Identification, molecular cloning and biophysical characterization of channel forming proteins in *Caulobacter crescentus* and *Legionella pneumophila*

by

Farhan Younas

A Thesis submitted in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy in Biotechnology

Approved Dissertation Committee

Prof. Dr. h. c. Roland Benz
Jacobs University Bremen gGmbH

Prof. Dr. Mathias Winterhalter
Jacobs University Bremen gGmbH

Prof. Dr. Miguel Viñas Ciordia
University of Barcelona, Spain

Date of Defense: 02.11.2016

Department of Life Sciences and Chemistry

Statutory Declaration

(Declaration on Authorship of a Dissertation)

I, Farhan Younas, hereby declare, under penalty of perjury, that I am aware of the consequences of a deliberately or negligently wrongly submitted affidavit, in particular the punitive provisions of § 156 and § 161 of the Criminal Code (up to 1 year imprisonment or a fine at delivering a negligent or 3 years or a fine at a knowingly false affidavit).

Furthermore I declare that I have written this PhD thesis independently, unless where clearly stated otherwise. I have used only the sources, the data and the support that I have clearly mentioned.

This PhD thesis has not been submitted for the conferral of a degree elsewhere.

Bremen, Germany 06.10.2016
Place Date

Signature

Dedicated to

My late Abba and Amma ji

List of Contents

Abstract iv

Acknowledgments v

Aim and outline of the thesis vii

Chapter 1 1

Chapter 1. General Introduction 2

1.1.Porins: 2

1.1.1.Short history and structure. 2

1.1.2. Outer membrane integration 3

1.1.3. Roles of Porins 4

1.2. Membrane channels reconstitution and Black lipid bilayer assay 6

Chapter 2 8

Chapter 2. OmpW of *Caulobacter crescentus* functions as an outer membrane channel for cations 9

Summary 9

2.1. Introduction 11

2.1.1. *C. crescentus* and its life style 11

2.1.2. OmpW family 13

2.2. Materials and methods 15

2.2.1. Bacterial culture conditions 15

2.2.2. His-tagged OmpW expression 15

2.2.3. Dot blot 17

2.2.4. Purification of his-tagged OmpW 17

2.2.5. Dialysis of the purified his-tagged OmpW and his-tag cleavage 18

2.2.6. Cloning for expression of native OmpW 18

2.2.7. Expression, purification and refolding of the native protein 21

2.2.8. Maintenance of *C. crescentus* Cultures, knockout of its native *ompW* and extraction of cell wall extracts. 22

2.2.9. Biophysical characterization using black lipid bilayer assay 23

2.3. Results 25

2.3.1. Biophysical characterization of PBS/EDTA extracts from *C. crescentus* 25

2.3.2. Purification of 20-22 kDa protein from extracts and biophysical characterization 26

2.3.2. Partial sequencing of the 20-22 kDa protein and alignments 28

2.3.3. Sequence alignments and homology model of OmpW *C. crescentus* 29

2.3.4. his-tagged overexpression in *E. coli* 31

2.3.5. Signal peptide prediction and overexpression of OmpW in inclusion bodies 32

2.3.6. Overexpression of OmpW in the outer membrane 33

2.3.7. MALDI confirmation 35

2.3.8. Knockout of *ompW* from wildtype *C. crescentus* 36

2.4. Discussion 38

Chapter 3 40

Chapter 3. Identification, molecular cloning and biophysical characterization of Lpg1974, a homologue of hVDAC-1 in *Legionella pneumophila.* 41

Summary 41

3.1. Introduction 43

3.2. Materials and methods 46

3.2.1. Bacterial cells and culturing conditions 46

3.2.2. Sequence analysis, secondary structure prediction and homology model of Lpg1974. 46

3.2.4. Cloning of *lpg1974*Δ1-21, expression, purification and biophysical characterization of the encoded protein by black lipid bilayer assay 49

3.3. Results 51

3.3.1. Initial sequence alignments and molecular dynamics simulations of Lpg1974 and hVDAC-1. 51

3.3.2. Over expression of Lpg1974-GST and GST tag cleavage 53

3.3.3. Biophysical characterization of Lpg1974 55

3.3.4. Signal peptide prediction for Lpg1974 and hVDAC-1 57

3.3.5. Expression and purification of Lpg1974Δ1-21 59

3.3.6. Biophysical characterization of Lpg1974 Δ1-21 60

3.4. Discussion 63

Outlines and Conclusions 66

List of Publications 67

References 68

List of Figures 80

Abstract

Caulobacter crescentus is well-known for its unique dimorphic life style. It is used as a model organism to study cell division and differentiation. A range of interesting features constitute its unique nature. It is normally found in dilute organic environments and was believed to be lacking any genes coding for porin like proteins. We found channel forming activity in the enriched cell wall extracts of the organism. The protein responsible for the porin like activity was found to be a member of the OmpW family. The protein formed small cation selective channels in artificial lipid bilayers. In order to confirm that the studied protein is responsible for the channel forming activity, an *ompW* knockout strain of *C. crescentus* was developed. Enriched outer membrane extracts from the mutant strain did not show channel forming activity.

We also identified and characterized a homologue of hVDAC-1 in *Legionella pneumophila*. *L. pneumophila* has genes coding for a range of eukaryotic like protein. We were especially interested in the gene *lpg1974* which codes for Lpg1974, a protein which had reasonable similarity to hVDAC-1. The protein was found to produce large anion selective channels in artificial lipid bilayers. We also developed a homology structure for the protein which had remarkable similarity to hVDAC-1. Here we further studied the properties of the protein, by expressing the protein without its predicted N-terminal signal peptide (Lpg1974Δ1-21). There are a series of diverse reports about the importance of the N-terminal sequence. The truncated protein formed large anion selective channels. The voltage sensitivity of the protein was not affected by the signal peptide deletion.

Acknowledgments

The duration of my PhD was the most challenging period of my life. I faced challenges every day, overcoming them and at the end of the day, keeping the motivation and optimism up was not an easy job. However, the hard work and troubles I faced during the time are nothing, compared to what I have gained from the experience.

Of course, I could not have done this alone. There are many people who I want to acknowledge for their never-ending support and motivation.

The very first person coming to my mind when I think about the acknowledgments is undoubtedly **Prof. Dr. h.c. mult. Roland Benz**. I can never acknowledge him enough in words. I am heartedly grateful to him for granting me an opportunity to be a part of his team. Throughout the years, he was a never-ending source of inspiration, knowledge and optimism for me. Every single meeting with him was like a door to new ideas.

I am also thankful to **Prof. Dr. Mathias Winterhalter** and **Prof. Dr. Miguel Viñas Ciordia** for their time-to-time suggestions, comments and being a part of my thesis committee. I would also like to thank Prof. Dr. Mathias Winterhalter for having me as a part of his research group and providing an excellent working environment.

I would also like to thank all my previous and current colleagues: Dr. Ivan Barcena Uribarri, Dr. Narges Abdali, Dr. Nafiseh Soltanmohammadi, Dr. Daniel Pletzer, Dr. Naresh Niranjan Dhanasekar, Dr. Giuseppe Federico Amodeo, Dr. Jules Philippe, Dr. Remi Terrasse, Satya Pratyusha Bhamidimarri, Ishan Ghai, Lorraine Benier, Usha Lamichane, Funda Citak, Eva Waltenberger and Gawri shankar for the friendly environment and support during my PhD.

I would also like to thank all my friends in Bremen for the great gatherings, support and fun. I have many nice memories to cherish

.

Funding

This PhD thesis has received support from the Innovative Medicines Initiative Joint Undertaking under Grant Agreement No. 115525, resources that are composed of financial contributions from the European Union´s seventh framework programme (FP7/2007-2013) and European Federation of Pharmaceutical Industries and EFPIA companies´ in-kind contribution.

The research PhD stipend was provided by the work group budget of Prof. Dr. Roland Benz / Prof. Dr. Mathias Winterhalter in Jacobs University Bremen, Bremen, Germany.

Aim and outline of the thesis

Caulobacter crescentus is well-known for its unique life style, having dimorphic cell division with daughter cells completely different from one another. The bacterium living in dilute organic environments was believed to be lacking any genes coding for porin like proteins. We further investigated the lack of porins in the organism and found a channel forming protein in its outer membrane. This was very interesting as most probably this is the only porin in *C. crescentus*. Here we further characterize the porin by using biophysical techniques and molecular dynamics simulations.

Legionella pneumophila is a Gram-negative pathogenic bacterium. It can infect both water living amoebae as well as humans. A range of eukaryotic like proteins has been hypothesized in this bacterium. These proteins most probably help them adapt to the human host. Here we investigate the presence of a human hVDAC-1 like channel in *L. pneumophila*. We characterize the protein by using biophysical approaches and molecular dynamics simulations.

The thesis is organized as follows:

Chapter 1 is about general introduction about porins, their roles in antibiotic resistance, and their assembly in the outer membrane. Chapter 2 states the identification, cloning and characterization of OmpW of *C. crescentus*. The chapter includes detailed materials and methods with all the results. Chapter 3 presents the characterization of hVDAC-1 like protein in *Legionella pneumophila*. It also contains the materials and methods used for the study and the results obtained during the study.

Appendix presents a short summary of some side projects completed during the PhD studies.

Chapter 1

Chapter 1. General Introduction

1.1.Porins:

1.1.1.Short history and structure.

The outer membrane of Gram-negative bacteria acts as an impermeable barrier for the entry of toxic and large charged molecules. Water filled porins act as the major pathway for passive transport of hydrophilic solutes. Porins in the outer membrane are of vital importance for the survival of Gram-negative bacteria because they serve as the major pathway for the entrance of hydrophilic nutrients into the cell [1]. They have previously been called by several names i.e. “peptidoglycan associated protein” [2], “peptidoglycan-associated general diffusion pore proteins” [3], or “matrix proteins” [4] but their part in diffusion led “Nakae” to name these proteins as “porins” [5].

Over the past several years, bacterial porins have been studied extensively. However, the very first study of porin reconstitution using electron microscopy was performed in 1988 and clarified several aspects of porin architecture [6]. The major outer membrane protein of *Rhodobacter capsulatus* was the very first porin whose structure was reported in high resolution using X-ray crystallography. The detailed structure showed external loops and periplasmic turns connecting 16 tilted β strands [7], [8]. Mostly, porins were found form trimers. Since then, several porin structures have been studied in detail. It was the common belief for quite a long time that except smaller changes mostly in the loop topology and surface charges, the porins share a very similar architecture [9], [10], [11], [12]. From structural and functional analyses of several of these porins, they were grouped as either having 16 or 18 strands [13], [14]. According to this classification, sugar specific porins such as ScrY or LamB were classified in the 18 stranded class, whereas 16 stranded group contained porins responsible for the transport of low molecular mass molecules i.e. Omp32, OprP and PhoE [15], [16], [17]. However, due to recent advances in the knowledge about porins and outer membranes, it is difficult to maintain this classification, as some of the porins discovered later showed a different type of architecture [18], [19], [20]. As the knowledge about porins widened, several porins which were previously classified as general diffusion were found to be specific for small negatively charged organic acids or phosphates [15], [16]. The porin family now also contains some unique proteins such as P100 from *Thermus thermophilus*. Earlier, this protein was believed to be responsible for the formation of S-layer

of this bacterium [21]. It was only found out later that P100 is actually assembling as densely packed porins in the outer membrane [22], [23].

1.1.2. Outer membrane integration

Our knowledge about the integration and assembly of α helical membrane proteins is largely contributed to, by the studies of bacteriorhodopsin [24]. Although, our understanding of the complex folding, oligomerization and integration of β barrel proteins is increasing, the process is still not completely clear. The outer membrane proteins (OMPs) must undergo several rearrangements of their secondary structures and side chains in order to be able to form pores in the outer membrane. Outer membrane proteins after being expressed are carried through the inner membrane and the periplasmic space in order to finally be able to incorporate into the outer membrane [25]. Most OMPs, with some exceptions are expressed in the cytoplasm with a signal sequence directing their transport to the periplasm. Their low hydrophobicity helps this export. The Sec pathway exports all the outer membrane proteins in *E. coli* with Sec translocase providing the path across the inner membrane. However, the question: do they refold into their mature structure before incorporation into the outer membrane or afterwards remain to be answered. Most of our knowledge about the folding mechanisms of OMPs is coming from the studies of OmpA [26], [27], [28], [29]. Although, OMPs can refold *in vitro* into their mature structures, the process is a bit slower, suggesting the presence of catalysts aiding in their efficient folding and integration into the outer membrane *in vivo* [30]. Insertion of OmpA in the outer membrane is facilitated by a proteocomplex consisting of BamA and four lipoproteins (BamBCDE) [31], [32], [33]. The complete catalyzing function of Bam complex in the successful integration of the OMPs in the outer membrane is not fully understood. BamA and BamD are of vital importance and their deletion studies have shown to induce cell death by causing fast accumulation of unassembled OMPs in the periplasm. Some OMPs also possess a C-terminal signal sequence aiding in their successful integration into the outer membrane. The C-terminal sequence is composed of several hydrophobic residues and ending with an aromatic residue (mostly phenylalanine) [34]. The Bam complex recognizes the C-terminal signal sequence. The C-terminal of Bam undergoes conformational changes after interacting with the OMP which allows the insertion of the protein into the outer membrane. The Bam complex dissociates and final conformational changes of the OMP may be catalyzed by the lipopolysaccharides.

1.1.3. Roles of Porins

Porins can be categorized as important inducers of biological activities in host-cell interactions. They have important effects in several pathogenic mechanisms, as shown by several studies analyzing the immunobiological activities of porins. Structural and functional features of porins have been extensively studied but their importance in the pathogenesis of several bacterial infections was revealed only during the last fifteen years. The role of bacterial porins is manifold: a) immunological activity [35], [36], [37], [38], b) inducing signaling pathways [39], [40], [41], [42] , c) their significance in the emergence of antibiotic resistant strains of pathogenic bacteria. Our studies are mainly concerned about the antibiotic resistance.

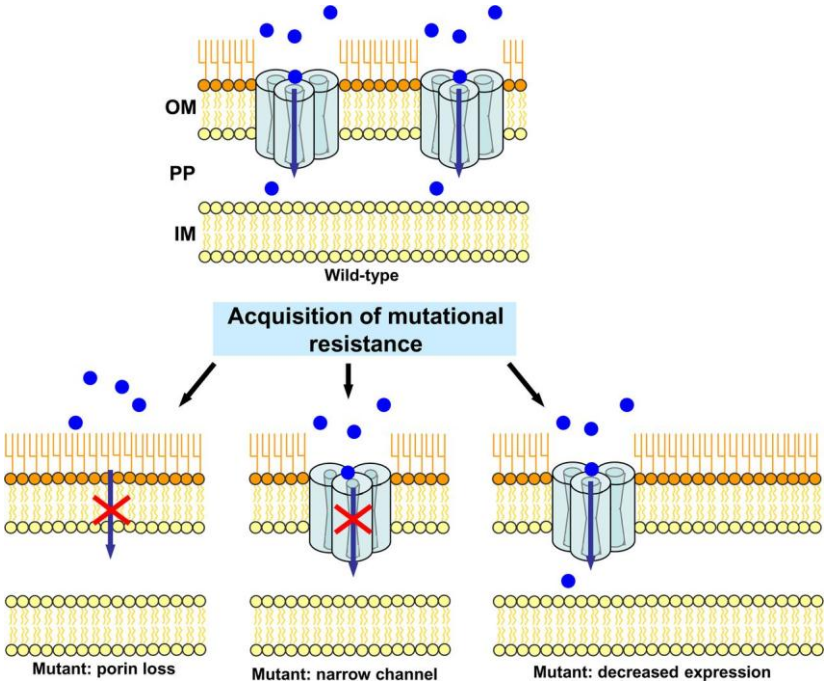


Figure 1. **A Schematic overview of all the potential roles of porins in acquisition of multidrug resistance in bacteria** [43]. OM: outer membrane, PP: periplasmic space, IM: inner membrane. Blue circles indicate antibiotic molecules, blue arrow shows transport through the porin and the red cross shows inhibition of the drug transport through the porin.

Multidrug resistance (MDR) is becoming one of the major threats of the modern times. Due to multidrug resistance in clinical Gram-negative bacteria the options available for treatment are limited which can lead to mortality when acquired as a nosocomial infection [44], [45]. MDR is prevalent in major Gram-negative clinical pathogens such as *Escherichia coli*, *Salmonella spp*, *Acinetobacter spp*, *Pseudomonas spp*, *Enterobacter spp*, *Campylobacter spp*, and *Klebsiella spp.* There are several hypotheses regarding the appearance of antibiotic resistance. During treatment, a patient may encounter an unrelated strain, which possesses a low range of

antibiotic resistance. The infecting strain might acquire genetic manipulations leading to the development of antibiotic resistance. Higher concentrations of antibiotics in hospital favor the antibiotic resistant strains. Low *et al.* collected several *E. coli* strains for 2 years, from a person who was under therapy with a range of antibiotics, including ciprofloxacin + ceftazidime, ciprofloxacin + cefotaxime, trimethoprim + ceftazidime, gentamicin + cefotaxime + meropenem, gentamicin + imipinem + meropenem and meropenem + gentamicin [46]. Seven different isolates that were characterized from the patient´s blood or liver abscesses showed significant increase in antibiotic resistance. Two of the strains showed remarkable decrease in OmpC expression and showed R124H substitution in the proteins´s loop 3 [46].

The hydrophobic lipid bilayer in combination with pore forming proteins having specific cut off values constitute the outer membrane of Gram-negative bacteria which functions as a highly selective sieve. In order for the antibiotics to reach their intracellular targets, the permeability properties of the OM are of vital importance. Porins in the OM serve as transport gateways for the transport of small hydrophilic drugs, such as β-lactams, as well as tetracycline, chloramphenicol and fluoroquinolones, however, macrolides and other hydrophobic drugs diffuse directly through the lipid bilayer. Reports showing modifications in the composition of the OM leading to MDR in several bacterial species, point out the importance of the OM in antibiotic resistance. Loss or functional impairment of porins in several organisms such as *E. coli*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Enterobacter aerogenes* and *Klebsiella pneumoniae* leads to antibiotic resistance [47], [48], [49], [50]. Resistance because of functional impairment of porins is mainly because of two mechanisms: 1) deletion or drastic down regulation of porins or replacement of a major porin by another; 2) specific mutations in the porins, resulting in their reduced permeability. *E. coli* can develop antibiotic driven tetracycline resistance by exposure to increasing concentrations of the antibiotic, which lead to a chromosome mediated multiple antibiotic resistance (Mar phenotype). Up regulation of OmpF results in inhibition of OmpF RNA translation [51]. Periplasmic accumulation of other OM proteins such as TolC and OmpX can also cause down regulation of OmpF [52]. Another mechanism for acquiring antibiotic resistance is the replacement of a general-diffusion porin with a narrower porin. This can be seen in *K. pneumonia,* where OmpK35 and OmpK36 are replaced by OmpK37 which is known to be forming smaller pores [53]. In some bacteria, antibiotic resistance may be achieved by reduced permeability of solute through a porin due to mutations. This is seen in the case of *Enterobacter aerogenes* where a glycine is replaced by aspartate [54]. This single point

mutation causes a reduction in the lumen of the pore [55] which leads to reduced cephalosporin sensitivity and also a decrease in the porin conductance. In *P. aeruginosa,* mutations in the L2 loop of the OprD makes the organism resistant to carbapenems [56]. These examples highlight the role of porins in the emergence of antibiotic resistance. This could help us to identify new strategies in order to design more efficient antibiotics.

1.2. Membrane channels reconstitution and Black lipid bilayer assay

Cells in either unicellular form or multicellular form are separated from their environment or their neighboring cells by membranes. While unicellular organisms maybe surrounded by a single outer membrane, in multicellular organisms several other organelles inside the cells are also surrounded by membranes. Whatever their structure or localization, they all serve a range of complex functions including protection of the cell and allowing the cells an exchange between the interior and exterior of the cells. The transport of material from inside of the cell to the outside or vice versa must be selective and optimally controlled. This is mostly accomplished by the presence of transport systems present in either the outer membrane or inner membrane.

The best way to study membrane proteins normally is reconstitution. The term reconstitution refers to the process of extracting a single protein from its complex, purifying it and inserting it into some measureable system where it can be studied. Bilayers formed by a single lipid or a mixture of lipids has been successfully used for a few decades in order to mimic the outer membrane and study membrane channels by inserting them into these lipid bilayers. The purpose of the ion channels reconstitution is to be able to incorporate the channel into an artificial system and be able to study the current flow through the channel at high resolution.

Black lipid bilayer assay is one of the most prominent approaches to characterize the biophysics of membrane channels. The first description of the technique goes back to 1962 by Mueller and his colleagues [57]. Physical properties of planar bilayers were nicely described by White [58]. A bilayer is formed either from a single lipid or a mixture of purified phospholipids in a non-polar solvent, mostly *n*-decane. Although, the setups vary a lot from lab to lab but the general assembly is always the same, a cuvette having two chambers with a small hole connecting the two chambers and an electrode inserted in each chamber. Before formation of the bilayer across the hole separating the two chambers the hole is pre painted with a small amount of phospholipid dispersion. Then a bilayer is formed across the pore, once the chambers are filled with an electrolyte solution. As mentioned before [58] the

formed membrane will be several micrometers thick, but due to Plateau-Gibbs border suction the membrane will finally thin into a bilayer. Van der Waals interactions between the electrolyte solutions on both sides of the bilayer also help in thinning of the membrane into a bilayer. Thinning of the film can be observed under reflected light through a low power telescope. If the bilayer is correctly formed, then the membrane will appear to be black, hence the name black lipid bilayer assay. However if the membrane is thick and multilayer, then several rainbow colors will be seen due to refraction of the light.

Here we report the presence of channel forming proteins in *C. crescentus* and *L. pneumophila*. We used molecular and biophysical approaches including bilayer to identify and characterize these proteins. The identified OmpW *C.c* in *C. crescentus* is most probably the only porin in its outer membrane. In *L. pneumophila* we report the identification and characterization of a homologue of hVDAC-1.

Chapter 2

Chapter 2. OmpW of *Caulobacter crescentus* functions as an outer membrane channel for cations

(NOTE: This chapter is partly derived from the contents of the following manuscript)

Benz R, Jones MD, Younas F, Maier E, Modi N, Mentele R, Lottspeich F, Kleinekathöfer U, Smit J. OmpW of *Caulobacter crescentus* Functions as an Outer Membrane Channel for Cations. PLoS One. 2015 Nov 25;10(11):e0143557. doi: 10.1371/journal.pone.0143557.

Individual Contributions

Caulobacter crescentus strains were maintained by Jones MD and the enriched outer membranes were extracted by him. *ompW* knockout strain was developed by him. I analyzed the enriched outer membrane extracts from both wild type and ompW knockout strain. I expressed the tagged OmpW in *E. coli* and purified it. I also cloned the gene coding for OmpW without its native signal sequence, expressed the protein, purified and refolded it. I also cloned the *ompW* gene with an *E. coli* OmpT signal sequence for the expression in the outer membrane of *E. coli*. I purified this protein and also confirmed the OmpW protein by MALDI-TOF.

Summary

The genome of *C. crescentus* has been completely sequenced but interestingly, no general diffusion porins of the OmpF/C type or substrate specific porins are coded in the genome. Instead, genes coding for more than 60 TonB dependent outer membrane receptors have been identified. Against this common belief that no general or substrate specific porin is present in the cell wall of *C. crescentus*, here we report a protein in the outer membrane showing high channel forming activity. Enriched cell wall extracts of *C. crescentus* had an ion permeable channel with a single channel conductance of 125 pS in 1 M KCl. The protein responsible for the activity had a molecular mass of about 20 kDa. Partial sequencing of the protein showed that it belongs to the well-known OmpW family of outer membrane proteins of Gram-negative bacteria.

In order to confirm that this is the particular protein responsible for the channel forming activity in the outer membrane of *C. crescentus*, we expressed the protein heterogeneously in several different conformations but unfortunately it never resulted in properly folded and active protein. So finally, the gene coding for OmpW was knocked out and enriched cell wall extracts of the mutant strain were checked again in lipid bilayer assay for its channel forming

activity. The enriched cell wall extracts from the Δ*ompW* strain did not show channel-forming activity, so we can conclude that the 20 kDa OmpW was responsible for the channel formation and most probably, it is the only porin in the outer membrane of *C. crescentus*. The OmpW *C.c* had a higher conductance as compared to two other members of the same family i.e. OmpW of *E. coli* and OprG of *P. aeruginosa*. In order to understand the reason behind the higher conductance of OmpW *C.c* we developed a homology structure for the protein and calculated the channel radius as well. OmpW *C.c* has a larger channel radius as compared to the other two members of the same family. This can be one of the reasons behind the larger conductance of the channels formed by OmpW *C.c.*

2.1. Introduction

2.1.1. *C. crescentus* and its life style

C. crescentus is a Gram-negative, Alphaproteobacterium living in dilute organic environments such as fresh water lakes and soil. Presence of some species has also been reported in nutrient rich environments [59], highly toxic gold mines [60] and even in contaminated water or sediments [61]. Different organisms develop different strategies, in order to survive the competition for food. *C. crescentus* is one of the organisms that utilize a type of development, which helps it survive the competition for food in dilute organic environments where it lives. Among several distinctive features of this organism, two of its features that are being extensively studied, are its dimorphic cell division and the secretion of holdfast glue. The dimorphic cell division that results in two morphologically completely different daughter cells enable the organism to survive in dilute organic environments. One of the daughter cells is free swarmer cell that can swim freely in water, so can detect suitable habitat by swimming around, while the other daughter cell is a stalked cell, which is attached to a substrate and helps establishing biofilms with the help of holdfast secreted at its tip. A polysaccharide composed partly of N-acetyl glucosamine is a constituent of the holdfast and is absolutely essential to the adhesive nature of the glue [62]. All the developed mutants, which lack the holdfast for adhesion, have also been found to be lacking N-acetyl glucosamine [63]. It was also reported that even the secretion of such adhesive holdfast glue is not enough for successful attachment of the cells. Other structures, such as flagella and pili help in the establishment of successful attachment to surfaces [64]. Asymmetric cell division makes this organism, a model organism to study cell division and cell differentiation [65].

