these bacteria within the host cell.

Recently, an *in silico* approach identified 24 putative Inc-proteins in the genome of the chlamydia-like bacterium *P. amoebophila* (47). Only three of the predicted Inc-proteins showed some sequence similarity to proteins of the *Chlamydiaceae* and one, pc0399, showed weak sequence similarity to an inclusion membrane protein, IncA, of *Chlamydiaceae* (47). For four of these proteins, pc0399, pc0156, pc0530 and pc1111, localization in the inclusion membrane is supported by halo-shaped signals observed in immunofluorescence analysis. In addition, no signal was detected for these proteins in highly-purified EBs where no inclusion membrane should be present. Although additional proof concerning the localization of these proteins is needed, the obtained results and the presence of a large bilobed hydrophobicity motif, a key feature of known Inc-proteins, point to a localization of these proteins to the inclusion membrane. Transmission electron microscopy studies are in progress to show the exact localization of these proteins.

Inc-proteins of *P. amoebophila* are of special interest, as this chlamydia-like bacterium resides within single-cell inclusions and the inclusions inhabited by this organism do not fuse in contrast to members of the *Chlamydiaceae* (19). As Inc-proteins and especially the IncA protein are thought to play an important role in the fusion of inclusions, probably by means of a SNARE-like motif recently identified in IncA (27) that is not present in pc0399, differences between Inc-proteins of *P. amoebophila* and *Chlamydiaceae* could help to a better understanding of the function of these proteins.

5. Summaries

5.1 Summary

Proteins in the outer membrane of chlamydiae are of great importance as they are involved in attachment to host cells, uptake of nutrients, removal of waste and also in the differentness of the two developmental stages of chlamydiae. Therefore and because of their potential as vaccine candidates, these proteins have been studied extensively for members of the *Chlamydiaceae*. Sequencing of the genome of the first member of the environmental chlamydiae, the amoeba endosymbiont *Protochlamydia amoebophila*, showed that no gene similar to the most abundant outer membrane protein of the *Chlamydiaceae*, the major outer membrane protein (MOMP), is encoded (19). In a recent study, a family of putative porins was identified in the genome of *P. amoebophila*. Two of these porins were found to be highly abundant on 2D gels of outer membrane preparations of this organism and predicted to form beta-barrels by *in silico* analysis (47).
In this study, the putative porin pc1489 was purified from elementary bodies of *P. amoebophila* and antibodies were raised against this protein. The localization of pc1489 to the outer membrane was confirmed by immunofluorescence analysis and immuno-transmission electron microscopy. It was shown that the protein is present throughout the whole developmental cycle, thereby indicating an important role of this protein for *P. amoebophila*. Its function as a porin was confirmed by lipid bilayer measurements using purified pc1489. These measurements showed that the pore formed by pc1489 is quite large and anion selective. These results suggest that pc1489 might be the functional replacement of MOMP in *P. amoebophila,* possibly together with other members of the putative porin family. However, in contrast to MOMP, preincubation of EBs with antibodies targeting pc1489 as well as antibodies against the immunodominant components of the outer membrane did not inhibit infection of host cells by *P. amoebophila*.
In the second part of this diploma thesis, the localization of putative inclusion proteins in *P. amoebophila* was studied. Four of the five proteins studied where shown to be localized to either the outer membrane or the inclusion membrane of *P. amoebophila* by immunofluorescence analysis. Although the function of these proteins is not known yet, they are suggested to play an important role in communication between symbiont and host and also in survival of *P. amoebophila* after internalization by amoebae.

5.2 Zusammenfassung

Außenmembranproteine spielen eine wichtige Rolle für Chlamydien, da sie für die Bindung an Wirtszellen, die Aufnahme von Nährstoffen in die Zelle und die Entfernung von Stoffwechselendprodukten benötigt werden und zusätzlich zu Unterschieden zwischen den beiden Entwicklungsstadien der Chlamydien beitragen. Da sie viel versprechende Kandidaten für die Entwicklung von Impfstoffen sind, sind die Außenmembranproteine der *Chlamydiaceae* gut erforscht. Im Gegensatz dazu ist wenig über diese Proteine in der kürzlich entdeckten Gruppe der Umweltchlamydien bekannt. Im ersten sequenzierten Genom eines Vertreters dieser Gruppe, *Protochlamydia amoebophila*, wurde kein Homolog für das häufigste Außenmembranprotein der *Chlamydiaceae*, das Major outer membrane protein, gefunden (19). Vor kurzem wurde eine Familie von putativen Porinen in *P. amoebophila* gefunden. Zwei dieser Proteine, pc1489 und pc1077, waren in großen Mengen auf 2D-Gelen mit Außenmembranfraktionen vorhanden (47).