The several stages in the life cycle of the organism are absolutely essential and they cannot be avoided. The swarmer cells have never been reported to undergo replication and division instead it must first lose the flagellum and develop a stalk. The protein localization necessary for the cell division only occurs in the stalked cell stage. Although there are some strains observed to be lacking any stalk, but in order to be able to divide they must develop a conformation close to the stalked cell so that polar localization of the important proteins can occur and the cell can divide [66], [67].

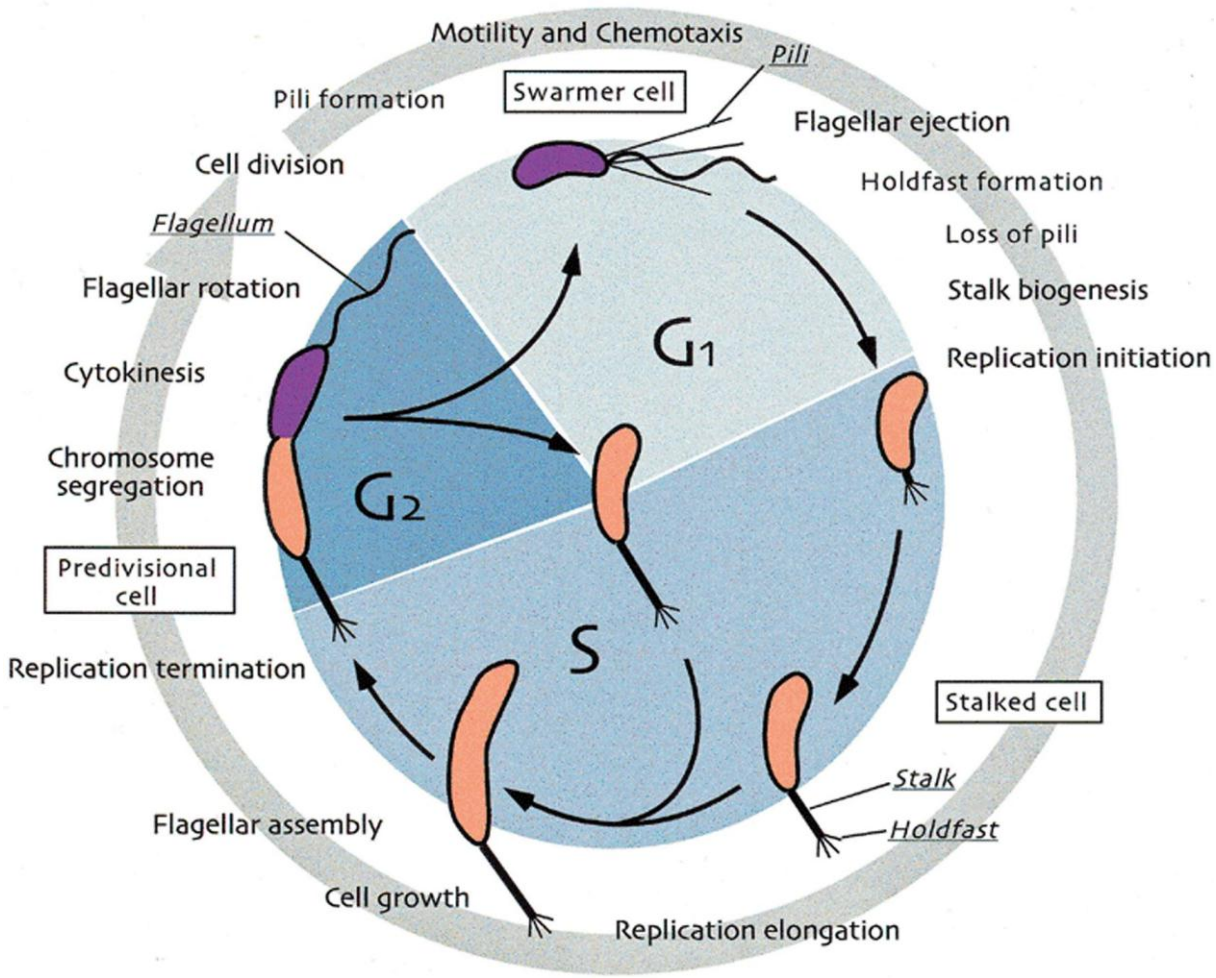


Figure 2. Life cycle of *Caulobacter crescentus.* The stalked cell in S phase is ready for DNA replication. As the cell grows and replicates its DNA, it becomes a pre-divisional cell. During this stage the cells cannot perform DNA replication, entering the G2 phase. In the late pre-divisional stage, a flagellum develops at the swarmer cell pole. After compartmentalization pili are extruded. One cell is a stalked cell which reenters the cyclic developmental program and S phase, completing the circle. The other cell is a swarmer cell. The swarmer cell cannot replicate, that's why they have been assigned to a separate phase G1 [68].

Although, *C. crescentus* differs sharply from *E. coli* in its life cycle and development but it is not the only organism having this unique life cycle. Dimorphic cell cycle as well as the secretion of the holdfast glue and a stalk is also observed in several other members of Alphaproteobacteria, especially some quite prominent organisms belonging to the order *Caulobacterales*. The consistency of these features among this specific group also hints their importance for organisms living in oligotrophic environments.

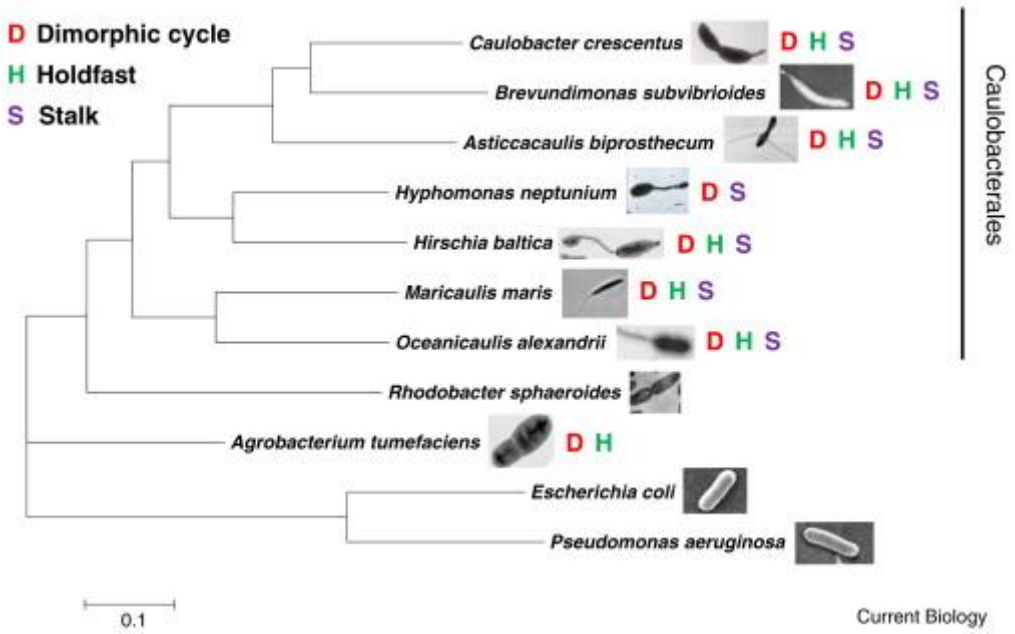


Figure 3. A phylogenetic tree pointing out the existence of dimorphic cycle, holdfast and stalk secretion in different bacteria [69].

Even after about fifteen years since the whole genome of *C. crescentus* was sequenced [70], so far no general diffusion porins of the OmpF / C type of Gram-negative bacteria have been reported in its genome [71]. Similarly, LamB or Tsx type specific porin sequences are also absent in the genome. Active uptake of nutrients from the dilute environments might be the prominent uptake system as genes coding for more than 60 TonB dependent receptors have been identified in the genome sequence of *Caulobacter crescentus* [71].

2.1.2. OmpW family

OmpW is a large and well known family of bacterial outer membrane proteins. Normally, proteins belonging to this family are predicted to have eight stranded beta barrels. Although, functions for most of the members of the family still need to be discovered, but recent research shows that they might have a role in protecting the bacteria against various forms of environmental stress. The family has its members widespread in gram negative bacteria. Two most widely and extensively studied are OmpW of *E. coli* and OprG of *Pseudomonas aeruginosa.* The crystal structures of these two proteins have been developed [72], [73], which helps in understanding the structure of other members of this family, like OmpW from *Caulobacter crescentus*. OmpW of *Vibrio cholera* has gained attention after its discovery about twenty years ago for its role in vaccine development [74] . OmpW of *E. coli*, which is a

protein with low expression under normal conditions has no known function so far, although it has been reported to be a receptor for Colicin S4 [75]. It was recently found out that OmpW of *E. coli* is crucial for its resistance to phagocytosis [76].

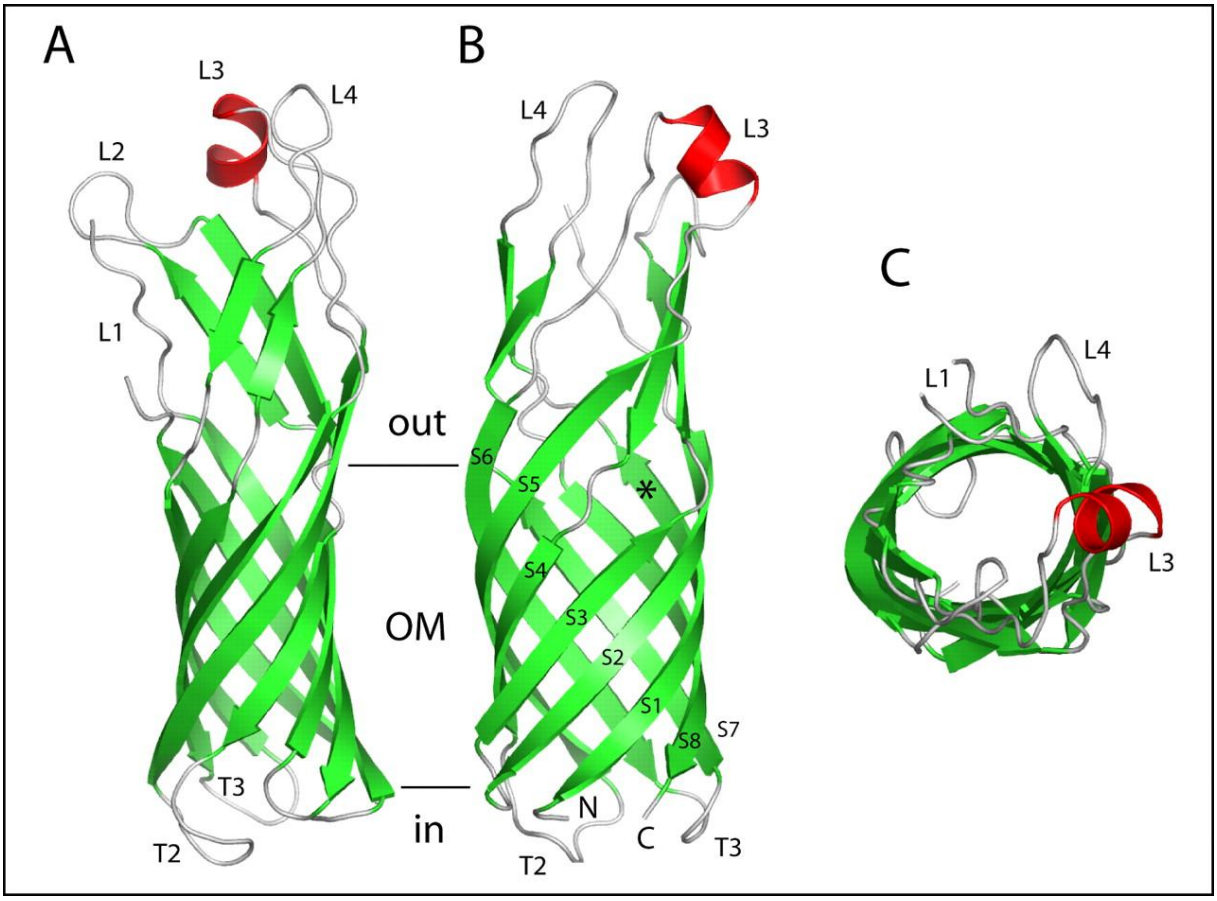


Figure 4. Schematic overview of OmpW from *E. coli*. L1-L4: the loops, S1-S8: Eight beta strands. N: N terminus, C: C terminus [72].

In this project we report the presence of an OmpW homologue in the outer membrane of *C. crescentus*. Against the common belief that the organism does not have any porin like channels, we found a channel forming protein in the cell wall extracts of the organism, which formed a cation selective channel, having a conductance of about 120 pS in 1 M KCl. The protein was found to be a member of the well-known OmpW family. The protein was further characterized and its properties compared to the other well characterized members of the group such as OmpW of *E. coli* and OprG of *Pseudomonas aeruginosa*.

2.2. Materials and methods

2.2.1. Bacterial culture conditions

Different types of cells were used for cloning and expression purposes. For cloning pruposes *E. coli* DH5 alpha cells were used, while for expression purpose *E. coli* BL21 DE3 Omp8 Δ*ompW* (the Δ*ompW* cells were developed in our lab, unpublished work) cells were used. The Omp8 cells lacked genes coding for some of the major porins like OmpF and OmpC. The cells were grown in LB medium at 37 °C/250 rpm. Ampicillin (100 µg/ml) and Kanamycin (25 µg/ml) were added to the liquid broth or agar plates whenever required.

2.2.2. His-tagged OmpW expression

The gene coding for OmpW of *Caulobacter crescentus* was previously cloned successfully with an N-terminus histidine tag. The plasmid, harboring the gene was an expression plasmid, PAraJS2. The plasmid has an ampicillin resistance and an arabinose inducible promoter. The plasmid was chemically transformed into a bacterial strain, lacking major porins, *E. coli* Bl21 (DE3) Omp8.

The bacterial cultures were grown in LB medium with 100 µg/ml ampicillin and 25 µg/ml kanamycin at 37 °C/250 rpm in a shaker incubator. The cells were grown till $O.D_{600}$ of 0.5-0.8 and then induced with 0.2 % final concentration of arabinose and incubated at 37 °C/250 rpm for 3 hours. After induction, the cells were harvested at 4,000×g, washed once with 10 mM Tris-HCl buffer and then resuspended in the same buffer. The cells were lysed by passing three times through French press at 1,500 psi. Unbroken cells were removed by centrifugation at 4,000×g for 30 minutes. The cell lysate from the lysed cells was ultra-centrifuged at 170,000×g for 1 h at 4°C (Beckman ultracentrifuge, rotor 70.1Ti). Membrane proteins formed the pellet while all the cytoplasmic proteins remained in the supernatant. The pellet was resuspended in 0.4 % LDAO in 10 mM Tris-HCl buffer pH 8, crushed completely and incubated at room temperature for 1 hour on a table top shaker. The mixture was then ultra-centrifuged again at 170,000×g for 30 minutes at 4°C. The supernatant contained the solubilized membrane proteins.

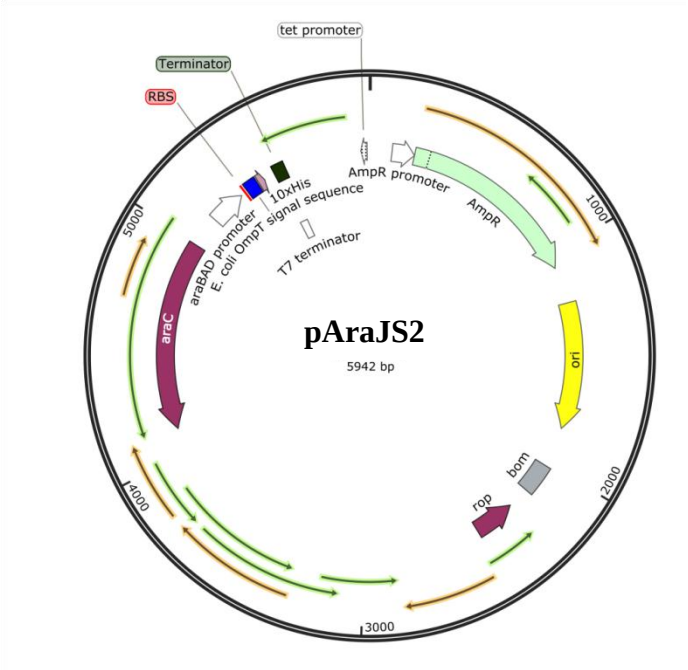


Figure 5. Plasmid map of the expression vector pAraJS2. The map indicates all the significant features of the plasmid, including signal sequence for OmpT of *E. coli*, arabinose inducible promoter (araBAD) and 10x histidine tag. The map was designed with SnapGene® software (from GSL Biotech; available at snapgene.com).

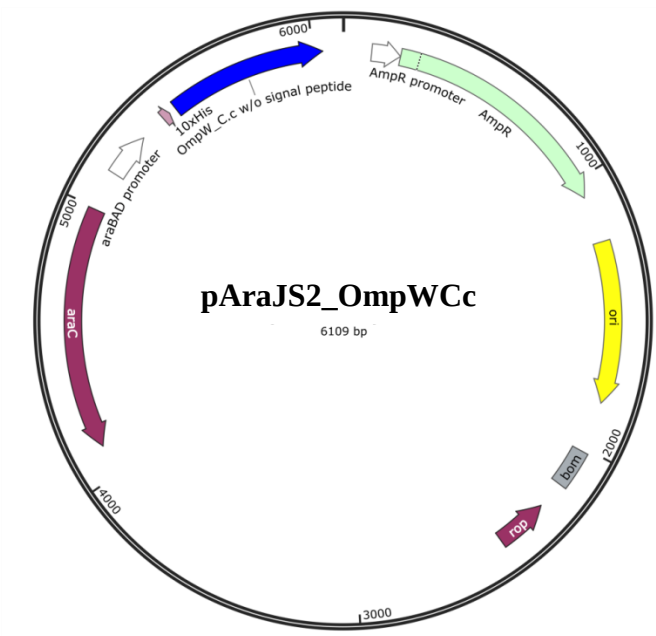


Figure 6. Map of the plasmid designed to express OmpW *C. crescentus* with an N-terminally his-tagged in the outer membrane of *E. coli* host

2.2.3. Dot blot

After expression of the gene, the different samples were tested through dot blot for the confirmation of his-tagged protein expression. A small piece of nitrocellulose membrane was cut and small circles were drawn with a pencil for each of the sample to be tested. 2µl sample was loaded gently in the middle of each circle. The membrane was allowed to dry for 5-10 minutes in a petri plate. Once the samples diffused completely into the membrane, the membrane was blocked with blocking buffer which is 10 % skimmed milk in tris buffer saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.6). The membrane was incubated with 1:3000 dilution of the anti-mouse anti his (Amersham Biosciences) for 90 minutes at room temperature. Post washing was done three times with blocking buffer, each time 10 minutes. Secondary antibody used was Anti mouse IgG alkaline phosphatase (Sigma) at a dilution of 1:30,000. The pH of the membrane was optimized by three times washing with alkaline phosphate buffer (100 mM NaCl, 100 mM Tris-HCl, 50 mM $MgCl_2$). The substrate used for the coloring reaction at the end was BCIP/NBT (Sigma Aldrich). The membrane was incubated with the substrate for 2-3 minutes or longer until the coloring reaction was observed.

2.2.4. Purification of his-tagged OmpW

Once his-tagged OmpW expression was confirmed by dot blot, the positive sample was used to get purified OmpW. There are several approaches possible to purify his-tagged proteins. We used the well-known and widely used, IMAC (Immobilized metal affinity chromatography). The used purification protocol was exactly the same as mentioned in the manual of the TALON® magnetic beads. All the buffers for washing and elution were the same as mentioned in the protocol (http://wolfson.huji.ac.il/expression/local/Talon_Products.pdf).

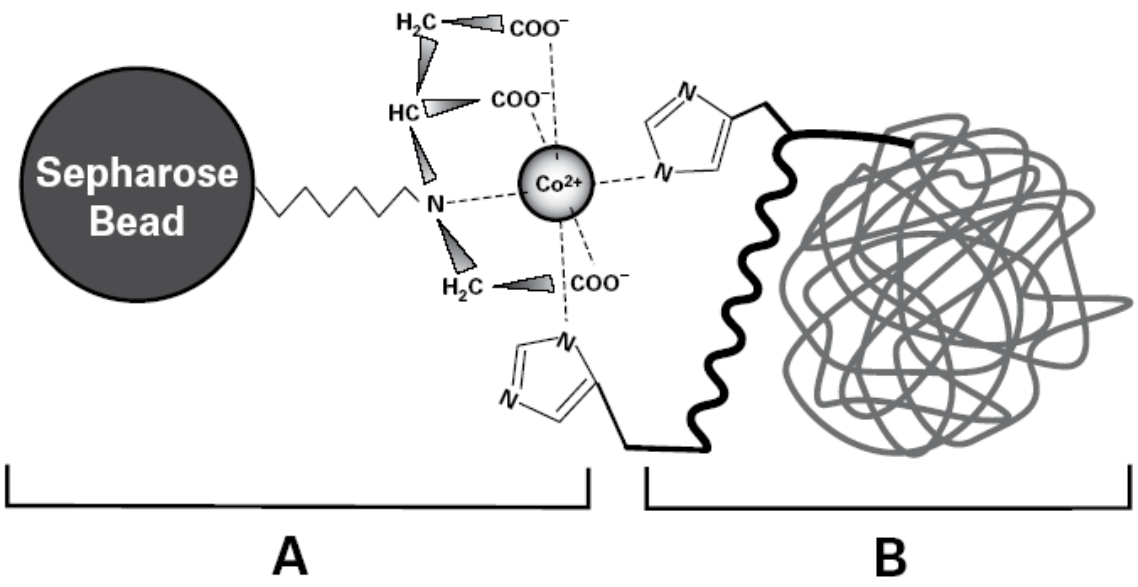


Figure 7. Schematic diagram of the TALON® IMAC System. Part A: TALON® Metal Affinity Resin; a Sepharose bead bearing the tetradentate chelator of the Co2+ metal ion. Part B: The polyhistidine-tagged recombinant protein binds to the resin. Source (http://wolfson.huji.ac.il/expression/local/Talon_Products.pdf).

2.2.5. Dialysis of the purified his-tagged OmpW and his-tag cleavage

The purified protein got after IMAC had a high concentration of imidazole so it could not be used directly for his-tag cleavage through thrombin, as thrombin is sensitive to higher concentrations of imidazole. In order to get rid of the excess imidazole, the protein was dialyzed overnight against a dialysate buffer (20 mM Tris-HCl, 50 mM NaCl, 1 mM $CaCl_2$, 0.4 % LDAO, pH 6.5). The membrane used for dialysis had a MWCO (molecular weight cutoff) value of 12 kDa. The cleavage was tried at a range of different conditions but no significant results were obtained.

2.2.6. Cloning for expression of native OmpW

Plasmid, pBluescript OmpW, containing the *ompW* gene was kindly provided by Michael D. Jones (Fig 8). The gene was PCR amplified using the primers OmpW_*Nde1*: 5´-CTCAACATATGCAAGACTTTACGCCGAAC and OmpW_*Xho1*: 5´GCCCCTCGAGTTAGAATTTGCGCGACAGG aimed for the expression of the gene in the inclusion bodies of the expression host. The primers were designed to have restriction sites for *Nde1* and *Xho1* respectively. The conditions used for the amplification of the *ompW* gene without its native signal sequence were as follows: Initial denaturation at 94 °C, final

denaturation at 94 °C for 1 min, annealing at 58 °C, extension at 72 °C for 3 min and final extension at 72 °C for 5 minutes.

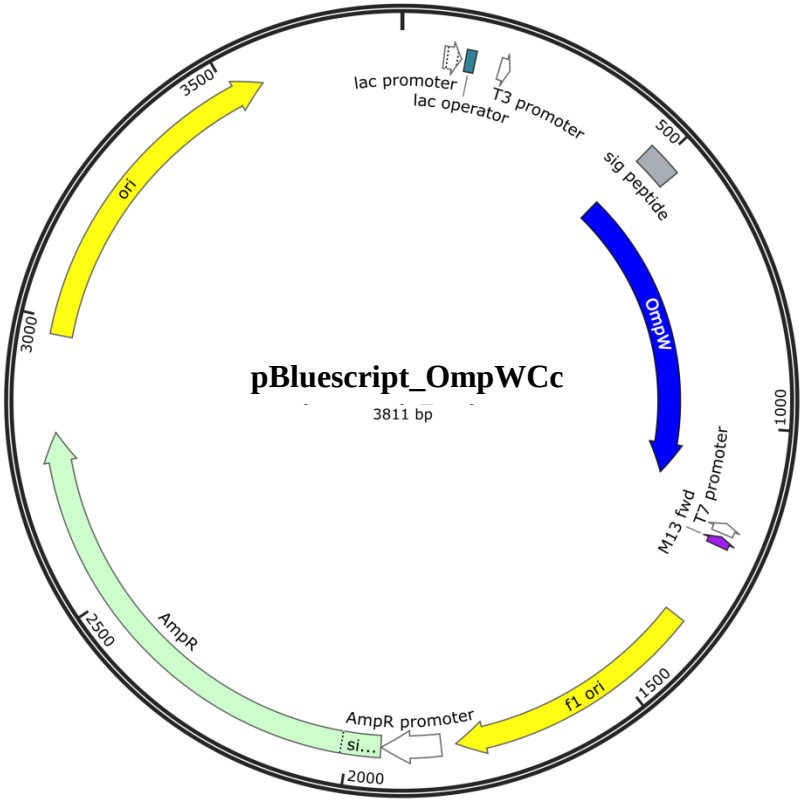


Figure 8. The parent plasmid provided by our collaborators harboring the *ompW C. crescentus* with its native signal peptide.

The amplified gene was cleaned up from excess of salts by using QIAGEN PCR cleanup kit. Both amplified PCR product and the expression plasmid pAraJS2 (Fig 5) were double digested using restriction enzymes *Nde1* and *Xho1*. The digested products for both restriction reactions were run separately on an agarose gel and the desired double digested products were cut out using a clean sterile scalpel. The gel cut DNA fragments were extracted according to the instructions from QIAGEN using QIAGEN gel extraction kit. The double digested gene and the expression plasmid were ligated overnight at 4 °C in the molar ration of 1:3. The ligation mixture was once more cleaned up using QIAGEN PCR cleanup kit and transformed into chemically competent *E. coli* XL1 blue cells. The cells were then plated on LB agar plates containing 100 µg/ml of ampicillin. Plasmids (Fig 9) were extracted from the cells using Thermo Fisher Scientific plasmid miniprep kit, and were sent for sequencing. After the gene in its proper orientation was confirmed by sequencing, then the plasmid was transformed into chemically competent expression host cells *E. coli* BL21 (DE3) Omp8 Δ*ompW*.

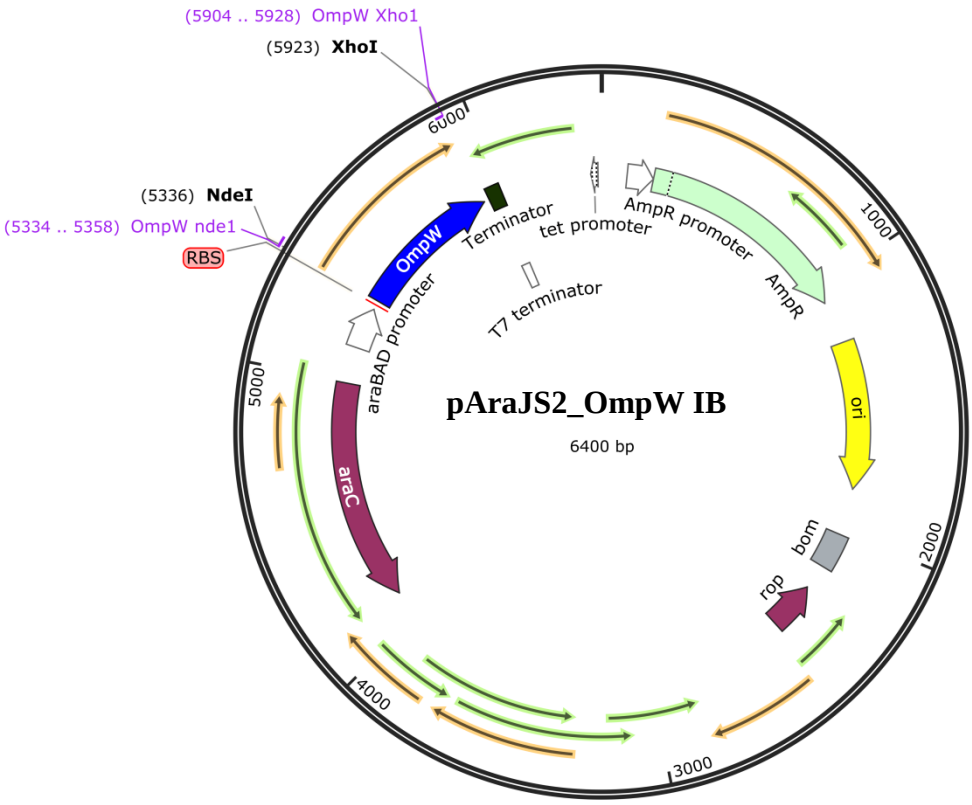


Figure 9. Map of the plasmid designed to express OmpW *C. crescentus* in the inclusion bodies of the host cell. There is no signal peptide with the gene sequence of the OmpW. The primers used to amplify the gene are also mentioned as *ompW Nde1* and OmpW *Xho1*. RBS: ribosome binding site.

Expression of OmpW in the inclusion bodies did not yield properly folded protein, so the protein was expressed in the outer membrane of the expression host in order to have the protein in its native conformation. The gene was amplified from pBluescriptOmpW, using the primers fwd SgrD1: 5´- TCAACGTCGACGCAAGACTTTACGCCGAAC and rev Sac1: 5´- TGTAGAGCTCTTAGAATTTGCGCGACAGGC harboring restriction sites for SgrD1 and Sac1 respectively. The aim of the cloning was to clone the *ompW* gene in the expression vector pAraJS2 in a way that the gene is expressed with the signal peptide of OmpT of *E. coli*. The plasmid already has this sequence. The gene was amplified as mentioned earlier in section (2.2.6) with annealing temperature of 67 °C. The ligation and transformation used was also the same. The expression plasmid developed (Fig 10) has an arabinose inducible promoter, and *ompW* cloned with the *E. coli* OmpT signal sequence at the 5´ terminus.

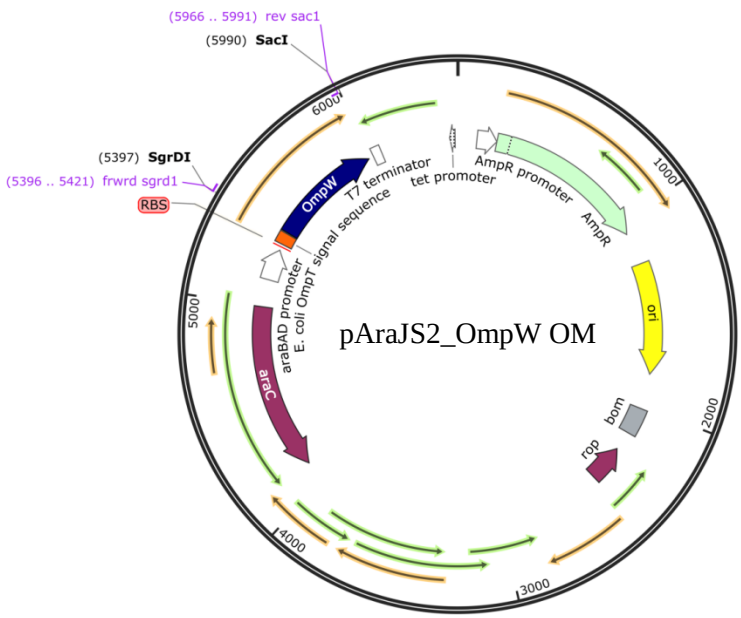


Figure 10. Plasmid map destined to express the *ompW* gene in the outer membrane of the host cell without any tag. *E. coli* OmpT signal sequence is attached to *ompW* to export the protein to the outer membrane. Fwd *SgrD1* and rev *Sac1* are the primers which were used to amplify the gene from its parent plasmid.

2.2.7. Expression, purification and refolding of the native protein

For expression of OmpW in inclusion bodies and their purification, the used protocol used was modified from [77], [78]. The cell cultures were grown as mentioned before, induced with 0.2 % Arabinose at an OD600 of 1.0 for 3 hours, and harvested at 4,000*g. Cells were resuspended in a lysis buffer composed of 50 mM Tris-HCl pH 8, 100 mM Nacl, 5 mM EDTA, 0.1 % sodium azide, 0.5 % Triton X-100, 0.1 mM PMSF and 1 mM DTT. Resuspended cells were sonicated at amplitude of 80 % at least 8 times, with each cycle of about 30 sec. Cells were incubated on ice for 20 sec after every cycle to avoid overheating of the sample. The EDTA in the cell lysate after sonication was chelated by adding 10 mM $MgSO_4$ and then incubated at RT for 20 mins with 0.01 mg/ml DNase and 0.1 mg/ml lysozyme. Inclusion bodies were collected by centrifugation at 4,000*g for 15 mins, and were washed twice more with the same buffer as above but without adding DNase and lysozyme. The final wash was done in the buffer without Triton (50 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM EDTA and 0.1 % NaN_3). The inclusion bodies were collected by centrifugation. The pure inclusion bodies were dissolved in 1/10th the initial culture volume of 100 mM Tris-HCl pH 8 with 50 mM glycine. The pellet was completely dispersed by sonication and the suspension was dissolved by adding it dropwise to 10 mM Tris-HCl pH 8 with 50 mM

glycine and 8.5 M urea with vigorous stirring. After the dispersed inclusion bodies are added completely to the buffer, then 5 mM reduced glutathione and 0.5 mM oxidized glutathione is added and stirred overnight at 4 °C. Refolding of the protein from inclusion bodies was done by rapid dilution method. The solubilized inclusion bodies from the previous step were added dropwise into a refolding buffer (0.1 M Tris-HCl pH 8, 0.4 M L-Arginine, 1 µg/ml pepstatin, 0.2 mM PMSF, 0.5 mM oxidized glutathione and 5 mM reduced glutathione.

Proteins from the inclusion bodies were checked on an analytical gel and the OmpW protein was purified by cutting the protein from a preparative SDS-PAGE gel.

The cell cultures were grown, induced and lysed as mentioned earlier (section 2.2.2). The strategy designed to have OmpW overexpressed in the outer membrane was successful and the protein was found in the cell wall proteins when checked on 15 % SDS-PAGE gels. The band corresponding to OmpW at about 22 kDa was gel cut from a preparative SDS-PAGE gel. Both analytical and preparative SDS-PAGE gels were run according to the instructions from Laemmli [79]. Preparative SDS-PAGE gels were run in the absence of strong reducing agents such as DTT and β mercaptoethanol. The gel cut OmpW was incubated with 0.4 % LDAO in 10 mM Tris-HCl pH 8 overnight at 4 °C for refolding. Refolding of the protein was checked again by running the sample on 15 % SDS-PAGE gel, as the protein is found to be heat modified. Unboiled OmpW runs at two different sizes on SDS-PAGE gel i.e. native protein at about 20 kDa and denatured protein at 22 kDa. This is also quite an efficient way to quickly check the refolding process of the protein.

2.2.8. Maintenance of *C. crescentus* Cultures, knockout of its native *ompW* and extraction of cell wall extracts.

We used the strain *C. crescentus* CB15 NA 1000 353ϕ (JS1013), which lacks S-layer because of an amber mutation in *rsaA* [80] for the identification of *ompW* and initial characterization. For the knockout of *ompW*, wildtype *C. crescentus* NA1000 was used. The same strain was used for comparison with the *ompW* knockout strain.

The cells were grown till OD600 of 0.8 in PYE (peptone yeast extract) [81] at 30 °C. This culture was used to inoculate larger volume cultures with M16HIGG medium [82], containing 0.3 % glucose, 0.09 % glutamate, 1.25 mM sodium phosphate, 3.1 mM imidazole, 0.05 % ammonium chloride and 0.5 % modified Hutner´s Mineral Base and shaken at 100 rpm.

The gene CCNA_01475 (C_1409) coding for OmpW was knocked out from wildtype *C. crescentus* NA1000 according to the protocols published previously [80]. The details of the procedure of developing a strain with a large internal deletion in *ompW* can be found in the published manuscript [18].

Enriched outer membrane extracts of the wildtype *C. crescentus* and *ompW* knock-out strain were obtained through a procedure which is reported in detail previously [18].

2.2.9. Biophysical characterization using black lipid bilayer assay

Black lipid bilayer assay, as mentioned earlier is one of the most prominent techniques to study reconstituted purified channel forming proteins. The setup we employed has a Teflon chamber, having two chambers connected through a small aperture having surface area of about 0.5 mm^2. Both of the chambers were filled with an electrolyte solution. An electrode is submerged into each of the chamber. The electrode on *cis*-side was connected to a voltage supplier. The electrode on the trans-side was connected in series to a homemade current to voltage amplifier. The resulting amplified signals were read on a strip chart recorder or a digital oscilloscope depending on the experiments. Lipid bilayers were formed by painting 1 % (w/v) solution of diphytanoylphosphatidylcholine (PC) in *n*-decane over the aperture with a help of a handmade loop. Salts used were of analytical grade and obtained from Applichem. All bilayer experiments were performed at 20 °C, unless mentioned otherwise.

Single channel conductance of porins in pure form and diluted in a detergent solution can be studied using the above mentioned setup. The protein was added in minor amounts to either one or both sides of the chamber and allowed to be integrated into the constituted lipid bilayer. Conductance of the channels, marked by the flow of ions through the pore was studied on the strip chart recorder. The reconstitution of porin molecules to the lipid bilayer resulted in a step wise increase in the conductance of the membrane. The most frequent conductance value corresponds to the single channel conductance of the purified protein. All the different conductance values for the purified protein were plotted in the form a histogram, telling us the most frequent conductance value which is also the conductance of the channel.

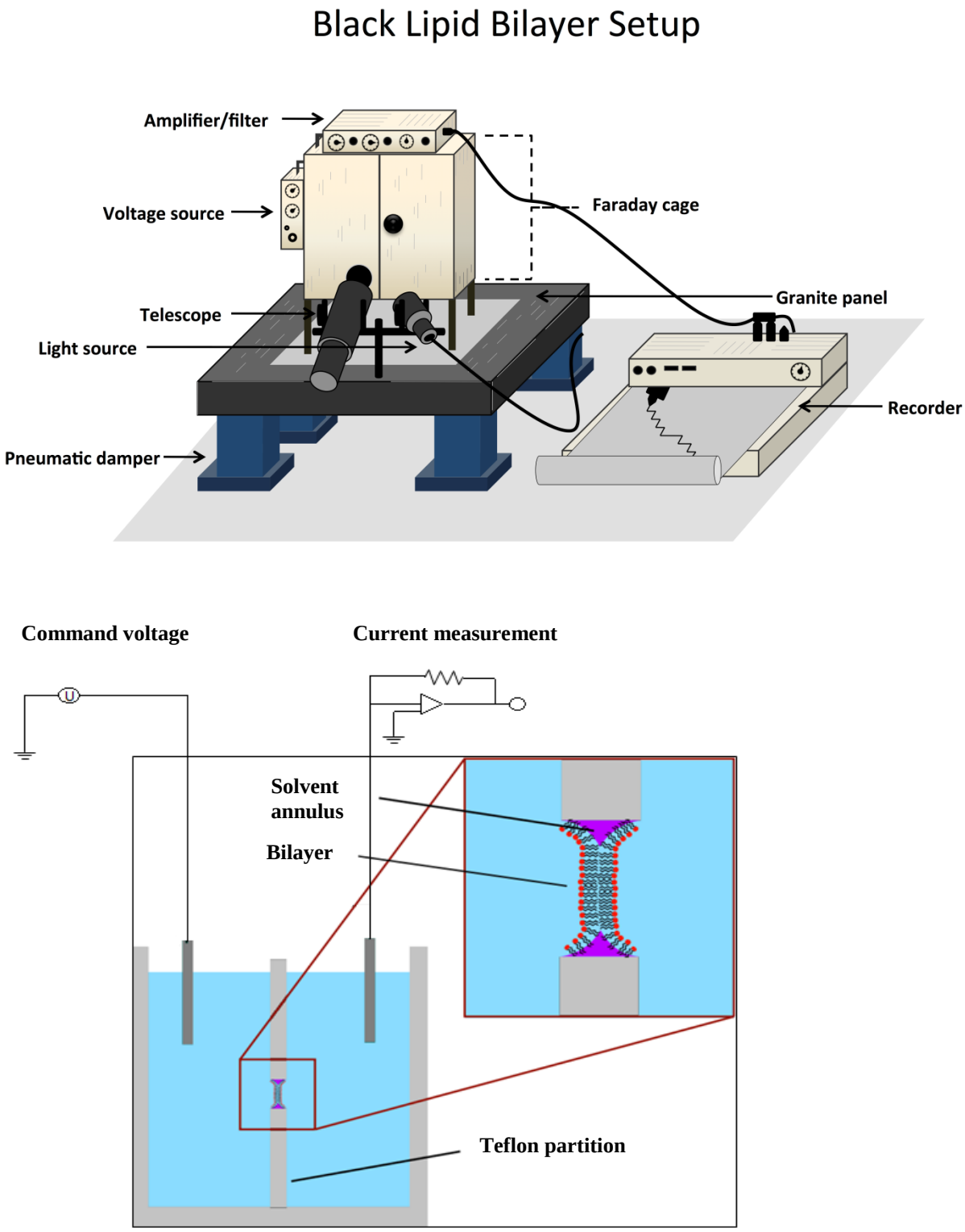


Figure 11. A schematic representation of the setup we use for biophysical characterization of membrane proteins using black lipid bilayer assay. Courtesy of Hashem Al Ghaili. Schematic representation of bilayer formation and the electrical circuit.

2.3. Results

2.3.1. Biophysical characterization of PBS/EDTA extracts from *C. crescentus*

Cell wall proteins from *C. crescentus* were extracted as mentioned in the materials and methods section. Addition of minor amounts (50 ng) of the proteins extracted with 0.5 % LDAO resulted in continuous and step wise increase in the conductance of the black lipid bilayer (Fig 12). Negative control experiments with just the detergent did not result in any increase in the conductance of the membrane. The bathing solution was 5 ml of KCl on both sides of the membrane. All the recorded channels were plotted as a histogram which showed that the most frequent conductance was 120 pS, although there were some channels with higher conductance of about 250 pS. The higher conductance might be because of a simultaneous insertion of two channels into the membrane. This data showed that the outer membrane of *C. crescentus* contained a porin having smaller conductance, as compared to porins from other Gram-negative bacteria.

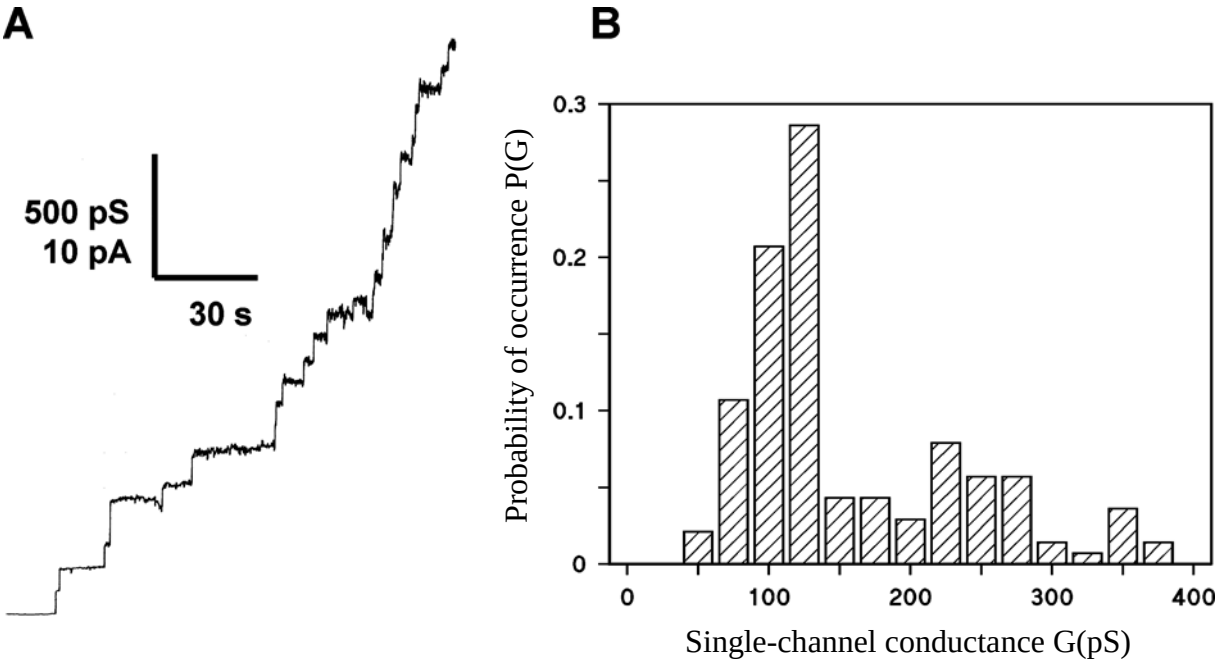


Figure 12. Single channel recording for enriched outer membrane extracts of *C. crescentus*. A: Single channel recording of a membrane formed of PC/*n*-decane after addition of enriched outer membranes of *C. crescentus* to 1 M KCl solution bathing the membrane. The applied membrane potential was 20 mV and temperature 20 °C. B: P(G) is the probability of occurrence for a specific conductance increment. It was obtained by the division of number of channels for the specific conductance and the total number of channels. Applied membrane potential was 20 mV and T= 20 °C.

Analysis of the PBS/EDTA cell wall extracts from *C. crescentus* on SDS-PAGE gel showed a range of different proteins (Fig 13). One protein at about 22 kDa was of specific interest as it

was running at two different sizes in un-boiled sample incubated at room temperature and sample boiled with sample buffer at 95 °C (Fig 13).

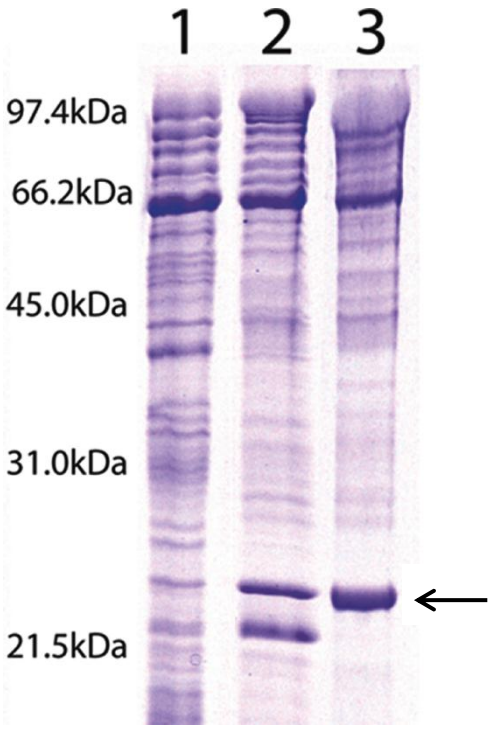


Figure 13. Coomassie stained 12 % SDS-PAGE gel with different extracts of *C. crescentus*. Lane1: Crude membrane preparations, Lane2: un-boiled PBS/EDTA cell wall extracts, Lane3: Boiled PBS/EDTA extracts.

2.3.2. Purification of 20-22 kDa protein from extracts and biophysical characterization

In order to identify the protein responsible for the channel forming activity in the cell wall extracts of *C. crescentus*, enriched cell wall extracts were run on a preparative SDS-PAGE gel. Proteins at different sizes were cut, crushed and eluted by overnight incubation with 1 % Genapol in 10 mM Tris-HCl pH 8 at 4 °C. Most frequent channel forming activity was observed for protein eluted between 20-22 kDa. The specific protein was subjected to analytical SDS-PAGE gel and it was found to be heat modified as was also seen in the gel with extracts from *C. crescentus* (Fig 14). Boiling of the protein at 95 °C for 10 min in the presence of sample buffer completely denatures the protein and it run at about 22 kDa, but when the sample is just incubated at room temperature in the presence of sample buffer for 10 min, the protein is able to maintain its native state and it runs at 20 kDa.

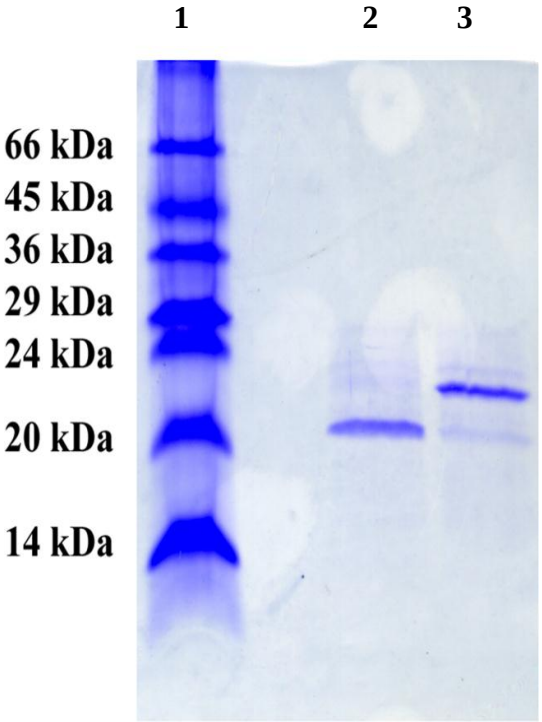


Figure 14. Coomassie stained 15 % SDS-PAGE gel of OmpW *C. crescentus* purified by preparative SDS-PAGE gel. Lane 1: Protein marker, 2: un-boiled purified OmpW, 3: Boiled purified OmpW.

The purified protein was subjected to black lipid bilayer assay for its biophysical characterization. The conditions used were the same as were for the biophysical characterization of the enriched cell wall extracts from *C. crescentus*. Addition of the protein to either one or both sides of the membrane in minor amounts resulted in fast step wise increase in the conductance of the membrane (Fig 15A). A histogram of all the observed channels showed the most frequent conductance value to be about 120 pS in 1 M KCl (Fig… 15B). Compared to the single channel measurements with cell wall extracts, the purified protein resulted in less frequent occurrence of channels with other conductance values.