In dieser Diplomarbeit wurde das putative Porin pc1489 aus Elementarkörpern aufgereinigt und Antikörper gegen dieses Protein hergestellt. Unter Verwendung dieser Antikörper wurde mittels Immunfluoreszenzanalyse und Transmissionselektronenmikroskopie gezeigt, dass pc1489 in der Außenmembran von *P. amoebophila* lokalisiert ist. Pc1489 konnte während des gesamten Entwicklungszyklus in der Außenmembran von *P. amoebophila* detektiert werden, was auf eine wichtige Rolle dieses Proteins hinweist. Lipid Bilayer Messungen zeigten, dass pc1489 tatsächlich die Funktion eines Porins besitzt und große, anionen-selektive Proteine in Lipidmembranen formt. Diese Ergebnisse deuten darauf hin, dass pc1489 in *P. amoebophila* das Major outer membrane protein ersetzen könnte, möglicherweise gemeinsam mit anderen Mitgliedern der putativen Porinfamilie.

Im zweiten Teil dieser Diplomarbeit wurden putative Inklusionsproteine von *P. amoebophila* bezüglich ihrer Lokalisation untersucht. Immunfluoreszenzanalyse mit Antikörpern gegen diese Proteine zeigte, dass 5 der 4 untersuchten Proteine entweder in der Außenmembran oder der Inklusionsmembran von *P. amoebophila* lokalisiert sind. Obwohl die Funktion dieser Proteine noch nicht bekannt ist, wird vermutet, dass sie eine wichtige Rolle in der Kommunikation mit der Wirtszelle spielen und dadurch auch den Verdau dieser Bakterien durch Amöben verhindern könnten.

6. List of abbreviations

λ	Wavelength
16S rRNA	Small ribosomal RNA subunit of prokaryotes
1D	One dimensional
2D	Two dimensional
A	Adenine
A.	*Acanthamoeba*
ABC	Ammonium bicarbonate
ACN	Acetonitrile
Amp	Ampicillin
APS	Ammonium persulfate
ATP	Adenosine-5'-triphosphate
bp	Base pair(s)
BSA	Bovine serum albumin
C	Cytosine
C.	*Chlamydia*
CLSM	Confocal laser scanning microscope
COMC	Chlamydia outer membrane complex
Cy2	5,5`-di-sulfo.1,1`-di-(X-carbopentynyl)-3,3,3`,3`-tetra-methylindol-Cy2.18-derivative N-hydroxysuccimidester
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
DAPI	4',6-Diamidino-2-phenylindol
DIC	Differential interference contrast
DDT	Dithiothreitol
DNA	Desoxyribonucleic acid
dNTP	Desoxynucleotide triphosphate
E.	*Escherichia*
EB(s)	Elementary body (-ies)
e.g.	example given

et al.	Et alterti (lat. and others)
EtOH	Ethanol
Fig.	Figure
FITC	Fluorescein isothiocyanate
g	Gram(s); gravity
GTP	Guanosine-5'-triphosphate
h	Hours
H_2O_{dd}	Double distilled water
h p.i.	hours post infection
HCl	hydrochloric acid
His	Histidine
HRP	Horseraddish-Peroxidase
Hsp	Heat shock protein
Ig	Immunglobulin
Inc	Inclusion
IPTG	Isopropyl-β-D-thiogalactopyranosid
k	Kilo
KCl	Kalium chloride
L	Liter(s)
LB	Luria Bertani
LC	Liquid chromatography
LiCl	Lithium chloride
µ	Mikro
m	Meter(s); milli
M	Molar
MALDI	Matrix-assisted laser desorption/ionization
mg	Milligramm
min	Minute(s)
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
n	Nano
NaCl	Sodium chloride

NaOH	Sodium hydroxide
P.	*Parachlamydia; Protochlamydia*
P_a	Permeability coefficient for an anion
P_c	Permeability coefficient for a cation
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMP	Polymorphic outer membrane protein
RB(s)	Reticulate body (-ies)
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
S	Siemens
SDS	Sodium dodecyl sulfate
sec	Second(s); section
SNARE receptors	Soluble N-ethylmaleimide-sensitive factor attachment protein
sp.	Species
T	Thymine
TEMED	N,N,N´,N´-tetramethylethane-1,2-diamine
Tris	Trishydroxymethylaminomethane
TSY	Trypticase Soy Broth with Yeast Extract
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volt
v/v	Volume per volume
W	Watt
w/v	Weight per volume
w/w	Weight per weight

7. References

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9. Curriculum Vitae

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