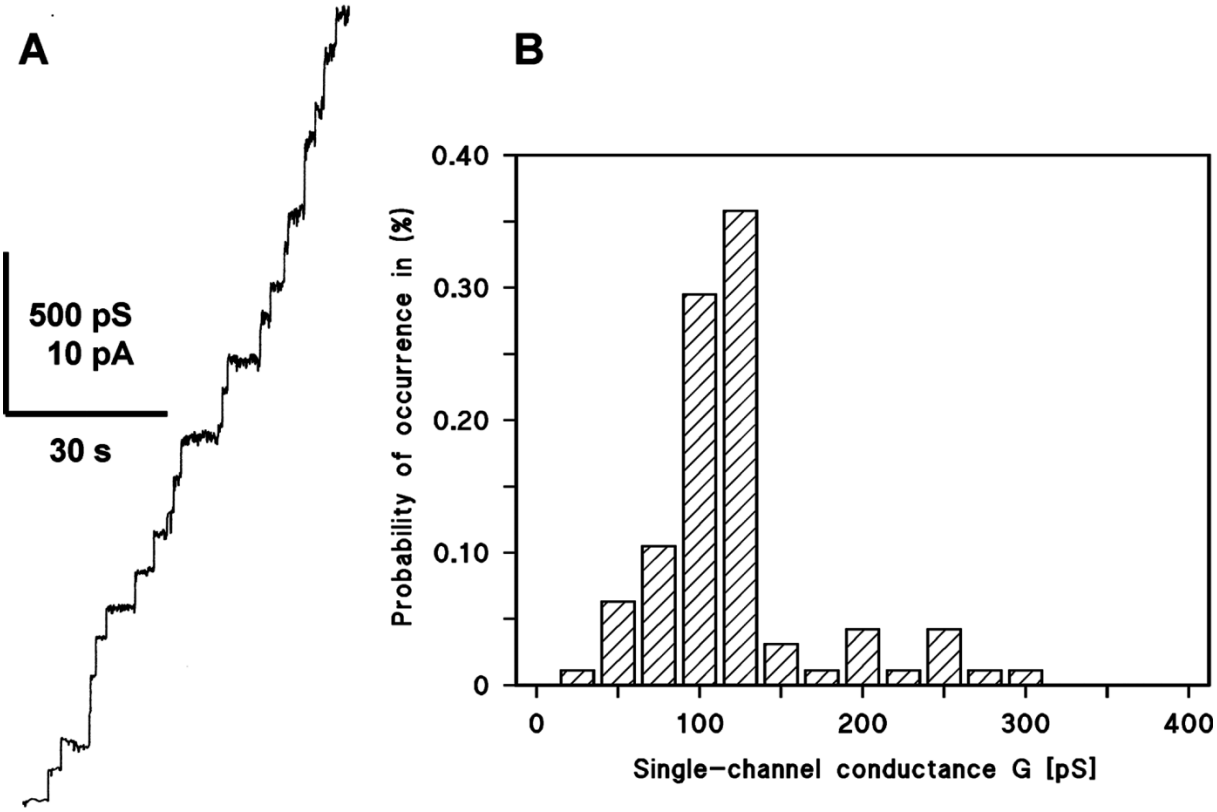


Figure 15. Single-channel recording of a PC/*n*-decane membrane caused by pure OmpW *C. crescentus*. A: The protein was added at 20 ng/ml dissolved in 1 % Genapol to the KCl solution bathing the membrane. The recording was performed at applied membrane potential of 20 mV at 20 °C. B: A histogram showing the probability of occurrence of a channel of specific conductance. The probability was calculated by dividing the number of insertions with a specific conductance by the total number of insertions observed. The aqueous phase was 1 M KCl, applied membrane potential of 20 mV and T of 20 °C.

2.3.2. Partial sequencing of the 20-22 kDa protein and alignments

The 20 kDa protein was digested by trypsin and the resulting peptides were separated. One of the prominent stretches of peptides with a molecular mass of 1764.8 could be identified by mass spectrometry. Mascot search (http://matrixscience.com) led to the identification of the peptide to be QDFTPNAKGDLIVHAR. The peptide was found to the N-terminal peptide of OmpW (CC_1409) of *C. crescentus* with the help of NCBI BLAST search [83], [84]. The same sequence was also identified in the protein at 22 kDa using the same procedure, which let us conclude that the protein at 22 kDa is a heat modified form of the protein at 20 kDa (OmpW).

2.3.3. Sequence alignments and homology model of OmpW *C. crescentus*

The homology of OmpW from *C. crescentus* to other members of the family such as OmpW from *E. coli* and OprG from *P. aeruginosa* is not very prominent but is still obvious. Identity of OmpW from *C. crescentus* to these two well characterized members of the family is about 32 % (Pôle BioInformatique Lyonnais) [72], [73] (Fig 16). A range of amino acids (41 amino acids) are highly conserved among these three members of the OmpW family. This list of conserved amino acids also includes several glycines and prolines.

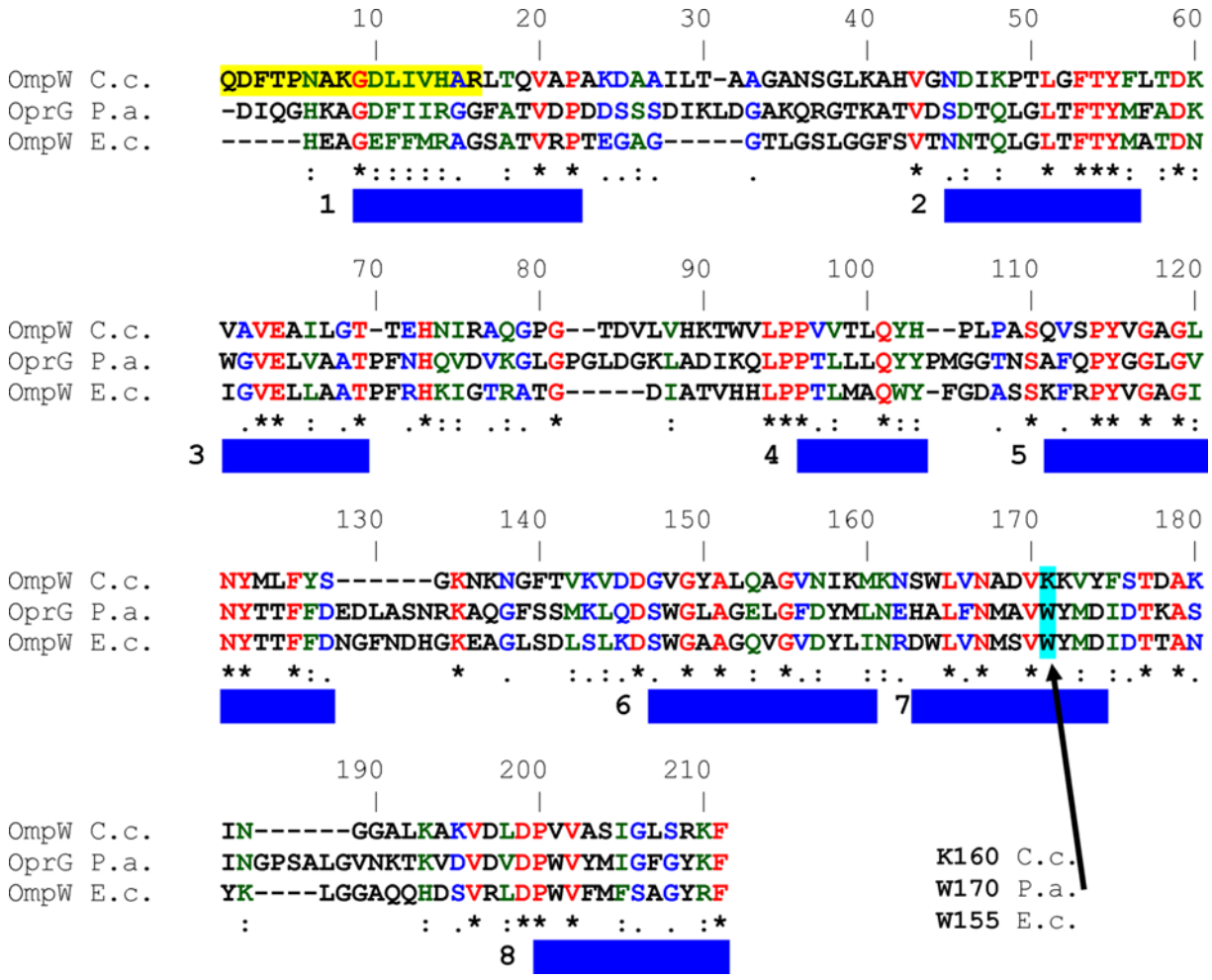


Figure 16. Amino acids sequence alignment of OmpW *C. crescentus* with the two well-known members of the OmpW family i.e. OmpW *E. coli* and OprG *P. aeruginosa*. Red color shows identical amino acids in all the three proteins (*), green colored are strongly similar residues (:), and blue colored are the weekly similar ones (.), The blue bars indicate the beta strands in OmpW *E. coli* and OprG *P. aeruginosa* as has been shown in their crystal structures [72], [73]. The yellow highlighted residues were identified after tryptic digestion of the protein and analysis of the resulting peptides by mass spectrometry. For alignment tools from "Pole Bioinformatique Lyonnaise Network Protein Sequence Analysis" (http://npsa-pbil.ibcp.fr) were used. The black arrow indicates the presence of lysine in OmpW *C. crescentus*, as compared to tryptophan in OmpW *E. coli* and OprG *P. aeruginosa*.

Although, the sequence homology between these members of the OmpW family is not very prominent, we were able to develop a 3-D structure of the OmpW of *C. crescentus* using homology modeling (Fig 17A) [85]. The availability of crystal structures of OmpW from *E. coli* and OprG from *P. aeruginosa* is of great advantage in developing the homology structure.

To find out the reason behind the higher conductance of OmpW *C. crescentus*, we also determined the channel radii for these three members of the OmpW family and we observed a slightly larger channel radius for OmpW *C. crescentus* as compared to the other two members. The channel radius was especially larger from the center of the channel towards the periplasm (Fig 17B).

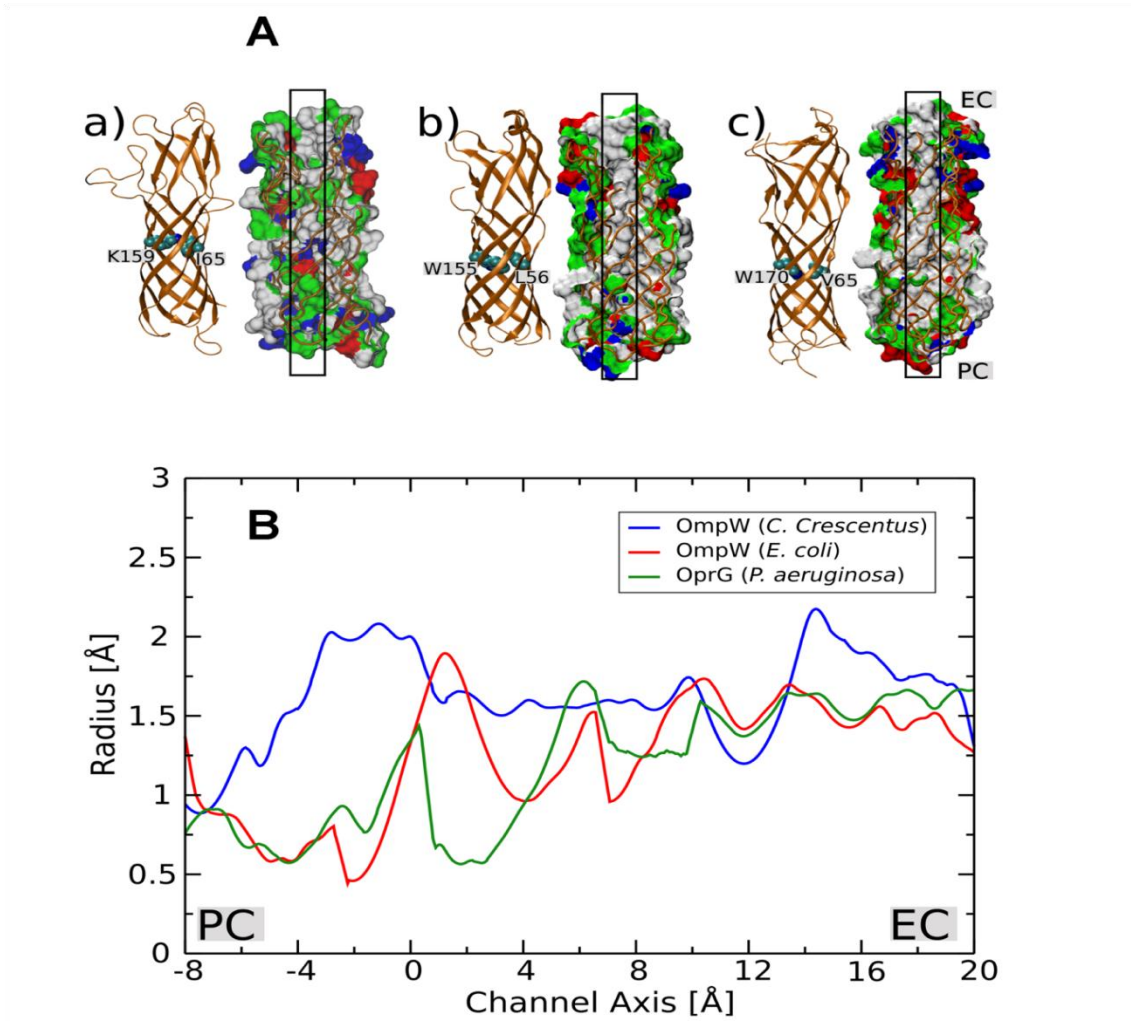


Figure 17. A:Structural comparison of a) OmpW *C. crescentus*, b) OmpW *E. coli* and c) OprG *P. aeruginosa*. Putative hydrophobic gate comprised of W155 and L56 in *E. coli*, W170 and V65 and the corresponding residues in *C. crescentus* K159 and K165 are shown as spheres. The representation of amino acids is color coded i.e. Green: hydrophilic, white: hydrophobic, red: acidic, blue: basic. The black box in the middle of the proteins represents the putative passage for the transport. EC: Extracellular side, PC: periplasmic side. The structures were modeled by homology modeling, taking OmpW *E. coli* as bait, through Modeller [85] B: Graphical representation of the channel radii of OmpW *E. coli* (red), OmpW *C. crescentus* (blue) and OprG *P. aeruginosa* (green) along the channel axis. The data is obtained using HOLE [86]

2.3.4. his-tagged overexpression in *E. coli*

The procedure for extraction and purification of his-tagged OmpW has been mentioned earlier in materials and methods section. Outer membrane proteins from lysed cells were separated using ultracentrifuge. The membrane proteins fraction was subjected to IMAC for the purification of overexpressed his-tagged OmpW. his-tagged OmpW conjugated to the magnetic beads was eluted in two steps. First elution was with 500 mM imidazole and the second and final elution was with 1 M imidazole. The eluted fractions were collected and analyzed on an analytical SDS-PAGE gel. Un-boiled protein samples ran on the gel in two different conformations, as seen with the native OmpW from *C. crescentus* cell wall extracts. The protein band at approximately 23 kDa shows the denatured OmpW and the lower protein band at approximately 20 kDa is the folded protein (Fig 18).

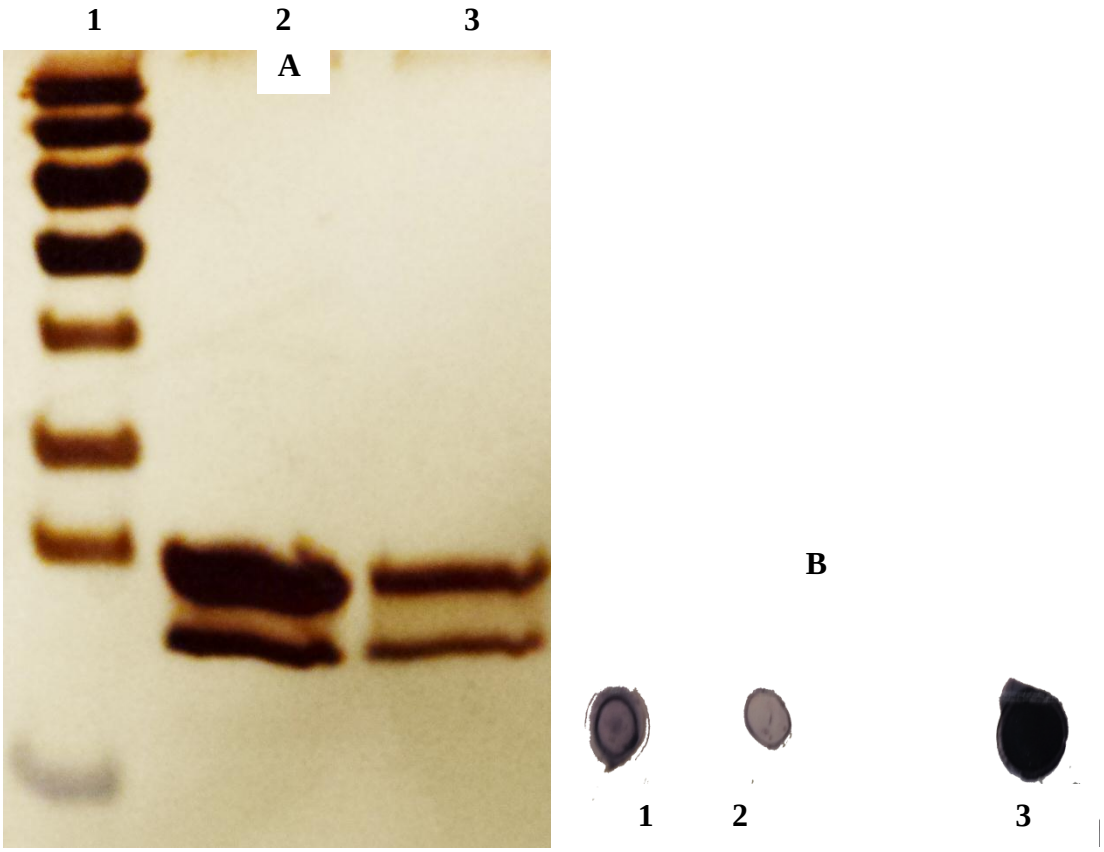


Figure 18. Purification of his-tagged OmpW *C. crescentus*. A: Silver stained 15 % SDS-PAGE gel with purified his-tagged OmpW. 1) pre stained protein ladder, 2) OmpW eluted from the beads using 500 mM imidazole, 3) OmpW eluted from the beads with 1 M imidazole. The samples were unboiled, so some of the protein can be seen in denatured (upper band) and a fraction as refolded form (lower). B: Dot blot of the purified his-tagged OmpW. 1) OmpW eluted with 500 mM imidazole, 2) OmpW eluted with 1 M imidazole, 3) A his-tagged protein as positive control. Mouse anti his antibody at 1:3000 dilution was used as a primary antibody and anti-mouse IgG alkaline phosphatase at 1:30,000 dilution was used as secondary antibody.

2.3.5. Signal peptide prediction and overexpression of OmpW in inclusion bodies

Cleavage of his-tag from his-tagged OmpW did not result in reasonable amount and concentration of the native protein. Signal peptide for the protein was predicted using sigp4.0 server. The *ompW* gene was cloned in the expression vector pAraJS2 without its native signal peptide. The plasmid has a signal peptide for OmpT of *E. coli*. The cloning strategy was designed in order to exclude both the signal peptides so that the protein can be expressed in the inclusion bodies. The expression was successful and the inclusion bodies were purified as mentioned in the materials and methods section. The sample was boiled in the presence of sample buffer and run on an analytical SDS-PAGE gel to check for the overexpression of OmpW (Fig 19).

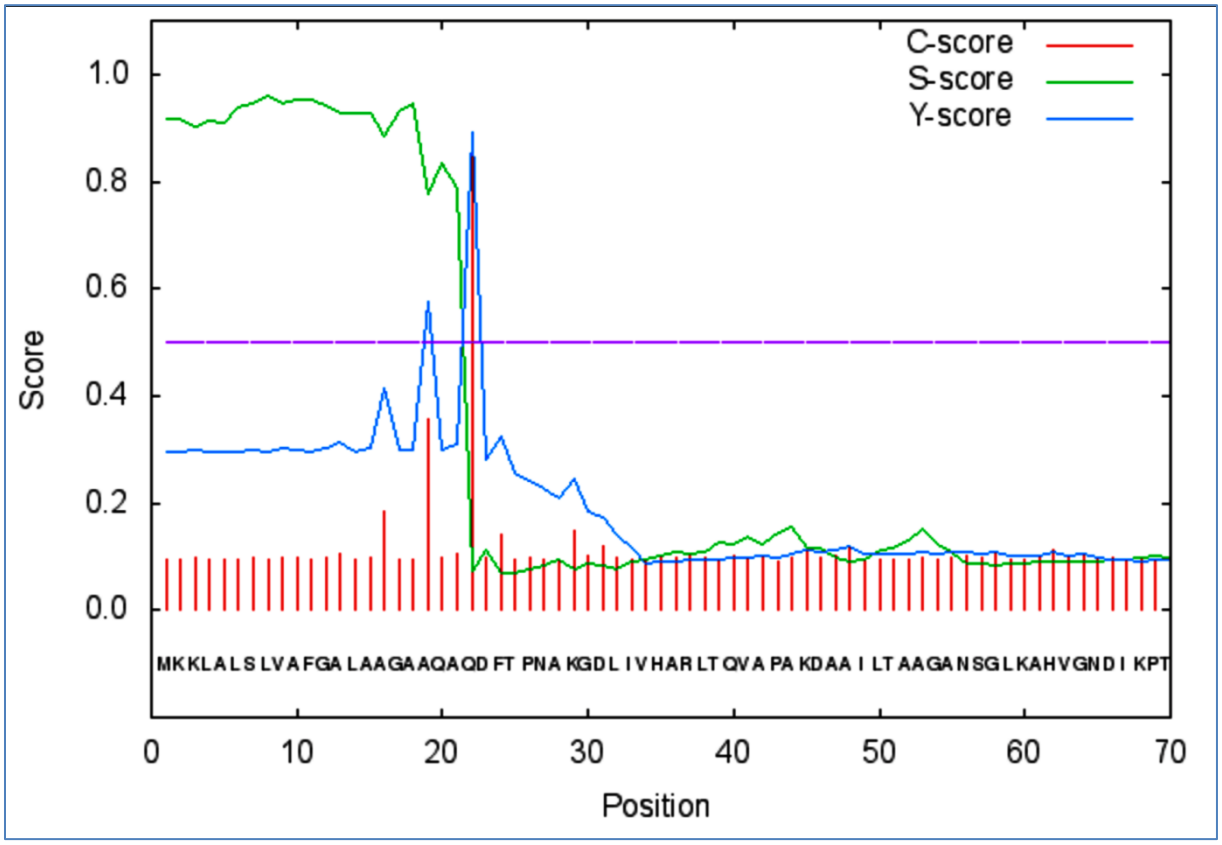


Figure 19. Signal peptide prediction for OmpW *C. crescentus*. The prediction was performed using the SignalP 4.0 prediction servers. The data suggests that the signal peptide will be cleaved between residues 21 and 22.

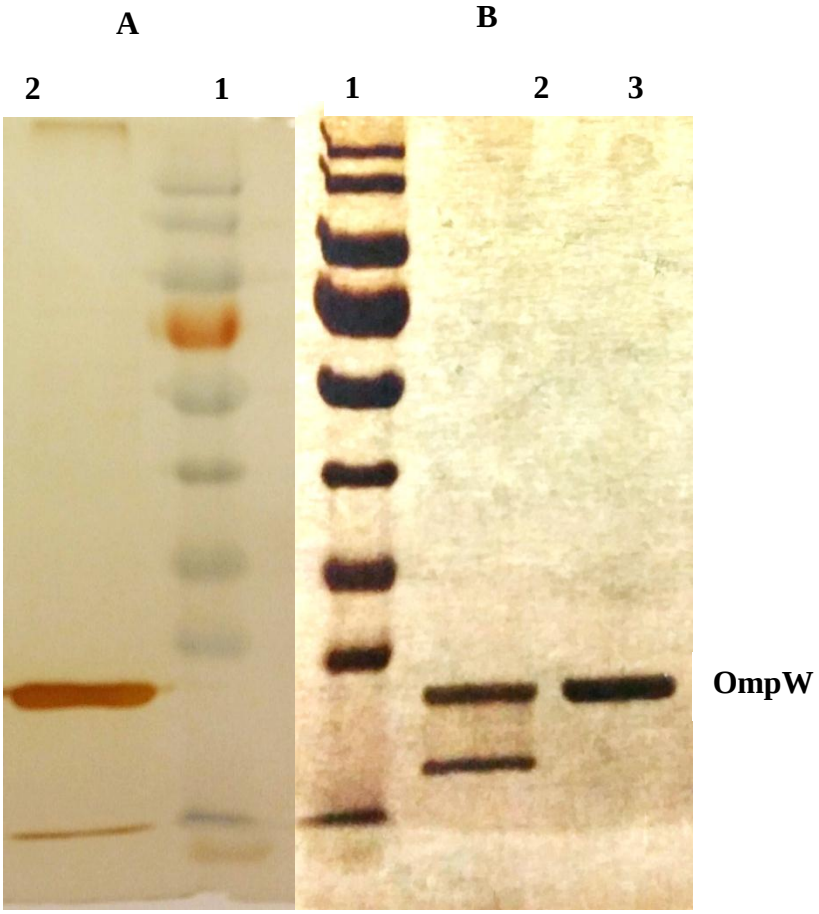


Figure 20. Silver stained 15% SDS-PAGE gels showing the proteins from purified inclusion bodies and purified OmpW. A: The proteins from inclusion bodies were mixed with sample buffer and boiled at 95 °C for 10 mins. Lane1: prestained protein ladder, Lane2: proteins from purified inclusion bodies. B: The gel shows the heat modifiability of OmpW extracted from inclusion bodies, as can be seen in un-boiled sample (Lane2) and boiled (Lane3). 1: prestained protein ladder

The overexpressed OmpW in inclusion bodies was also checked for its heat modifiability. The OmpW protein band was gel cut from a preparative SDS-PAGE gel, incubated with 0.4 % LDAO in 10 mM Tris-HCl overnight at 4 °C. The sample was then checked as boiled and unboiled in the presence of sample buffer on an analytical gel (Fig 20). The protein showed the same heat modifiability as shown earlier. The protein can be expressed in the inclusion and the final concentration of the purified protein can be reached up to more than 2 mg/ml.

2.3.6. Overexpression of OmpW in the outer membrane

Even after being able to get the OmpW in very high concentration and high purity from the inclusion bodies, the protein was still unable to be properly reconstituted into planar lipid bilayers. This might be because of the misfolding of the protein after purification or maybe the protein is not being expressed in its proper folding. So, a strategy was designed to express the protein in the outer membrane of *E. coli*. For this the signal peptide for OmpT of *E. coli*

was employed to transport the protein to the outer membrane after expression. After protein expression and cell lysis, the outer membrane extracts were separated from the cytoplasmic contents and checked on an analytical SDS-PAGE gel for the over expressed protein (Fig 22).

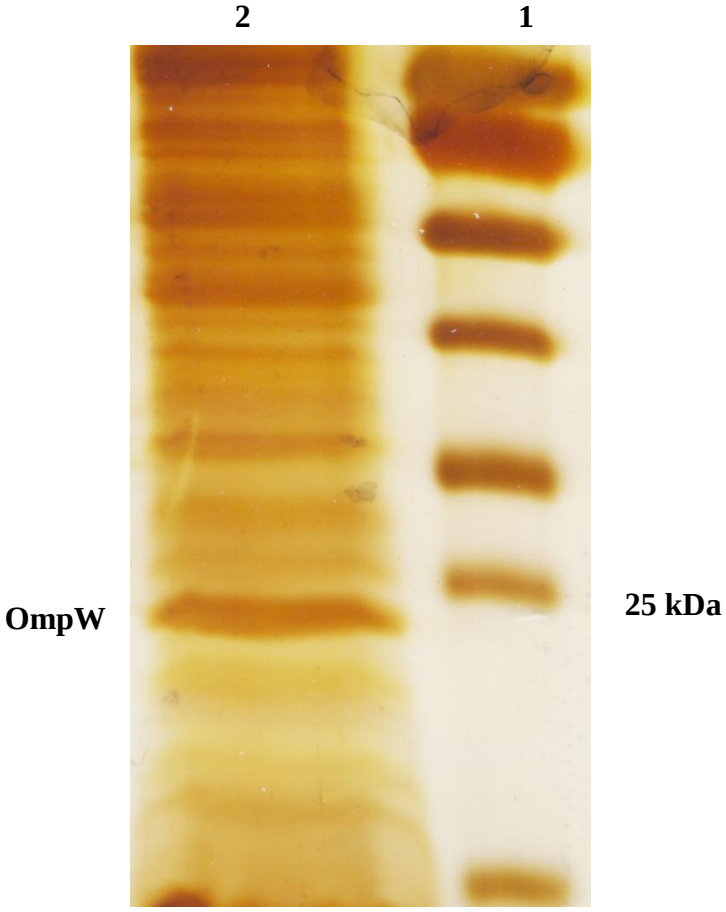


Figure 21. Silver stained 15% SDS-PAGE gel showing proteins from the outer membrane of the expression host harboring over expression plasmid for OmpW. Lane1: pre stained protein ladder, Lane 2: cell wall extracts of the expression host, with overexpressed OmpW shown.

As for the purification of the protein from inclusion bodies, the protein from the outer membrane extracts was also purified by running the sample on a preparative SDS-PAGE gel, cutting, crushing the slice and refolding the protein in 0.4 % LDAO in 10 mM Tris-HCl pH 8. After overnight incubation of the crushed gel slice with the detergent at 4 °C, the sample was checked as boiled and unboiled, as mentioned earlier. The protein was heat modified as can be seen by the two bands at approximately 22 kDa and 20 kDa on an analytical SDS-PAGE gel (Fig 23).

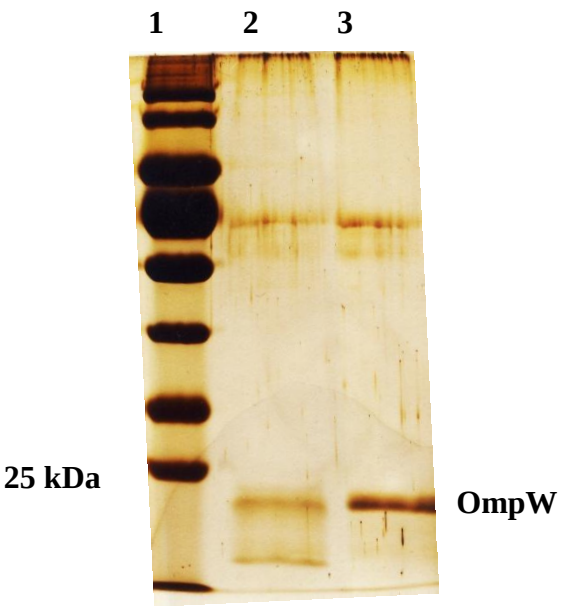


Figure 22. Silver stained 15% SDS-PAGE gel showing OmpW from the outer membrane of the expression host purified by cutting the protein band from a preparative SDS-PAGE gel. Lane1: pre stained protein ladder, Lane2: unboiled purified OmpW, Lane3: boiled OmpW

2.3.7. MALDI confirmation

The OmpW band was cut from a preparative SDS-PAGE gel under super clean conditions. The gel slice was crushed and tryptic digested. The tryptic digested sample was then analyzed on MALDI TOF and searched against Mascot servers. The most prominent hits were with OmpW of *C. crescentus* resulting from matching of several peptides from the tryptic digested sample to OmpW of *C. crescentus* (Fig 24).

Protein View

Match to: **gi|13422768** Score: **141** Expect: **6.6e-009**
outer membrane protein OmpW [Caulobacter crescentus CB15]

Nominal mass (M_r): **22488**; Calculated pI value: **9.57**
NCBI BLAST search of gi|13422768 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Caulobacter crescentus CB15
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|16125658 from Caulobacter crescentus CB15

Fixed modifications: Carboxymethyl (C)
Variable modifications: Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Number of mass values searched: **20**
Number of mass values matched: **10**
Sequence Coverage: **56%**

Matched peptides shown in **Bold Red**

```
  1 MKKLALSLVA FGALAAGAAQ AQDFTPNAKG DLIVHARLTQ VAPAKDAAIL
 51 TAAGANSGLK AHVGNDIKPT LGFTYFLTDK VAVEAILGTT EHNIRAQGPG
101 TDVLVHKTWV LPPVVTLQYH PLPASQVSPY VGAGLNYMLF YSGKNKNGFT
151 VKVDDGVGYA LQAGVNIKMK NSWLVNADVK KVYFSTDAKI NGGALKAKVD
201 LDPVVASIGL SRKF
```

Figure 23. OmpW protein confirmation using MALDI TOF. Peptides resulting from the tryptic digestion of the OmpW were checked with MALDI TOF and aligned against the databases of mascot servers (http://matrixscience.com). All the peptides that matched the OmpW from *C. crescentus* are marked in red. Over all match to OmpW was about 60 %.

2.3.8. Knockout of *ompW* from wildtype *C. crescentus*

As the over expression in *E. coli* did not result in properly folded and active OmpW, so a strategy was designed to knockout the *ompW* gene coding for OmpW protein in the wild type *C. crescentus*. The procedure is mentioned in details in the materials and methods section. After the knockout, cell wall extracts were extracted from both the knockout strain and the wild type using PBS/EDTA, and were checked on an analytical SDS-PAGE gel for the confirmation of the *ompW* knockout (Fig 25).

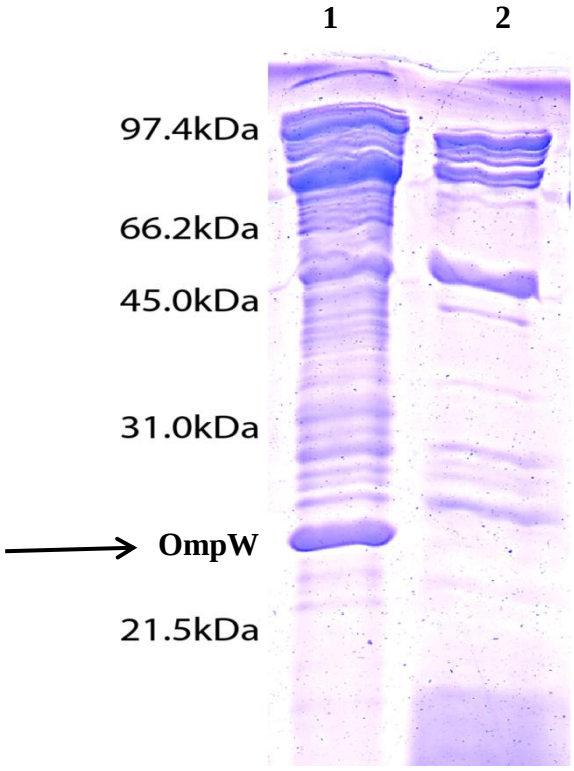


Figure 24. Coomassie stained 12 % SDS-PAGE gel with the extracts from wildtype and Δ*ompW C. crescentus*. Lane1: PBS/EDTA extracts from wildtype *C. crescentus*, Lane2: PBS/EDTA extracts from Δ*ompW C. crescentus*. Presence of OmpW is marked by the arrow.

2.3.9. Biophysical characterization of cell wall extracts of Δ*ompW C. crescentus*

The cell wall extracts from the *ompW* knockout strain of *C. crescentus* were extracted in the same way as from wild type strain. These extracts were tested in black lipid bilayer assay under the same condition, as for the wild type cell wall extracts. We could find out that the specific OmpW type activity which was observed with the wild type extracts was not there anymore (Fig 26).

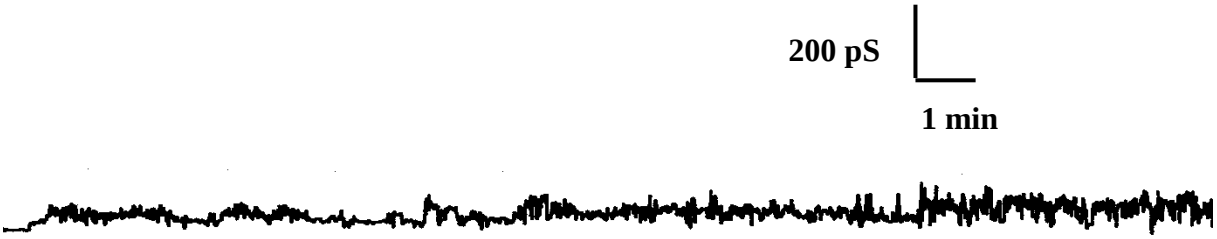


Figure 25. Single channel recording of PC/*n*-decane membrane in the presence of PBS/EDTA extracts from Δ*ompW C. crescentus*. The protein solubilized in 0.5 % LDAO was added to the 1 M KCl bathing solution at 100 ng/ml concentration. Applied membrane potential was 50 mV and T=20 °C.

2.4. Discussion

The genome of *C. crescentus* has been known for almost 15 years now but interestingly, no genes coding for classical outer membrane porins have been reported in its genome [70]. In this study we found channel forming activity of the enriched outer membranes of *C. crescentus*, which hinted the presence of a porin like protein in its outer membrane. Analyzing proteins at different molecular ranges on preparative SDS-PAGE showed that the enriched outer membrane extracts contained at least one porin like protein. By further purification steps the protein responsible for the channel forming activity was found to be a 20 kDa protein. Partial sequencing led us to conclude that the protein is indeed a member of the well-known OmpW family. The protein was having a conductance of 120 pS in 1 M KCl in artificial lipid bilayers. No significant activity was found for any other protein from the enriched outer membrane extracts, which shows that most probably the OmpW is the only porin in the organism. Although there are some functions attributed to some members of the OmpW family in several species, but their exact role is still a topic of debate.

The two well characterized members of the OmpW i.e. OmpW of *E. coli* and OprG of *P. aeruginosa* are having much lesser conductance in 1 M KCl as compared to the OmpW of *C. crescentus*. The crystal structures of these two porins suggest that the channel is plugged by tryptophan (W115 in OmpW *E. coli* and W170 in OprG *P. aeruginosa*), hindering the passage of ions through the channel (Fig 17 A) [72], [73]. The reason for higher conductance of OmpW *C. crescentus* might be because of the replacement of the tryptophan by a lysine (K160). The lysine compared to tryptophan is unlikely to hinder passage of ions. Presence of more hydrophilic residues in the channel of OmpW *C. crescentus*, compared to OmpW *E. coli* and OprG *P. aeruginosa* probably also has a role in the higher conductance of the channel (Fig 17 A). OmpW *C. crescentus* is also having larger channel radius compared to the counter parts from *E. coli* and *P. aeruginosa* (Fig 17B).

The protein was over expressed as N-terminally his-tagged in *E. coli* BL 21(DE3) Omp8 and was purified from the outer membrane of the expression host. This his-tagged OmpW *C.c* did not show clear channel forming activity in artificial lipid bilayers. This might be because of the his-tag. The his-tag might be blocking the channel. his-tag cleavage was challenging as we could not get enough cleaved protein and most of the time, the Factor Xa protease chopped off the whole protein. To overcome this issue the protein was expressed in inclusion bodies but still the protein did not reconstitute successfully into bilayer. This might be because of the improper refolding of the protein from the inclusion bodies. To avoid this issue, the protein

was expressed in its native state in the outer membrane of the expression host. The protein was successfully expressed and purified but the reconstitution experiments still proved to be challenging. Most probably the *E. coli* host is expressing the protein in a different folded state compared to its natural host. Although, this hypothesis will need further detailed studies in order to be confirmed.

To confirm that OmpW is the protein responsible for the porin like activity in the enriched cell wall extracts of *C. crescentus*, gene coding for OmpW was knocked out in the wild type. Comparing the enriched extracts from the *ompW* knockout strain and the wild type confirmed the hypothesis, as the extracts from the knockout strain did not show the channel forming activity as was seen with the extracts from the wild type strain.

Chapter 3

Chapter 3. Identification, molecular cloning and biophysical characterization of Lpg1974, a homologue of hVDAC-1 in *Legionella pneumophila.*

NOTE: The chapter contains contents from the manuscript in preparation

Identification, molecular cloning and biophysical characterization of Lpg1974, a homologue of hVDAC-1 in *Legionella pneumophila.*

Younas F., Soltanmohammadi N., Modi N., Kleinekathöfer U., Knapp O., Benz R.

Individual contributions

I expressed and purified the GST tagged Lpg1974. Biophysical characterization of the GST tagged Lpg1974 was done by Nafiseh Soltanmohammadi. I analyzed the data. I predicted the presence of a signal peptide in Lpg1974. I cloned the gene coding for Lpg1974 without its predicted signal peptide, over expressed it in *E. coli* and purified the encoded protein and refolded it. I characterized the protein in artificial lipid bilayers and confirmed its voltage dependent closure. I performed all the experiments, analyzed the data and also wrote the manuscript.

Summary

Legionella pneumophila is the causative agent of Legionnaires´ disease. It can infect both water-borne protozoans and human alveolar macrophages. Infection always starts with an interaction of the bacterium with the host cell, and interestingly, it uses same infection mechanism to infect these two very diverse hosts. The ability of *L. pneumophila* to infect macrophages has always been a topic of interest. Genome analyses revealed presence of a range of genes coding for eukaryotic like proteins in *L. pneumophila*. These eukaryotic like proteins most probably help the bacteria survive in complex eukaryotic hosts and have a role in the pathogenicity of the organism. We were interested in studying a homologue of hVDAC-1, Lpg1974 in *L. pneumophila*. hVDAC-1 is a member of a mitochondrial permeability transition pore (MPTP) complex in mitochondria. Similar complex has been hypothesized in *L. pneumophila*. Lpg1974 had 15 % identity and 35 % conservative replacement of amino acids, when compared to hVDAC-1. Lpg1974 was found to be forming large voltage gated channels in lipid bilayers.

Another distinguishing feature of Lpg1974 was the presence of an N-terminal signal peptide, while hVDAC-1 lacked any signal peptide. In order to study the significance of this predicted signal peptide, a truncated version of the protein Lpg1974Δ1-21 was over expressed in *E. coli* and purified. The protein was studied using several biophysical approaches. The results showed that the signal peptide has no role in the voltage gating of Lpg1974. It most probably has a role in the stability of the protein. Lpg1974Δ1-21 formed large anion selective and voltage gated channels in 1 M KCl in artificial lipid bilayers.

3.1. Introduction

The genus *Legionella* belongs to Gram-negative, non sporulating Gamma proteobacteria. Majority of the 48 species of the genus *Legionella* are harmless, aquatic saprophytes. Their most favored habitats are natural fresh water reservoirs [87], but they can also be found in hot water systems made by humans [88]. One of the unique features of this organism, which sparked new prospects in the field of science, is their survival and replication not only in water borne protozoa but also in human macrophages. Interestingly, they use the same virulence mechanism for infecting these two very different host types [89]. Only a small number of *Legionella* species are human pathogens; among them the most important one is *Legionella pneumophila* which causes Legionnaires´ disease. If not properly diagnosed and treated in the early stages, it can lead to an often fatal pneumonia. An outbreak at an American legion conference in Philadelphia in 1976 led to the recognition of *Legionella* as a pathogen [90]. Immuno-compromised persons, elderly and smokers are much more susceptible to *Legionella* as compared to a normal healthy person. *Legionella pneumophila* is the most dominant pathogen of all the pathogenic species of genus *Legionella*, being responsible for more than 98% of Legionnaires´ disease worldwide [91]. It is commonly believed that human alteration of the natural environment, partly led to the emergence of Legionnaires´ disease in the second half of the 20th century.

Out of the many forms of programmed cell death, apoptosis is the best understood and well characterized. During apoptosis the mitochondrial membrane permeability is significantly elevated because of the activation of mitochondrial permeability transition pore (MPTP), which allows the release of several apoptogenic factors i.e. cytochrome c into the cytoplasm [92], [93]. *Legionella*, most likely is evolutionarily not adapted to humans but it is able to infect alveolar macrophages and possibly epithelial cells, inducing apoptosis [94]. It finds its way to inside of the human body through inhalation of contaminated aerosols. Direct contact with the mammalian cell is absolutely necessary for the infection to be initiated [95], [96], [97]. *L. pneumophila* in their transmissive form is ingested by phagocytes. Within a short time single membrane phagosomes are formed which do not follow the normal endolysosomal pathway. These phagosomes which harbor *L. pneumophila* are resistant to phagolysosomal fusion and also to phagosomal acidification [98]. In nutrient rich environments such as the pathogenic vacuoles merged with lysosome, the transmissive phase is suppressed. After a decline in the nutrients level, the bacteria starts its transmissive phase again, where they express several virulence traits that lead to transmission to new hosts [99]. *L. pneumophila* is

reported to be causing apoptosis in several myeloid cell lines as well as epithelioid cell line [100].

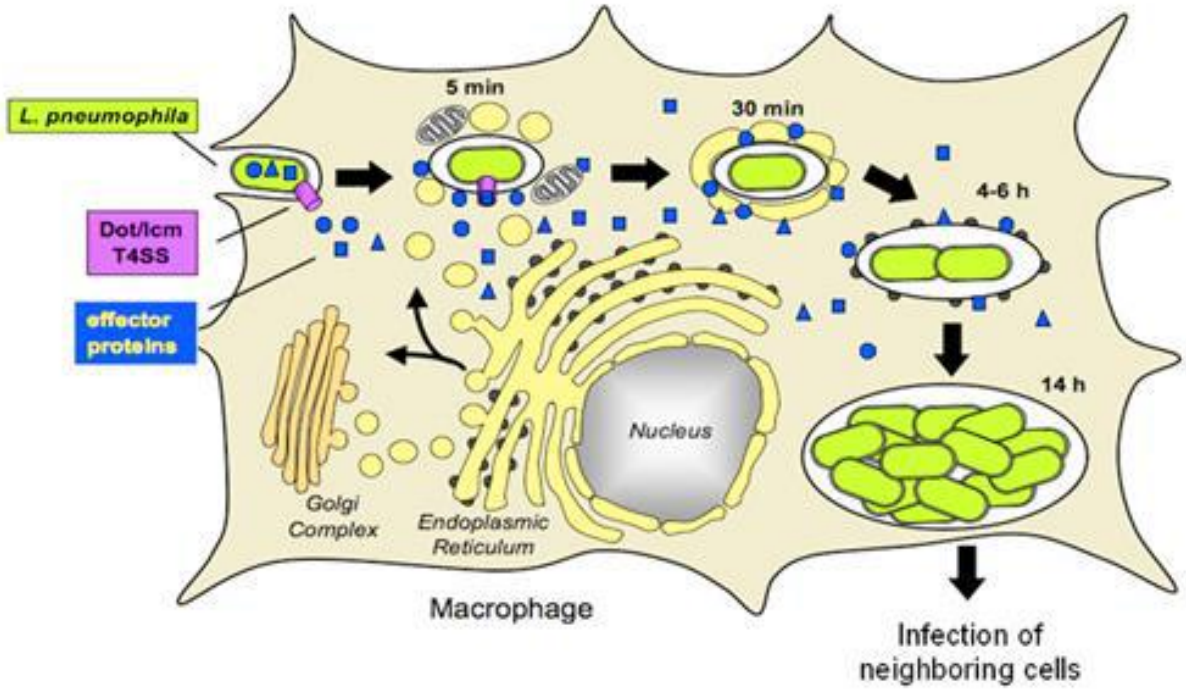


Figure 26. *Legionella pneumophila* intracellular replication cycle. *L. pneumophila* uses the Dot/Icm T4SS (pink) to deliver a multitude of effector proteins (blue) into the cytosol of an infected macrophage. The pathogen recruits host cell transport vesicles to establish a replication vacuole. (National Institute of Health, 2009).

A number of eukaryotic like proteins have been identified in *L. pneumophila* [101], [102], [103]. Eukaryotic like proteins (ELPs) constitute almost 3 % of the putative proteomes of *L. pneumophila* strains [101]. Some of the several possible assumptions about the origin of these ELPs can be lateral gene transfer from eukaryotes, gene fragments acquisition, or bacterial genes that became eukaryotic like [101].

Presence of hVDAC-1 like proteins is rarely reported in bacteria [104], [105]. So far, proteins resembling almost all of the components of the MPTP complex of mitochondria have been reported only in *L. pneumophila* [106]. The proposed counter parts of peripheral benzodiazepine receptor (PBR) and hVDAC-1 of the MPTP complex are Lpg0211 and Lpg1974 respectively in *L. pneumophila*.

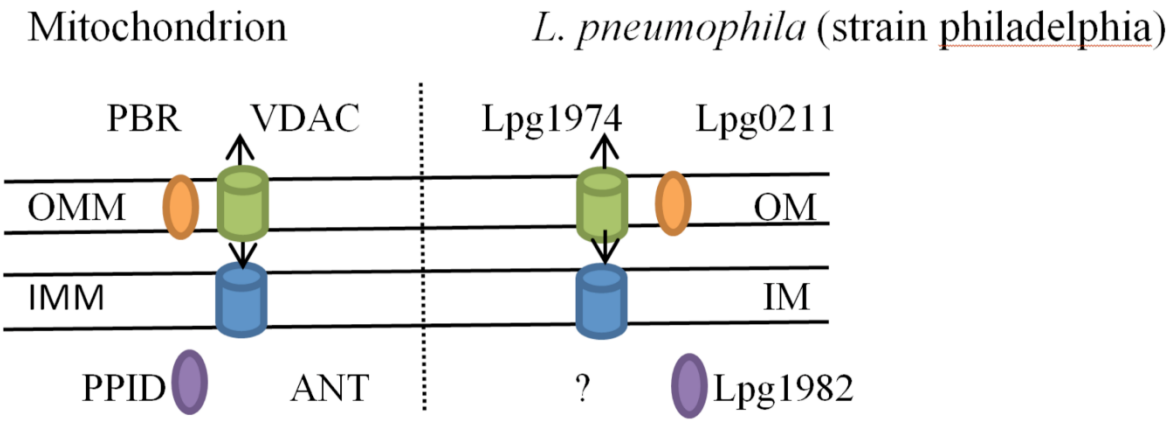


Figure 27. Schematic view of the selected components comprising the MPTP complex (left) and the corresponding components identified in the genome of *L. pneumophila*. OMM: outer mitochondrial membrane, IMM: inner mitochondrial membrane, PBR: peripheral benzodiazepine receptor, PPID, cyclophilin D (peptidyl prolyl isomerase D), ANT: adenine nucleotide translocator, IM: inner membrane, OM: outer membrane. Counter part of ANT in *L. pneumophila* is still to be known. Adapted from [106].

Here we confirm Lpg1974 to be a homologue of hVDAC-1 by its biophysical characterization in black lipid bilayer assay and molecular dynamics simulations. The protein was found to be having a conductance of 5.5 nS in 1 M KCl. The protein was further characterized by studying the role of its N-terminal signal peptide. The gen *lpg1974* coding for Lpg1974 was cloned in a way to have the protein expressed without its putative signal peptide (named here Lpg1974 Δ1-22).

3.2. Materials and methods

3.2.1. Bacterial cells and culturing conditions

For the purpose of N-terminally GST tagged Lpg1974 expression, the plasmid pGEX-2T *lpg1974* was designed previously in our lab. For cloning purposes *E. coli* DH5α or *E. coli* XL1 blue cells were used. *E. coli* K-12 KS26 *ΔompC, ΔompF, ΔlamB* [107] were used as an expression host for the expression of the GST tagged Lpg1974. The *lpg1974* was also amplified without the predicted signal peptide and cloned into the expression vector pAraJS2 in order to overexpress the protein without its native signal peptide without any tag. *E. coli* BL21 (DE3) Omp8 were used as host cells for the expression of Lpg1974 (Δ1-21). Cultures were grown under standard conditions in LB medium with 100 µg/ml ampicillin.

3.2.2. Sequence analysis, secondary structure prediction and homology model of Lpg1974.

Based on a hypothesis published earlier [106] about the presence of a homologue of hVDAC-1, Lpg1974 in *L. pneumophila*. We further analyzed the sequences, developed predicted secondary structures and also a homology model for the protein, using hVDAC-1 as bait.

Protein sequence of hVDAC-1, as obtained from UniprotKB with I.D of P21796.2 was as follows:

```
MAVPPTYADLGKSARDVFTKGYGFGLIKLDLKTKSENGLEFTSSGSANTE
1         10        20        30        40        50

TTKVTGSLETKYRWTEYGLTFTEKWNTDNTLGTEITVEDQLARGLKLTFD
          60        70        80        90       100

SSFSPNTGKKNAKIKTGYKREHINLGCDMDFDIAGPSIRGALVLGYEGWL
         110       120       130       140       150

AGYQMNFETAKSRVTQSNFAVGYKTDEFQLHTNVNDGTEFGGSIYQKVNK
         160       170       180       190       200

KLETAVNLAWTAGNSNTRFGIAAKYQIDPDACFSAKVNNSSLIGLGYTQT
         210       220       230       240       250

LKPGIKLTLSALLDGKNVNAGGHKLGLGLEFQA
         260       270       280   283
```

Figure 28. Protein sequence of hVDAC-1 as obtained from www.rcsb.org with PDB ID 2JK4.

The protein has 288 amino acid residues.

Protein sequence of Lpg1974 as obtained from NCBI database with accession number AAU28043.1 was as follows:

```
MFSLKKTTAAVFALGSSALFAGTMGPVCTPGNVTVPCERTAWDIGITALY
1         10        20        30        40        50

LQPTYDADWGYNGFTDVGGWRNWHDVDLEWDWGFKLEGSYHFNTGNDINV
          60        70        80        90       100

NWYHLDANTDHWAFVDQLHAYDITWDAVNAELGQFVDFSANKKMRFHGGV
         110       120       130       140       150

QYAYIKTDVNRHLNGFFLDNFNSKFNGFGPRTGLDMNYVFGNGFGIYAKS
         160       170       180       190       200

AVAILVGTSKFVDNCTVCGFSYGSKNAIVPEVEMKLGADYTYAMAQGDLT
         210       220       230       240       250

LDVGYMWFNYFNALHNTAAVNVGLGTSLETDFSASGPYIGLKYVGNV
         260       270       280       290   297
```

Figure 29. Primary amino acids sequence of Lpg1974. The sequence was retrieved from NCBI database with accession number AAU28043.1. The protein is composed of 297 residues.

Multiple sequence alignments of the two proteins were performed using clustal omega. Homology model of Lpg1974 was developed by Niraj Modi using modeler.

3.2.3. Expression and purification of Lpg1974-GST

Expression host *E. coli* K-12 KS26 harboring the expression plasmid were grown overnight at 37°C in LB medium containing 100 µg/ml ampicillin. This starter culture was then used to inoculate larger volume media. The cultures were grown at 37°C, until the OD600 reached 1. 1mM IPTG was used as inducer for the protein expression. Induced cultures were allowed to grow O/N at 37°C/250 rpm. Cells were harvested by centrifugation at 4000*g for 30 min at 4°C. Cell lysis was accomplished by sonicating the cells suspended in 10 mM Tris-HCl pH 8. Sonication was performed at 80 % amplitude for 8 min with 30 sec break after every 30 sec impulse. The sonicated sample was centrifuged at 4000*g for 15 min. As, the protein was getting expressed in the inclusion bodies, so the inclusion bodies were purified using the protocol mentioned earlier in the section 2.2.7. The inclusion bodies can also be checked for the over expression in a simpler and much more time efficient way. After centrifugation of the sonicated sample, the pellet was suspended in 0.5 % LDAO in 10 mM Tris-HCl pH 8. This resuspended pellet was mixed with 4x sample buffer lacking the strong denaturing agents such as DTT and β mercaptoethanol. The dye was added 4 times the volume of the suspension. This sample was then incubated for 10 min at room temperature and centrifuged at 13,000*g for 2 min to pellet the debris. The supernatant was then loaded directly on either an analytical or preparative SDS-PAGE gel. The over expressed Lpg1974-GST was purified by cutting the protein band from a preparative SDS-PAGE gel and refolding the protein either in 1 % Genapol or 0.5 %LDAO in 10 mM Tris-HCl pH 8 at 4 °C for 16-18 hrs.

In order to cleave the GST tag attached at N-terminus of Lpg1974, several condition were tried, but best results were obtained after incubating 1 mg/ml of the purified protein with 100 µl of the thrombin agarose resin in the presence of 1x final concentration of the cleavage buffer (500 mM Tris-HCl, pH 8.0, 100 mM $CaCl_2$). The cleavage mixture was run on a preparative SDS-PAGE gel and the cleaved protein was gel cut and refolded as mentioned earlier.

3.2.4. Cloning of *lpg1974*Δ1-21, expression, purification and biophysical characterization of the encoded protein by black lipid bilayer assay

When both Lpg1974 and hVDAC-1 were analyzed for their signal peptides using the signal peptide prediction server from SignalP4.0 [108]. Interestingly, the prediction was positive for Lpg1974, but not for hVDAC-1. In order to study the significance of the signal peptide the gene *lpg1974*, coding for Lpg1974 was cloned into an expression vector pAraJS2. The gene was amplified from pGEX-2T *lpg1974* using the primers 5´-TCAACATATGGGTACGATGGGTCCAGTATGTAC and 5´-TGTACTCGAGTTAGACATTGCCAACATATTTTAAGCC. The primers contained restriction sites for *Nde1* and *Xho1* (underlined in the primers sequences). The amplified pcr product and the expression plasmid were double digested with *Nde1* and *Xho1*. The double digested products were extracted from a preparative agarose gel and purified using QIAGEN gel extraction kit. The double digested amplified PCR product and the plasmid were ligated at 4°C O/N at the molar ratio of 1:3. The ligation product was chemically transformed into chemo competent DH5α cells. Transformed cells were grown on LB agar containing 100 µg/ml ampicillin. Plasmids were extracted from the transformed cells, confirmed by double digestion and sequencing. Once the correct orientation of the gene in the plasmid was confirmed, the plasmids were transformed into chemo competent expression hosts i.e. *E. coli* BL21 (DE3) Omp8. The protein was overexpressed in *E. coli* BL21 (DE3) Omp8 using the same protocol as mentioned above for the expression of the GST tagged protein, except that the cells were induced with 0.2 % arabinose only for 3 hours.

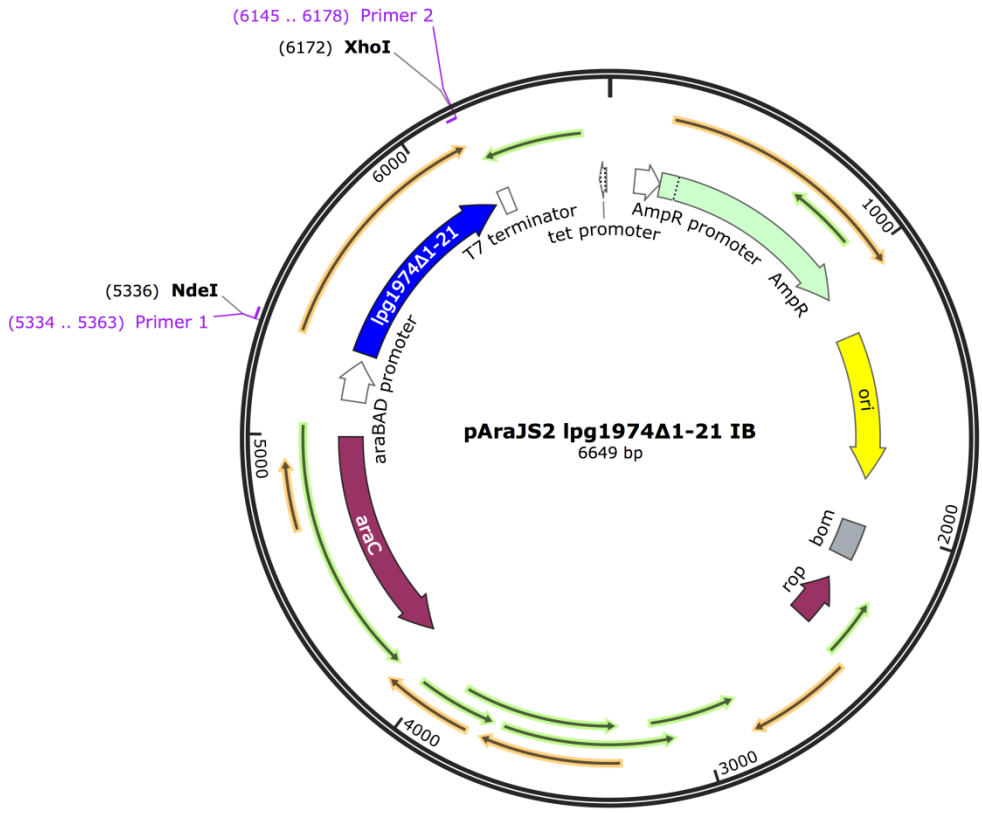


Figure 30. Plasmid map of pAraJS2 harboring the mutant gene coding for Lpg1974Δ1-21. The map shows the primers which were used to amplify the gene from the parent plasmid and also the restriction sites for *Nde1* and *Xho1* which were used to clone the gene into the plasmid. araBAD is an arabinose inducible promoter.

Black lipid bilayer assay was employed for the characterization of Lpg1974Δ1-21. The technique is mentioned in detail in chapter 2. Potassium chloride used in the experiments was of analytical grade and obtained from Applichem. 1 % (w/v) diphytanoyl phosphatidylcholine (PC) (Avanti Polar Lipids, Alabaster AL) in *n*-decane was used to form bilayers.

3.3. Results

3.3.1. Initial sequence alignments and molecular dynamics simulations of Lpg1974 and hVDAC-1.

The protein sequences of Lpg1974 and hVDAC-1 were aligned using Clustal W. The results were not very prominent but still obvious (Fig 32). Lpg1974 showed 15% identity and 17% strongly similar amino acids in comparison to hVDAC-1. hVDAC-1 has a molecular mass of 31 kDa with 283 amino acids.

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
Lpg1974     ------MFSLKKTTAAVFALGS-SALFAGTMGPVCTPGNVTVPCERTAWDIGITALYLQP
hVDAC1      MAVPPTYADLGKSARDVFTKGYGFGLIKLDLKTKSENGLEFTSSGSANTETTKVTGSLET
                    .* *::  **: *   .*:   : . .  *   ...  :  :   .:  *:.
                    70        80        90       100       110       120
                     |         |         |         |         |         |
Lpg1974     TYDADWGYNGFTDVGGWRNWHDVDLEWDWGFKLEGSYHFNTGNDINVNWYHLDANTDHWA
hVDAC1      KYR--WTEYGLTFTEKWNTDNTLGTEITVEDQLARGLKLTFDSSFSPNTGKKNAKIKTGY
            .*   *   *:* .  *.. : :. *     :*  . ::. ...:. *  : :*: .

                   130       140       150       160       170       180
                     |         |         |         |         |         |
Lpg1974     FVDQLHA-YDITWDAVNAELGQFVDFSANKKMRFHGGVQYAYIKTDVNRHLNGFFLDNFN
hVDAC1      KREHINLGCDMDFDIAGPSIRGALVLGYEG---WLAGYQMNFETAKSRVTQSNFAVG---
              ::::   *: :* ....:   : :. :    : .* *  : .:. .   ..* :.

                   190       200       210       220       230       240
                     |         |         |         |         |         |
Lpg1974     SKFNGFGPRTGLDMNYVFGNGFGIYAKSAVAILVGTSKFVDNCTVCGFSYGSKNAIVPEV
hVDAC1      YKTDEFQLHTNVNDGTEFGGSIYQKVNKKLETAVNLAWTAGNSNTR-FGIAAKYQIDPDA
             * : *  :*.:: .  **..:   .:. :   *. :  ..*...  *. .:*  * *:.

                   250       260       270       280       290       300
                     |         |         |         |         |         |
Lpg1974     EMKLGADYTYAMAQG-DLTLDVGYMWFNYFNALHNTAAVNVGLGTSLETDFSASGPYIGL
hVDAC1      CFSAKVNNSSLIGLGYTQTLKPGIKLT--LSALLDGKNVNAG-GHKLG---------LGL
             :.  .: :  :. *   **. *      :.** :   **.* * .*          :**


Lpg1974     KYVGNV
hVDAC1      EFQA--
            :: .
```

Figure 31. Amino acid sequence alignment of Lpg1974 and hVDAC-1. The alignment was performed using Clustal Omega. (*) shows amino acids identical in both the proteins. Strongly similar amino acids are shown by (:), and weakly similar ones are represented by (.).

The structure of hVDAC-1 is composed of 19 β strands with an α helix at the N-terminus. Lpg1974 has 297 residues and a molecular mass of approximately 33 kDa. A homology model for Lpg1974 was developed by using Modeller and taking hVDAC-1 as a substrate

(Fig 33). Structural alignment of hVDAC-1 and Lpg1974 showed amazing similarity between the two proteins (Fig 33).

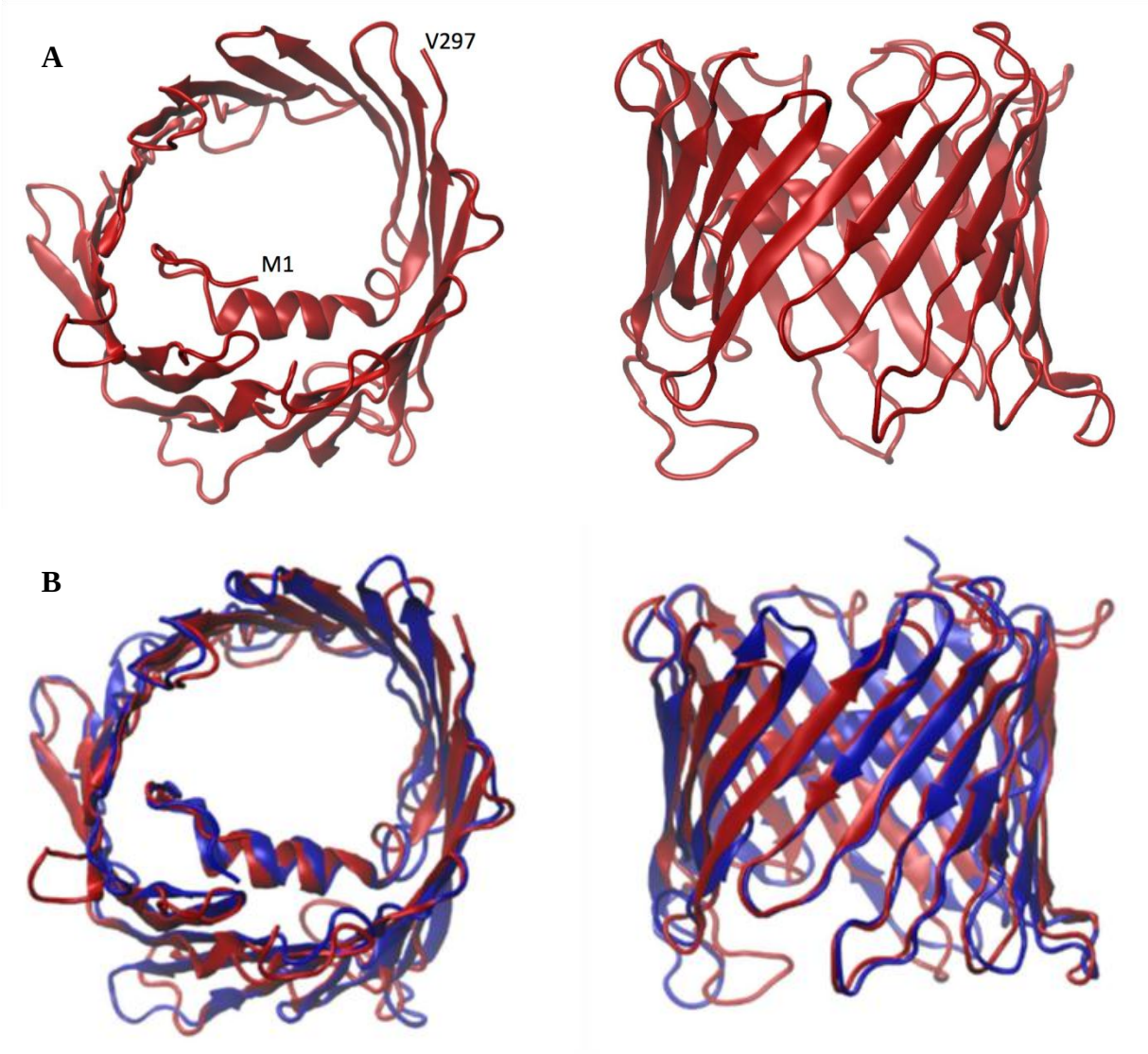


Figure 32. Homology structure of Lpg1974 and structural alignment with hVDAC-1. A: Views of homology structure of Lpg1974 using hVDAC-1 as bait. The structure was developed using Modeller. M1 is the first amino acid methionine, and V297 is the last amino acid valine. B: Structural alignment of Lpg1974 with hVDAC-1. Red colored structure is of Lpg1974 and blue colored is hVDAC-1. The structures were developed by Niraj Modi using Modeller.

3.3.2. Over expression of Lpg1974-GST and GST tag cleavage

lpg1974 coding for Lpg1974 was cloned into the expression vector pGEX-2T in order to have a GST tag at the N-terminus of the protein. The sequence also contained a thrombin cleavage site downstream of the GST tag to be used later for the cleavage of the tag by thrombin. The recombinant protein was expressed in *E. coli* KS26, a porin deficient mutant strain of *E. coli* K-12 (*ΔompC, ΔompF. ΔompC and ΔlamB*). The cells harboring the recombinant plasmid were induced at an OD600 of 1.0 with 1 mM IPTG. The protein was getting expressed mostly in the inclusion bodies. IMAC purification could not lead to pure protein, maybe because of the aggregation of the protein. So the protein was purified the cutting the over expressed Lpg1974-GST from a preparative SDS-PAGE gel and refolding it in 0.4 % LDAO in 10 mM Tris-HCl pH 8 (Fig 34).

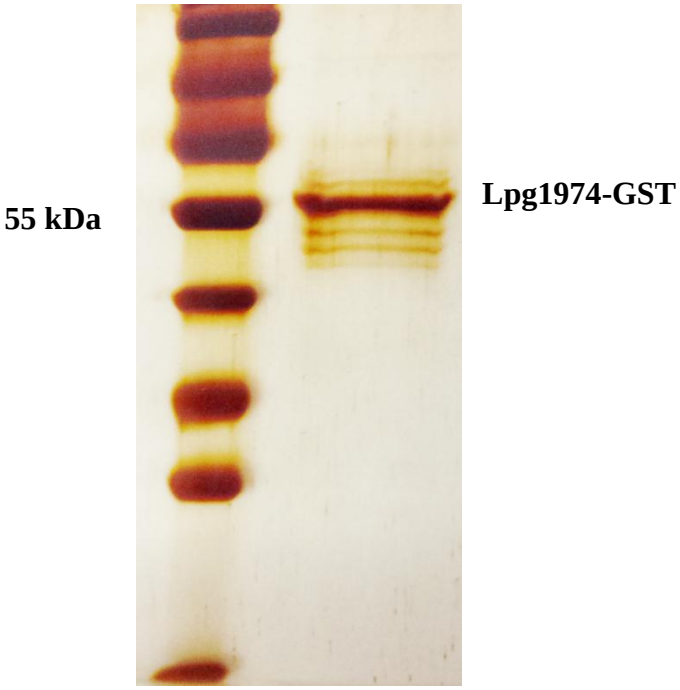


Figure 33. SDS-PAGE gel for purified Lpg1974-GST. A silver stained 12 % SDS-PAGE gel which shows the gel extracted pure Lpg1974-GST.

After the protein was purified in good enough concentration and purity the attached GST tag was to be cleaved. For cleavage 1mg of the protein was incubated with 100 units of thrombin at RT. The cleavage mixture was then run on an analytical SDS-PAGE gel to check for the cleavage efficiency (Fig 35). Cleavage was tried at several different conditions and resulted in different success rates.

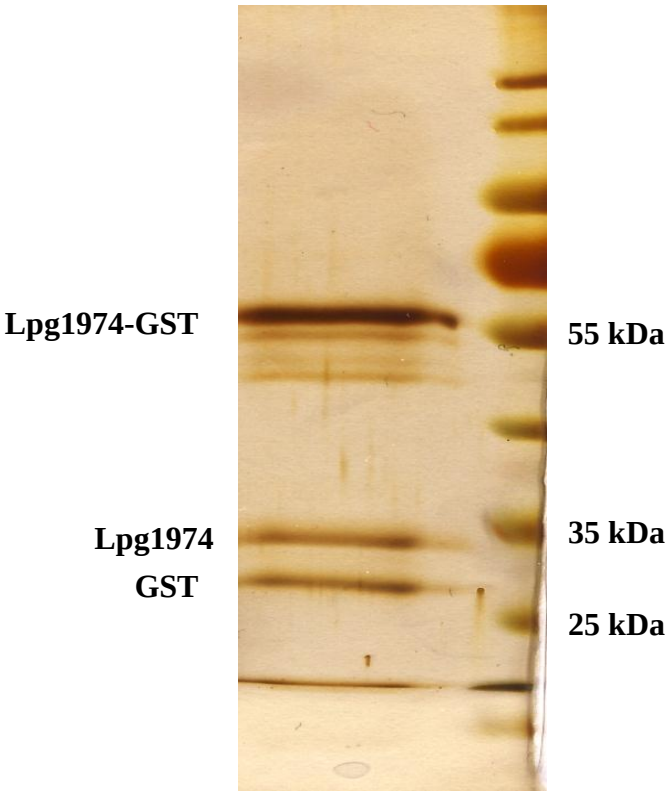


Figure 34. GST tag cleavage of Lpg1974-GST. A silver stained 15 % SDS-PAGE PAGE gel with the cleavage mixture of GST tag cleavage experiment. The upper band at about 60 kDa shows uncleaved protein. The band at about 33 kDa is the protein without GST tag, and the protein band at about 26 kDa is the cleaved GST tag.

As the protein could not be purified by IMAC, the cleaved Lpg1974 could also be not purified using the IMAC. The cleaved protein was cut from a preparative SDS-PAGE gel, refolded in a detergent solution and checked again on an analytical SDS-PAGE gel (Fig 36). Both the cleaved protein and the cleaved GST tag were cut from the gel and checked on the SDS-PAGE gel. The protein was pure, with no other significant contaminating proteins. The protein without the tag had a molecular mass of approximately 33 kDa. The cleaved off GST tag can be seen running at about 25 kDa.

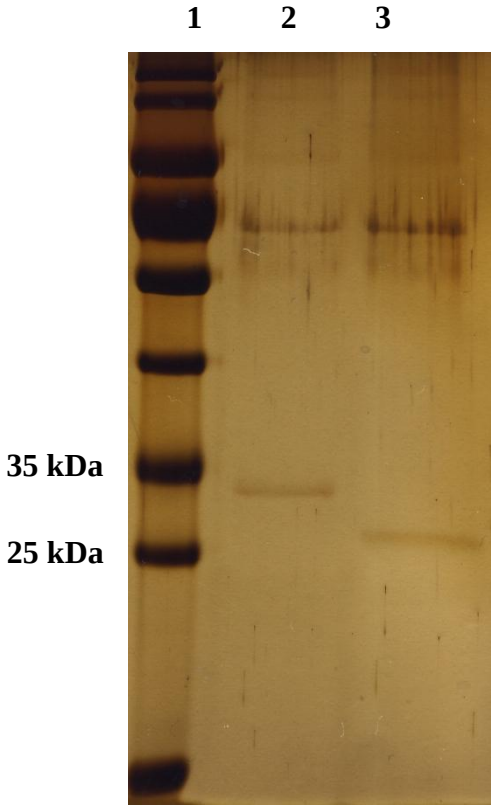


Figure 35. SDS-PAGE PAGE gel for purified Lpg1974 after GST tag cleavage. A silver stained 15 % SDS-PAGE gel showing the purified Lpg1974 after the cleavage of GST tag. 1: Pre-stained protein ladder, 2: purified Lpg1974, 3: Cleaved off GST tag.

3.3.3. Biophysical characterization of Lpg1974

3.3.3.1. Single-channel analysis

Addition of pure Lpg1974 refolded in 0.4 % LDAO to the either side of an artificial membrane formed from PC/*n*-decane led to gradual stepwise increase in the conductance of the membrane within a few minutes. The channel activity was exclusively because of the protein, as addition of just the dye did not result in conductance increase. The protein was added at 100 ng/ml concentration. The channels had a tendency to remain open at low voltage of 20 mV. Analysis of all the channels observed concluded that most frequent channels had a conductance of 5.5±0.5 nS in 1 M KCl.

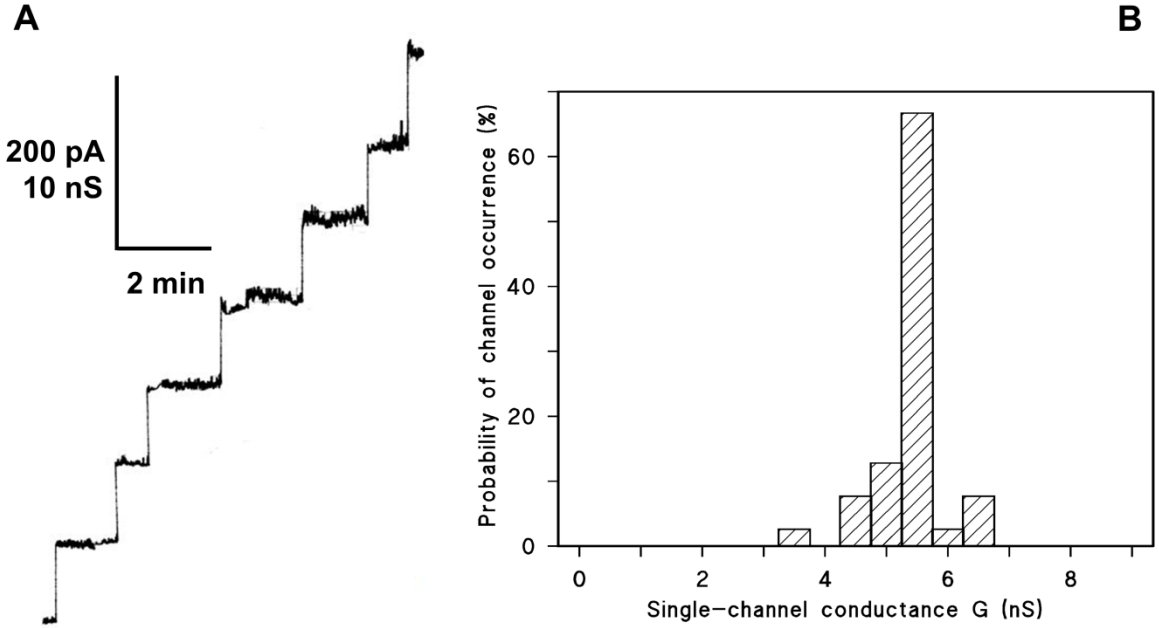


Figure 36. Single-channel recordings for Lpg1974. (A) Single-channel recording for a PC/*n*-decane membrane after addition of 100 ng/ml of the purified Lpg1974 to the 1 M KCl pH 7 bathing the membrane. Applied voltage was 20 mV and temperature 21 °C. (B) A histogram showing the probability of occurrence of channels with a specific conductance value. The most frequent channels are having a conductance of 5.5 nS. Adapted from the PhD thesis of Nafiseh Soltanmohammadi.

3.3.3.2. Lpg1974 is voltage gated

Voltage dependent behavior of Lpg1974 was studied in multi-channel experiments. Lpg1974 was added to either side of the black lipid bilayer formed from PC/*n*-decane which resulted in the reconstitution of a number of channels in the membrane. Once the conductance reached a stationary phase, increasing positive and negative voltages were applied to the *cis*-side of the membrane. The results showed symmetric voltage dependent closure of the channels at higher voltages.

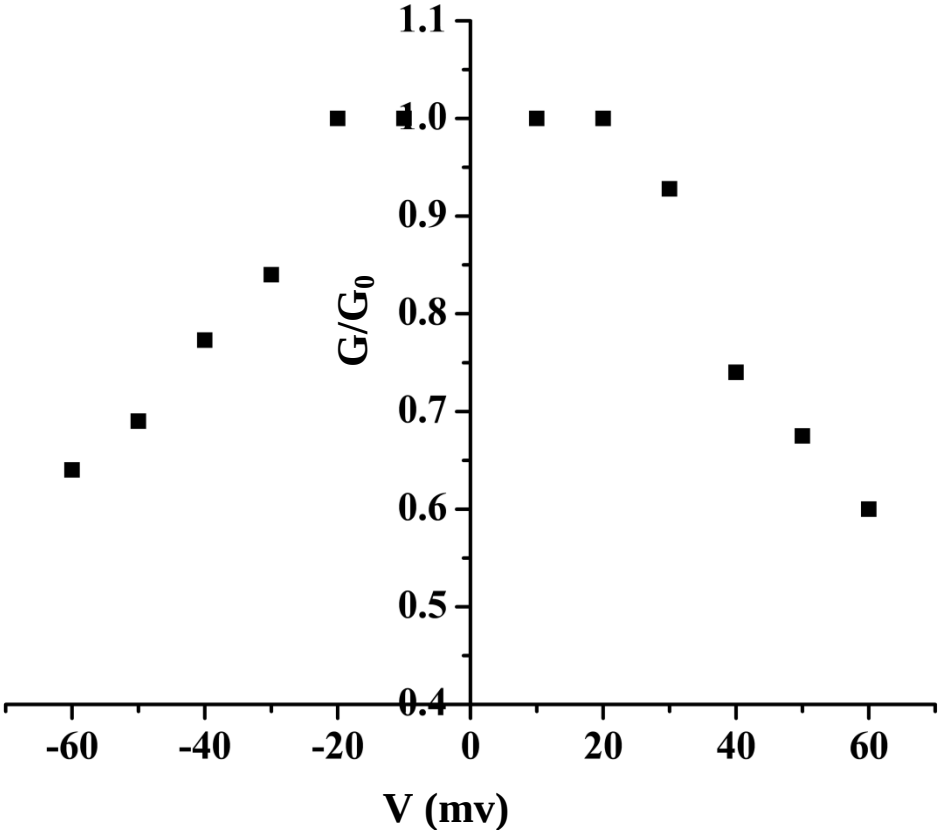


Figure 37. Graphical representation of voltage dependent behavior of Lpg1974. The aqueous phase contained 1 M KCl, 100 ng/ml of Lpg1974 refolded in 0.4 % LDAO. Lipid bilayer membrane was formed from PC/*n*-decane at a temperature of 21 °C. Conductance (G) at a specific membrane potential (V_m) divided by the conductance at 20 mV (G_0) as a function of the membrane potential. Figure made from data adapted from the PhD thesis of Nafiseh Soltanmohammadi.

3.3.4. Signal peptide prediction for Lpg1974 and hVDAC-1

Most membrane proteins are expressed with a signal sequence which will direct the protein to be transported to the outer membrane after being expressed, although there are some exemptions to this rule. For some proteins there are no signal peptides predicted but they are still being transported to the outer membrane with a system which is still not completely clear. The signal peptide is cleaved off once the transported protein reaches its destination.

hVDAC-1 is the most abundant protein in the outer membrane of mitochondria and is also known as mitochondrial channel. Lpg1974 is also a membrane protein in the outer membrane of *L. pneumophila*. We checked both of these proteins were checked for the presence of a signal peptide. For the predictions we used SignalP4.0 servers [108]. For prediction of signal peptide of Lpg1974 organism group selected was “Gram-negative bacteria” while for

hVDAC-1 "Eukaryotes". Interestingly the servers identified a signal peptide only in Lpg1974 (Fig 37), no signal peptide was hypothesized for hVDAC-1 (Fig 38)

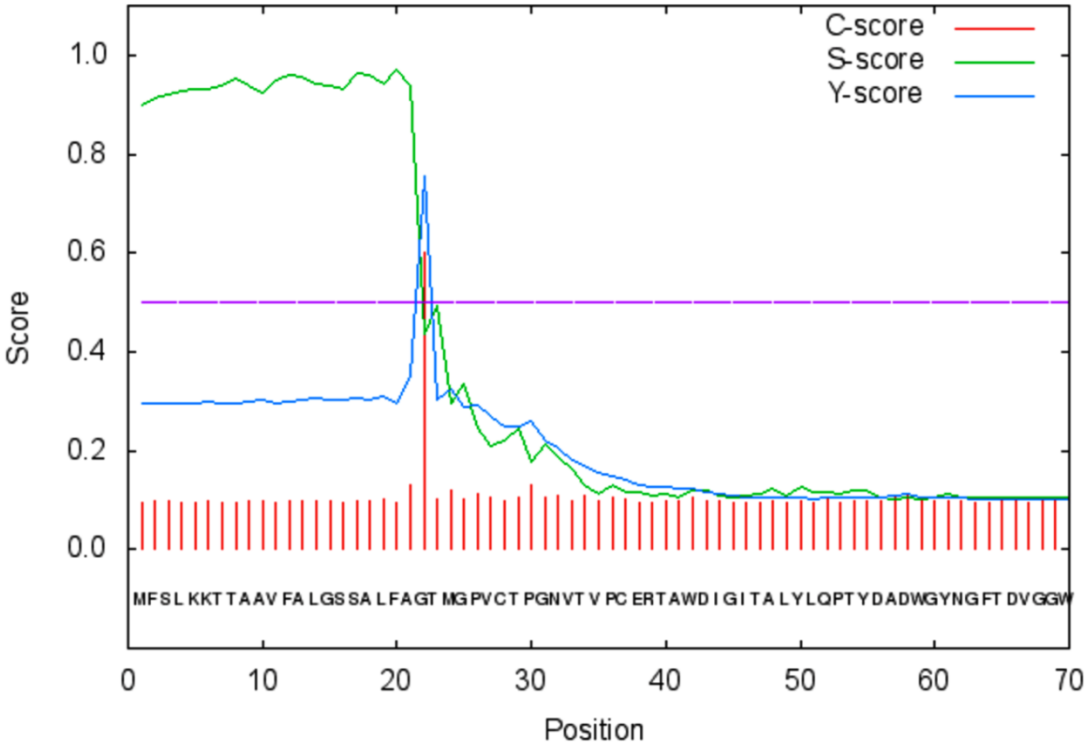


Figure 38. Signal peptide prediction for Lpg1974. Graphical representation of the predicted signal peptide for Lpg1974 using SignalP4.0 server. The cleavage position of the signal peptide is after amino acid no 21 which is alanine.

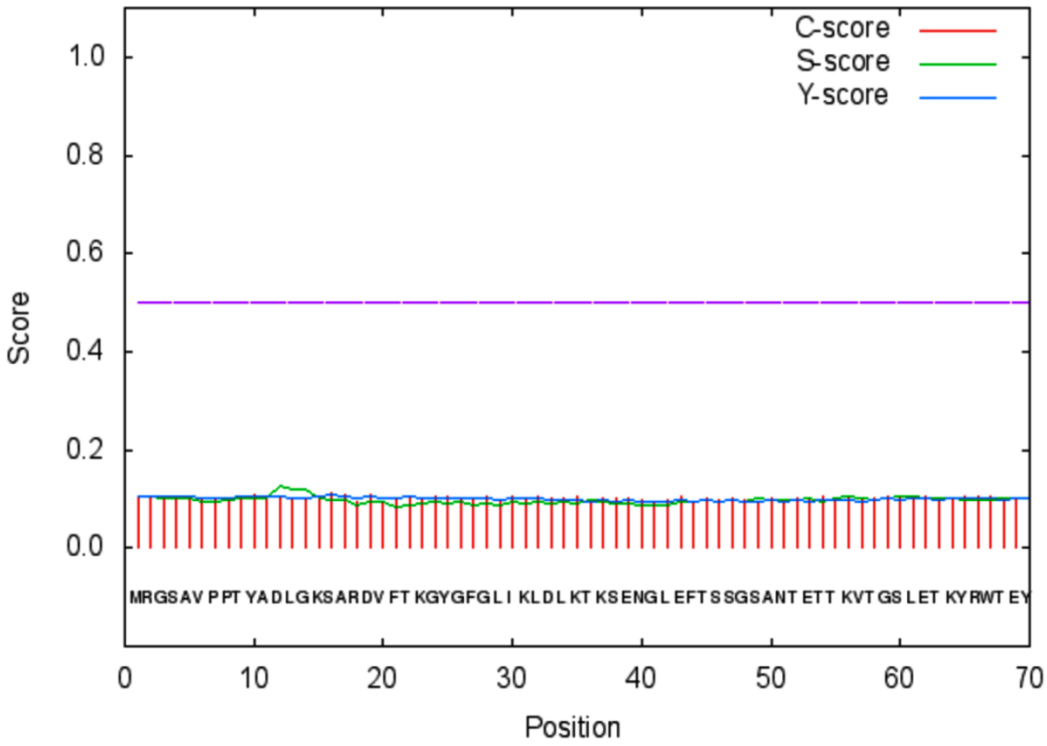


Figure 39. Signal peptide prediction for hVDAC-1. No signal peptide is predicted for the amino acid sequence of the protein.

3.3.5. Expression and purification of Lpg1974Δ1-21

In order to study the significance of the predicted signal peptide in Lpg1974, the encoding gene *lpg1974* was cloned into an expression vector pAraJS2 without any signal peptide. The transgenic plasmid was transformed into expression hosts *E. coli* BL21 (DE3) Omp8. The cells were induced at an OD600 of 1.0 with 0.2 % arabinose for three hours. Inclusion bodies containing the overexpressed Lpg1974Δ1-21 were purified using the protocol mentioned in the section (2.2.7). Purified inclusion bodies suspended in 0.5 % LDAO in 10 mM Tris-HCl pH 8 were mixed with SDS-PAGE sample buffer and incubated at RT for 10 min. The sample was centrifuged at 13,000*g for 2 min and the supernatant run on an analytical SDS-PAGE gel. The sample showed a clear and very high over expression of the Lpg1974Δ1-21 at approximately 30 kDa (Fig 39A). The overexpressed protein band was cut out from a preparative SDS-PAGE gel, refolded in 0.4 %LDAO in 10 mM Tris-HCl pH 8. In order to confirm the purity of the protein, the gel extracted was run again on an analytical SDS-PAGE gel (Fig 39B). The purified protein was in high purity and reasonable concentration.

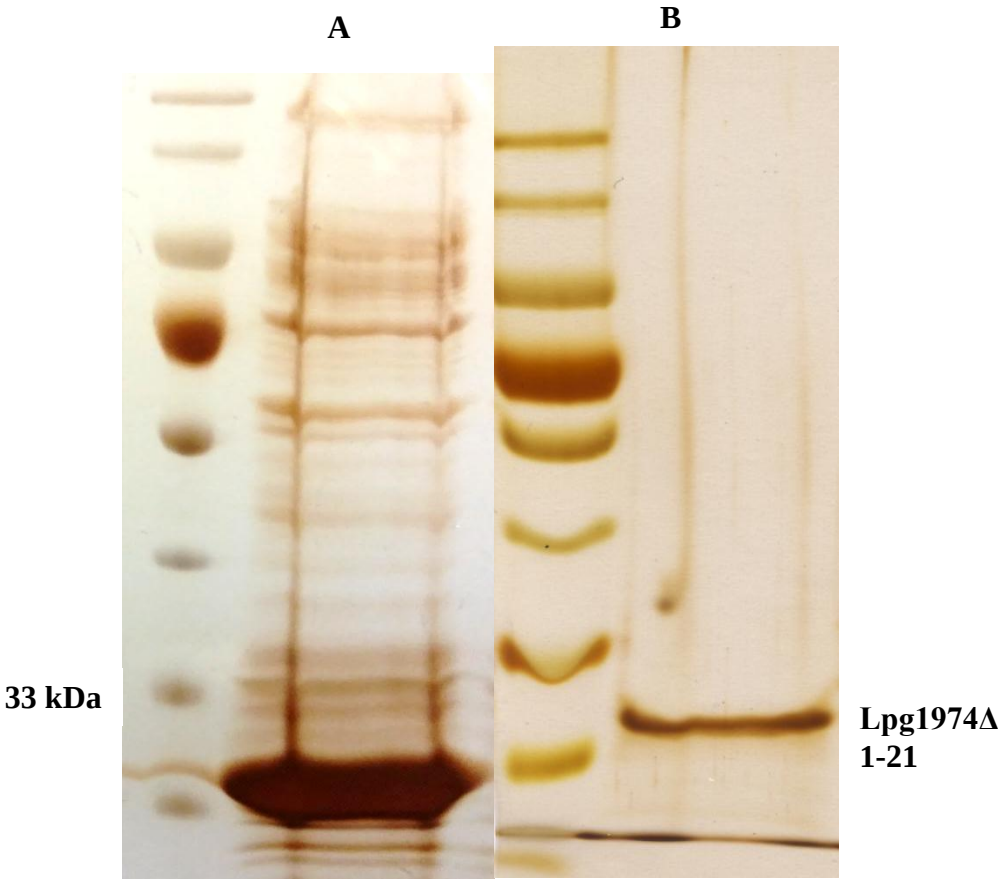


Figure 40. Silver stained 15 % SDS-PAGE gels showing the expressed and purified Lpg1974Δ1-21. A: Proteins from the inclusion bodies showing the over expressed Lpg1974Δ1-21 at about 33 kDa. B: Purified Lpg1974Δ1-21 after gel extraction and refolding.

3.3.6. Biophysical characterization of Lpg1974 Δ1-21

3.3.6.1. Single-channel analysis

Addition of the purified Lpg1974Δ1-21 in minor amounts (100 ng/ml) to either one or both sides of a bilayer formed of PC/*n*-decane resulted in a stepwise increase in the conductance of the membrane (Fig 40A). The increase in conductance was recorded on a chart recorder. The conductance increase was measured at 20 mV applied membrane potential in 1 M KCl. Plotting all the inserted channels (approximately 80 channels) as a histogram (Fig 40B) indicated that the most frequent conductance step was about 7.5 nS in 1 M KCl at applied membrane potential of 20 mV.

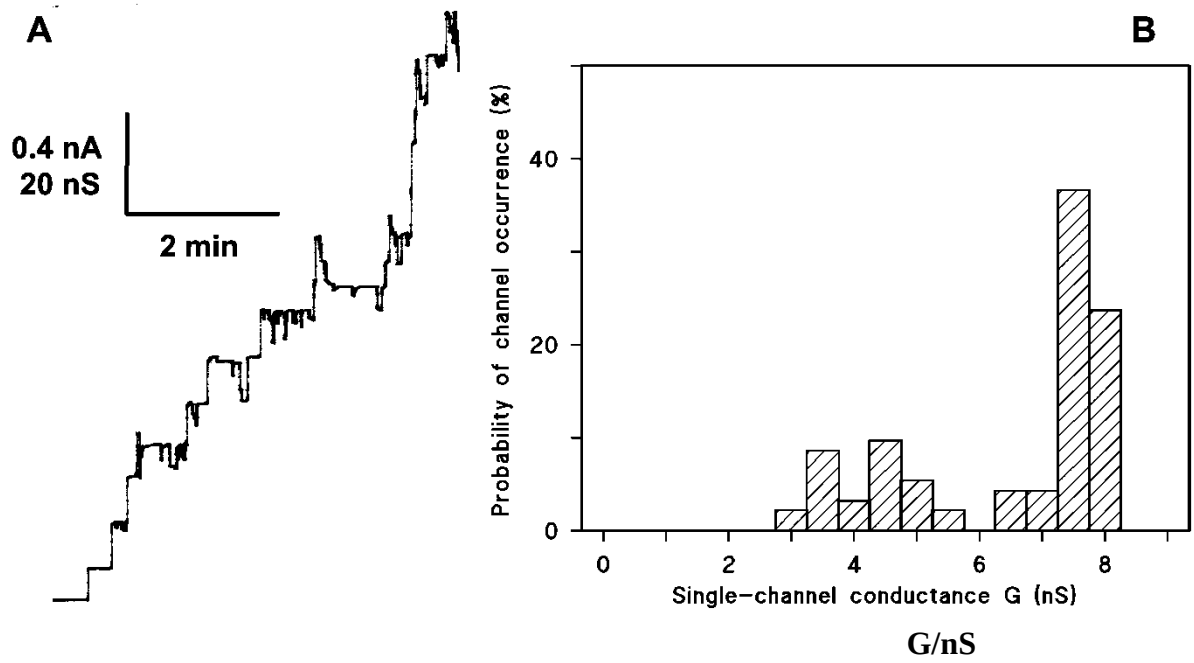


Figure 41. Single-channel recordings for Lpg1974Δ1-21. A: Single channel recording of a PC/*n*-decane membrane after addition of 100 ng/ml of the purified truncated Lpg1974 to the bath solution of 1 M KCl pH 7. B: A histogram showing the probability of occurrence of channels of a specific conductance. The most frequent conductance value is 7.5 nS.

3.3.6.2. Lpg1974Δ1-21 is voltage gated

Lpg1974Δ1-21 was studied for its voltage gating behavior in multi-channel experiments. Purified Lpg1974Δ1-21 refolded in 0.5 %LDAO in 10 mM Tris-HCl pH8 was added to either one or both sides of the black lipid bilayer membrane. Once, a number of channels were inserted in the membrane, increasing positive and negative membrane potentials showed that more and more channels were getting closed with increasing membrane potentials. Chart

recording of Lpg1974Δ1-21 showing its behavior to increasing positive and negative voltages is shown below (Fig 41)

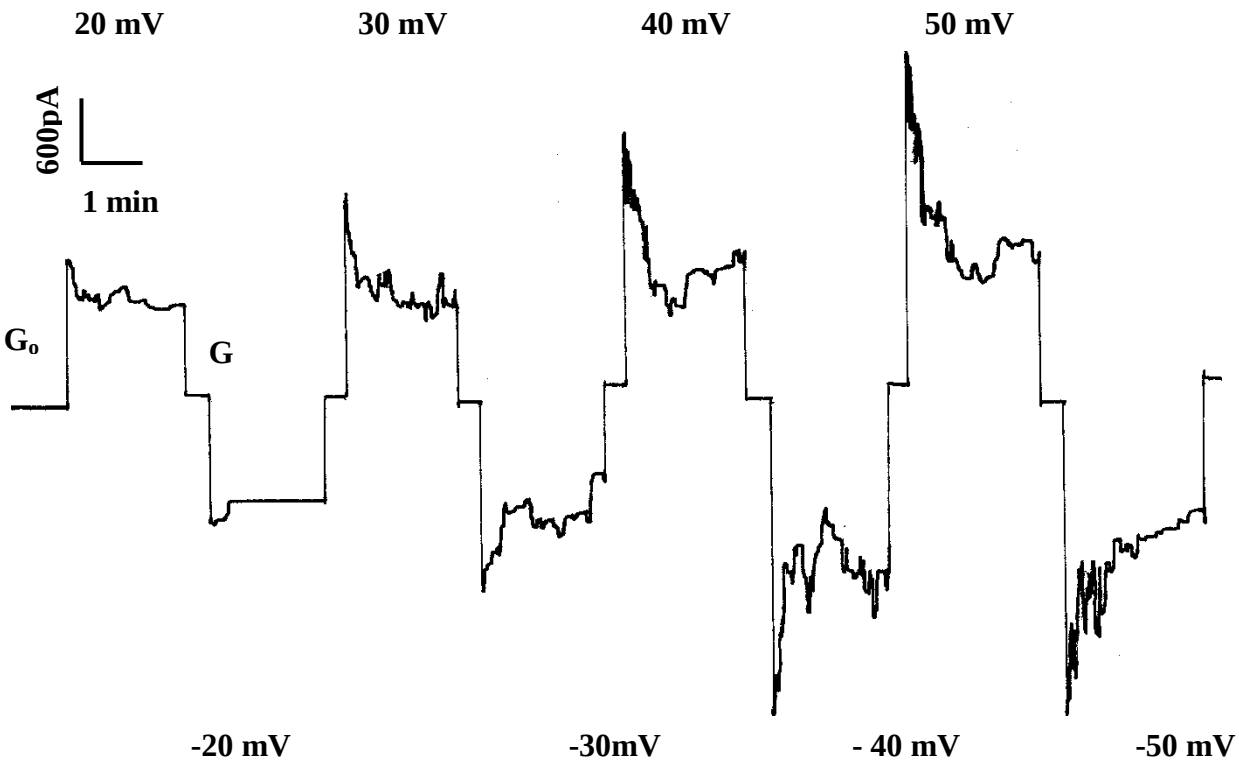


Figure 42. Chart recording of voltage gating for Lpg1974Δ1-21. Increasing positive and negative voltages were applied across a lipid membrane in which several channels were inserted and the recordings were followed for up to 2-3 mins for each voltage increment.

Frequency of channels remaining open using the above data was calculated and plotted as shown below [109]. The plot shows almost symmetrical gating on the application of positive and negative membrane potentials. For positive voltages at 40 mV almost 50 % channels are closed, while on negative voltages it takes 50 mV for 50 % of the channels to be closed.

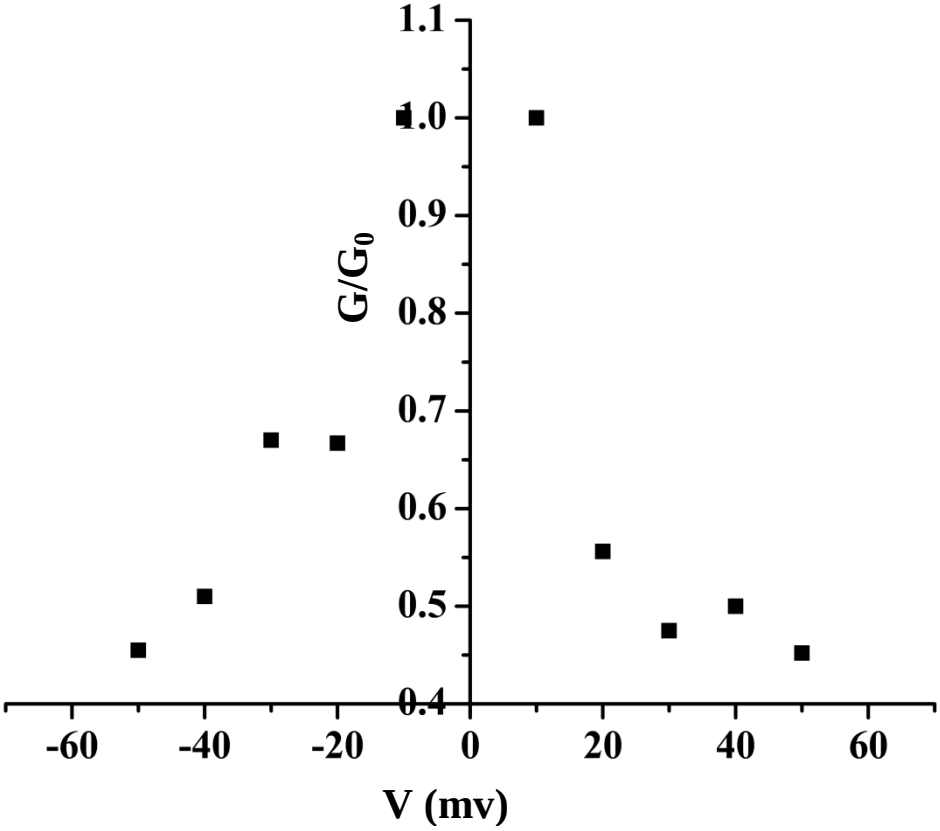


Figure 43. Graphical representation of voltage dependent closure of Lpg1974Δ1-21. Conductance (G) at a given membrane potential (V_m) divided by the conductance at 20 mV (G_0) as a function of the membrane potential. The aqueous phase contained 1 M KCl, and 100 ng/ml of truncated Lpg1974 refolded in 0.4% LDAO (*cis* side). The membranes were formed from PC/*n*-decane at a temperature of 21°C.

3.3.5.3. Ion selectivity measurements for Lpg1974Δ1-21

Preference of Lpg1974Δ1-21 for cations or anions was measured by zero current membrane potential. The experiment was performed by developing a salt gradient across the bilayer in which several channels were inserted. This gradient allowed ions to move through the porins from the concentrated towards the diluted side [110]. The negative value of zero current membrane potential (V_m) shows the preference of Lpg1974Δ1-21 channels for anions over cations. Permeability ratio (P_{cation}/P_{anion}) was calculated as mentioned before [111].

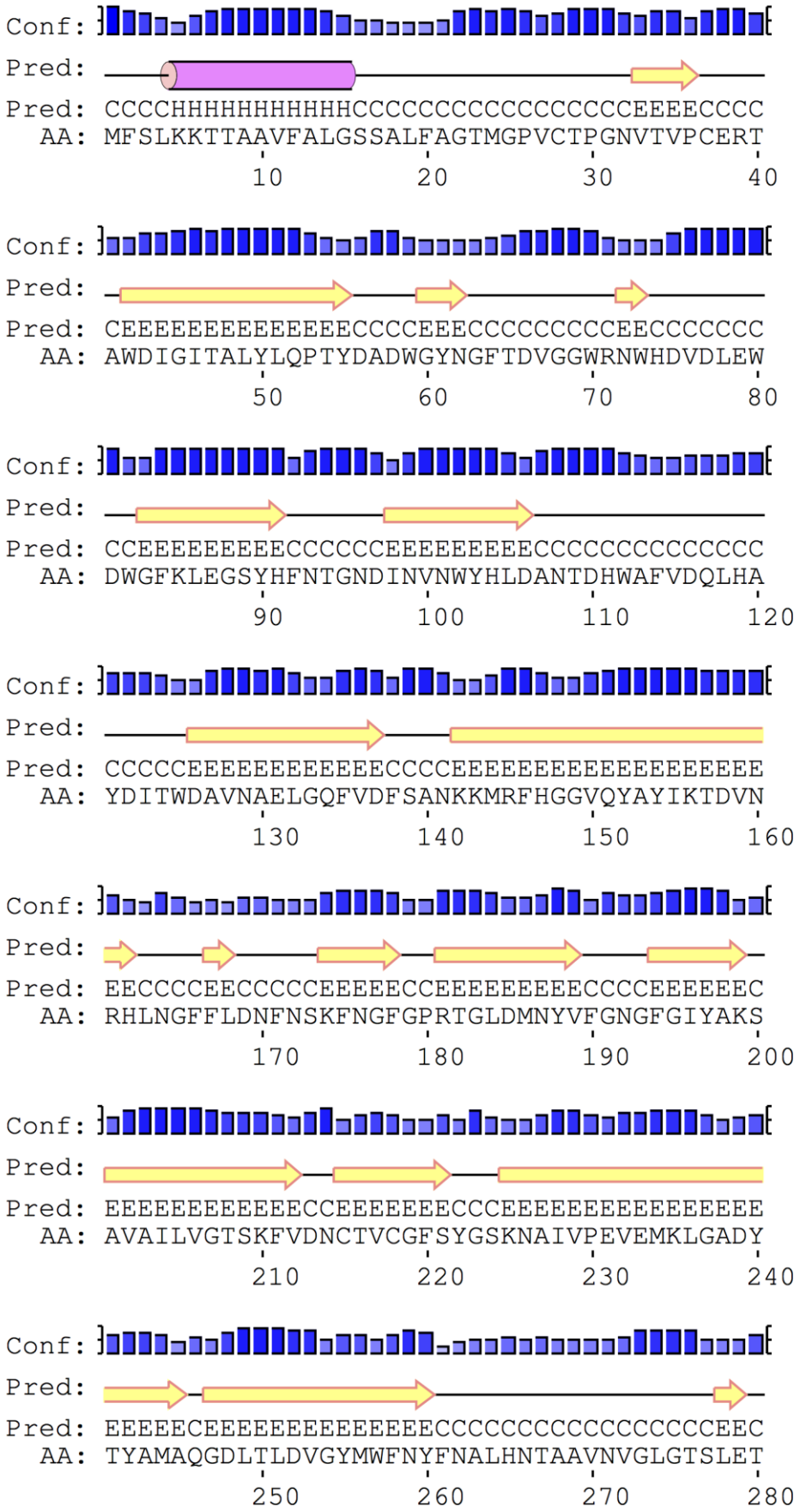
Electrolyte	**Permeability ratios** P_{cation}/P_{anion}	V_m **(mV)**
KCl	0.61	-(2.66±0.5)

Table 1. Ion selectivity ratios for Lpg1974Δ1-21. Zero current membrane potential (V_m) are measured for membranes formed by PC/*n*-decane with 1.5 fold salt gradient and several channels of Lpg1974 inserted. The aqueous solution had a pH 7 and temperature of 20°C. The data is an average of at least three independent measurements.

3.4. Discussion

The discovery of *L. pneumophila* marked new prospects for scientific research. This Gram-negative bacterium is able to infect both water-borne amoebae and humans and interestingly using the same invasion mechanism. It was thought that the organism would most probably need eukaryotic like proteins in order to survive inside human host. A series of such proteins have been hypothesized in the past years. One of such proteins was Lpg1974 which had a reasonable resemblance to hVDAC-1 of human mitochondria. In humans hVDAC-1 is a member of a complex called MPTP, involved in transport across the mitochondrial membrane. We studied the counter part of the hVDAC-1 in *L. pneumophila*. Protein sequence alignments of the two proteins were not very prominent but still obvious. However the secondary structure predictions showed that Lpg1974 has the same number of beta strands as in hVDAC-1 (Fig 43-44). Homology structure developed for the protein also showed a perfect match with hVDAC-1. The gene *lpg1974*, coding for Lpg1974 was cloned and expressed in two different conformations. In one case the protein was expressed as an N-terminally GST tagged in the inclusion bodies and purified.

In the second strategy in order to study the importance of the N-terminal predicted signal peptide, the gene was cloned without its native signal peptide and any tag. The Lpg1974Δ1-21 was also expressed in the inclusion bodies and then refolded. The N-terminal sequence in VDAC-1 which forms an α-helix has long been investigated for its role, and there have been diverse reports about its significance. There are several studies showing the importance of the N-terminal helix in voltage gating [112], [113], [114], [115]. In our case the Lpg1974Δ1-21 still showed voltage dependent behavior which agrees with a report in which affixing the N-terminal sequence to the wall of the channel did not prevent the voltage gating [116]. Another report differing with the theory of independent dynamic rearrangements of the N-terminus was from Wagner´s group [117]. They used nuclear magnetic resonance to study hVDAC-1 in both open and closed states. Although spectra varied a lot during the open and closed states, but the conformation of the N-terminus always remained stable in both the states which suggested a rather stable arrangement of the N-terminus in contrast to several other reports. Same conclusion was drawn by [118] using NMR to study the wild type protein and an N-terminus deletion mutant. Their results showed that N-terminus deletion had no effect on the voltage gating but they suggested its role in maintaining the β barrel conformation of the channel. The mutant protein resulted in noisy and unstable channels. Results from Popp et al. also agree with the above mentioned conclusions [119].


Conf:
Pred:
Pred: CCCCHHHHHHHHHHHCCCCCCCCCCCCCCCCCEEEECCCC
AA: MFSLKKTTAAVFALGSSALFAGTMGPVCTPGNVTVPCERT
10 20 30 40
Conf:
Pred:
Pred: CEEEEEEEEEEEEECCCCEEECCCCCCCCCCEECCCCCCC
AA: AWDIGITALYLQPTYDADWGYNGFTDVGGWRNWHDVDLEW
50 60 70 80
Conf:
Pred:
Pred: CCEEEEEEEEECCCCCCEEEEEEEECCCCCCCCCCCCCC
AA: DWGFKLEGSYHFNTGNDINVNWYHLDANTDHWAFVDQLHA
90 100 110 120
Conf:
Pred:
Pred: CCCCCEEEEEEEEEEEECCCCEEEEEEEEEEEEEEEEE
AA: YDITWDAVNAELGQFVDFSANKKMRFHGGVQYAYIKTDVN
130 140 150 160
Conf:
Pred:
Pred: EECCCCEECCCCCEEEEECCEEEEEEEECCCCEEEEEEC
AA: RHLNGFFLDNFNSKFNGFGPRTGLDMNYVFGNGFGIYAKS
170 180 190 200
Conf:
Pred:
Pred: EEEEEEEEEEEECCEEEEEECCCEEEEEEEEEEEEEEE
AA: AVAILVGTSKFVDNCTVCGFSYGSKNAIVPEVEMKLGADY
210 220 230 240
Conf:
Pred:
Pred: EEEEECEEEEEEEEEEEEECCCCCCCCCCCCCCCCCEEC
AA: TYAMAQGDLTLDVGYMWFNYFNALHNTAAVNVGLGTSLET
250 260 270 280

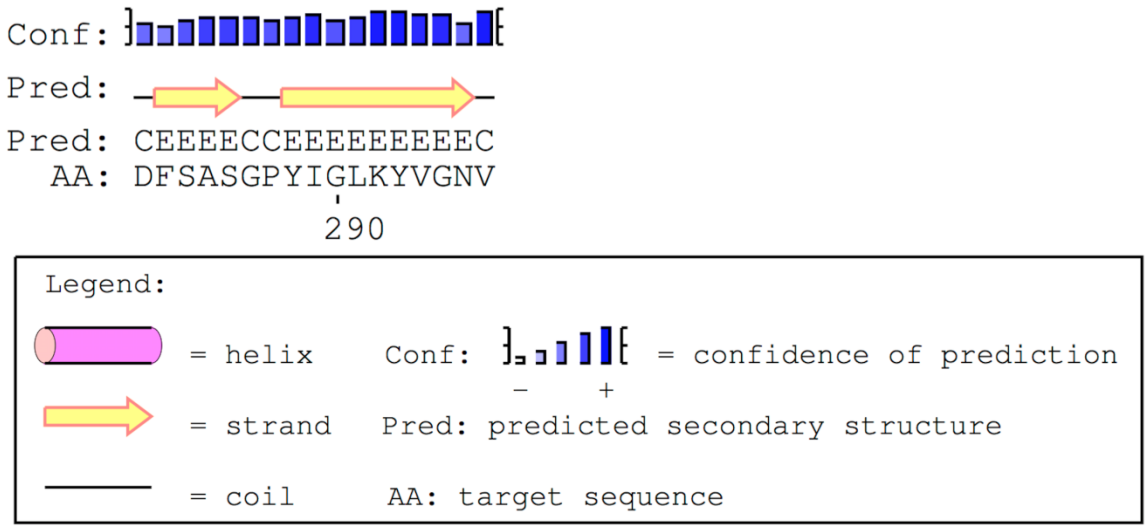


Figure 44. Secondary structure prediction for Lpg1974. Beta sheets and alpha helices in the amino acids sequence of the protein were predicted using http://bioinf.cs.ucl.ac.uk/psipred. The sequence has 19 beta strands predicted as shown by the yellow arrows, which is the same number of beta sheets as in hVDAC-1.

In our studies here, we report the role of the predicted signal peptide in Lpg1974. The mutant protein Lpg1974Δ1-21 was still voltage gated which shows that the voltage dependent behavior of the protein is independent of the N-terminal signal peptide. The signal peptide might have a role in stabilization of the protein channels. Lpg1974Δ1-21 formed large channels which had a preference for anions over cations. Detailed structural studies can help us better understand the properties of the protein and also the role of the N-terminal signal peptide

Outlines and Conclusions

My OmpW project contributes to the increasing knowledge about OmpW proteins in Gram-negative bacteria. Against the common belief that *C. crescentus* lacks any porins in its outer membrane. Here, I report the presence of a porin in this organism. Porin like channel forming activity was observed in the enriched outer membrane extracts of *C. crescentus*. The protein responsible for the channels forming activity was purified and was found to be OmpW homologue. The results were confirmed by developing *ompW* knockout mutant. Enriched outer membrane extracts from the mutant strain lost the channel forming activity in artificial lipid bilayers. The OmpW *C. c* is found to be highly cation selective so it can be the channel for cations to be transported in this organism. Although, a range of other OmpW homologues have also been identified in several other bacteria, but OmpW *C. c* is most probably having a different function compared to the other members of this family. The protein according to our homology structure has more hydrophilic channel axis and is highly selective for the transport of divalent cations. This is a function unique to the OmpW *C. c* and it has not been reported in any other member of the OmpW family. Over expression of the *ompW* gene in *E. coli* did not result in an active protein. The protein expressed in *E. coli* might have a different folding than the protein in its native state. This can be confirmed by developing crystal structure for the protein.

Legionella pneumophila is the only bacteria having all the hypothetical proteins resembling the proteins constituting MPTP complex in human mitochondria. One of the components of this complex is hVDAC-1which is the most abundant protein in the outer membrane of mitochondria. The components constituting this complex in mitochondria are highly debated. Here, I report that at least one protein Lpg1974 is sharing reasonable homology to hVDAC-1and I confirm experimentally using biophysical approaches that it is having behavior similar to hVDAC-1. It is voltage gated and is slightly anion selective. There are other proteins in *L. pneumophila* sharing resemblances to mitochondrial proteins constituting the MPTP complex. They can be studies as well and knockout mutants can be developed to confirm the presence of MPTP like complex in *L. pneumophila*.

List of Publications

1: OmpW of *Caulobacter crescentus* functions as an outer membrane channel for cations

Benz R, Jones MD, **Younas F**, Maier E, Modi N, Mentele R, Lottspeich F, Kleinekathöfer U, Smit J. OmpW of *Caulobacter crescentus* Functions as an Outer Membrane Channel for Cations. PLoS One. 2015 Nov 25;10(11):e0143557. doi: 10.1371/journal.pone.0143557.

2: Identification, molecular cloning and biophysical characterization of Lpg1974, a homologue of hVDAC-1 in *Legionella pneumophila*.

Younas F., Soltanmohammadi N., Modi N., Kleinekathöfer U., Knapp O., Benz R.

(Manuscript in preparation)

3: A single residue acts as gate in OpdH and related channels

Karunakar R Pothula, Naresh N Dhanasekar, Usha Lamichhane, **Farhan Younas**, Daniel Pletzer, Roland Benz, Mathias Winterhalter, Ulrich Kleinekathöfer.

(Manuscript submitted)

4: PorACur of pathogenic *Corynebacterium urealytiucm* DSM 7109 is the smallest cation selective porin reported.

Narges Abdali, **Farhan Younas**, Samaneh Mafakheri, Iván Bárcena-Uribarri, Karunakar Reddy Pothula, Urlich Kleinekathöfer, Andreas Tauch, Roland Benz

(Manuscript in preparation)

References

1. Benz, R., *Porin from bacterial and mitochondrial outer membranes.* CRC Crit Rev Biochem, 1985. **19**(2): p. 145-90.

2. Nikaido, H. and T. Nakae, *The outer membrane of Gram-negative bacteria.* Adv Microb Physiol, 1979. **20**: p. 163-250.

3. Lugtenberg, B. and L. Van Alphen, *Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria.* Biochim Biophys Acta, 1983. **737**(1): p. 51-115.

4. Rosenbusch, J.P., *Characterization of the major envelope protein from Escherichia coli. Regular arrangement on the peptidoglycan and unusual dodecyl sulfate binding.* J Biol Chem, 1974. **249**(24): p. 8019-29.

5. Nakae, T., *Outer membrane of Salmonella. Isolation of protein complex that produces transmembrane channels.* J Biol Chem, 1976. **251**(7): p. 2176-8.

6. Lepault, J., et al., *Three-dimensional reconstruction of maltoporin from electron microscopy and image processing.* Embo j, 1988. **7**(1): p. 261-8.

7. Weiss, M.S., et al., *The three-dimensional structure of porin from Rhodobacter capsulatus at 3 Å resolution.* FEBS Letters, 1990. **267**(2): p. 268-272.

8. Weiss, M., et al., *Molecular architecture and electrostatic properties of a bacterial porin.* Science, 1991. **254**(5038): p. 1627-1630.

9. Cowan, S.W., et al., *Crystal structures explain functional properties of two E. coli porins.* Nature, 1992. **358**(6389): p. 727-733.

10. Kreusch, A., et al., *Structure of the membrane channel porin from Rhodopseudomonas blastica at 2.0 Å resolution.* Protein Science, 1994. **3**(1): p. 58-63.

11. Hirsch, A., et al., *The structure of porin from Paracoccus denitrificans at 3.1 Å resolution.* FEBS Letters, 1997. **404**(2-3): p. 208-210.

12. Zeth, K., et al., *Crystal structure of Omp32, the anion-selective porin from Comamonas acidovorans, in complex with a periplasmic peptide at 2.1 Å resolution.* Structure, 2000. **8**(9): p. 981-992.

13. Schirmer, T., et al., *Structural basis for sugar translocation through maltoporin channels at 3.1 A resolution.* Science, 1995. **267**(5197): p. 512-514.

14. Forst, D., et al., *Structure of the sucrose-specific porin ScrY from Salmonella typhimurium and its complex with sucrose.* Nat Struct Mol Biol, 1998. **5**(1): p. 37-46.

15. Zachariae, U., et al., *High Resolution Crystal Structures and Molecular Dynamics Studies Reveal Substrate Binding in the Porin Omp32.* Journal of Biological Chemistry, 2006. **281**(11): p. 7413-7420.

16. Moraes, T.F., et al., *An arginine ladder in OprP mediates phosphate-specific transfer across the outer membrane.* Nat Struct Mol Biol, 2007. **14**(1): p. 85-87.

17. Korteland, J., J. Tommassen, and B. Lugtenberg, *PhoE protein pore of the outer membrane of Escherichia coli K12 is a particularly efficient channel for organic and inorganic phosphate.* Biochim Biophys Acta, 1982. **690**(2): p. 282-9.

18. Benz, R., et al., *OmpW of Caulobacter crescentusFunctions as an Outer Membrane Channel for Cations.* PLoS ONE, 2015. **10**(11): p. e0143557.

19. Pajatsch, M., et al., *Properties of a Cyclodextrin-specific, Unusual Porin from Klebsiella oxytoca.* Journal of Biological Chemistry, 1999. **274**(35): p. 25159-25166.

20. Yildiz, Ö., et al., *Structure of the monomeric outer-membrane porin OmpG in the open and closed conformation.* The EMBO Journal, 2006. **25**(15): p. 3702-3713.

21. Faraldo, M.L., M.A. de Pedro, and J. Berenguer, *Cloning and expression in Escherichia coli of the structural gene coding for the monomeric protein of the S layer of Thermus thermophilus HB8.* Journal of Bacteriology, 1991. **173**(17): p. 5346-5351.

22. Maier, E., et al., *Identification of the outer membrane porin of Thermus thermophilus HB8: the channel-forming complex has an unusually high molecular mass and an extremely large single-channel conductance.* J Bacteriol, 2001. **183**(2): p. 800-3.

23. Caston, J.R., et al., *S-layer protein from Thermus thermophilus HB8 assembles into porin-like structures.* Mol Microbiol, 1993. **9**(1): p. 65-75.

24. Booth, P.J. and A.R. Curran, *Membrane protein folding.* Curr Opin Struct Biol, 1999. **9**(1): p. 115-21.

25. Reusch, R.N., *Insights into the structure and assembly of Escherichia coli outer membrane protein A.* Febs j, 2012. **279**(6): p. 894-909.

26. Buchanan, S.K., *β-Barrel proteins from bacterial outer membranes: structure, function and refolding.* Current Opinion in Structural Biology, 1999. **9**(4): p. 455-461.

27. Kleinschmidt, J.H. and L.K. Tamm, *Folding intermediates of a beta-barrel membrane protein. Kinetic evidence for a multi-step membrane insertion mechanism.* Biochemistry, 1996. **35**(40): p. 12993-3000.

28. Surrey, T. and F. Jahnig, *Kinetics of folding and membrane insertion of a beta-barrel membrane protein.* J Biol Chem, 1995. **270**(47): p. 28199-203.

29. Tamm, L.K., A. Arora, and J.H. Kleinschmidt, *Structure and Assembly of β-Barrel Membrane Proteins.* Journal of Biological Chemistry, 2001. **276**(35): p. 32399-32402.

30. Schleiff, E. and J. Soll, *Membrane protein insertion: mixing eukaryotic and prokaryotic concepts.* EMBO Reports, 2005. **6**(11): p. 1023-1027.

31. Rigel, N.W., et al., *BamE modulates the Escherichia coli beta-barrel assembly machine component BamA.* J Bacteriol, 2012. **194**(5): p. 1002-8.

32. Rigel, N.W. and T.J. Silhavy, *Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria.* Curr Opin Microbiol, 2012. **15**(2): p. 189-93.

33. Ricci, D.P., et al., *Activation of the Escherichia coli beta-barrel assembly machine (Bam) is required for essential components to interact properly with substrate.* Proc Natl Acad Sci U S A, 2012. **109**(9): p. 3487-91.

34. de Cock, H., et al., *Role of the carboxy-terminal phenylalanine in the biogenesis of outer membrane protein PhoE of Escherichia coli K-12.* J Mol Biol, 1997. **269**(4): p. 473-8.

35. Di Donato, A., et al., *Do porins inhibit the macrophage phagocyting activity by stimulating the adenylate cyclase?* J Cyclic Nucleotide Protein Phosphor Res, 1986. **11**(2): p. 87-97.

36. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.

37. Galdiero, F., et al., *Activation of complement system by porins extracted from Salmonella typhimurium.* Infect Immun, 1984. **46**(2): p. 559-63.

38. West, A.P., A.A. Koblansky, and S. Ghosh, *Recognition and signaling by toll-like receptors.* Annu Rev Cell Dev Biol, 2006. **22**: p. 409-37.

39. Galdiero, M., M. Vitiello, and S. Galdiero, *Eukaryotic cell signaling and transcriptional activation induced by bacterial porins.* FEMS Microbiol Lett, 2003. **226**(1): p. 57-64.

40. Gupta, S., et al., *Involvement of signal transduction pathways in Salmonella typhimurium porin activated gut macrophages.* Mol Cell Biochem, 1999. **194**(1-2): p. 235-43.

41. Galdiero, M., et al., *Haemophilus influenzae porin contributes to signaling of the inflammatory cascade in rat brain.* Infect Immun, 2001. **69**(1): p. 221-7.

42. Galdiero, M., et al., *Porins from Salmonella enterica serovar Typhimurium activate the transcription factors activating protein 1 and NF-kappaB through the Raf-1-mitogen-activated protein kinase cascade.* Infect Immun, 2002. **70**(2): p. 558-68.

43. Fernandez, L. and R.E. Hancock, *Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance.* Clin Microbiol Rev, 2012. **25**(4): p. 661-81.

44. Rice, L.B., *Emerging issues in the management of infections caused by multidrug-resistant gram-negative bacteria.* Cleve Clin J Med, 2007. **74 Suppl 4**: p. S12-20.

45. Blot, S., et al., *Measuring the impact of multidrug resistance in nosocomial infection.* Curr Opin Infect Dis, 2007. **20**(4): p. 391-6.

46. Low, A.S., et al., *Protected environments allow parallel evolution of a bacterial pathogen in a patient subjected to long-term antibiotic therapy.* Mol Microbiol, 2001. **42**(3): p. 619-30.

47. Achouak, W., T. Heulin, and J.M. Pages, *Multiple facets of bacterial porins.* FEMS Microbiol Lett, 2001. **199**(1): p. 1-7.

48. Hancock, R.E. and A. Bell, *Antibiotic uptake into gram-negative bacteria.* Eur J Clin Microbiol Infect Dis, 1988. **7**(6): p. 713-20.

49. Poole, K., *Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria.* Curr Pharm Biotechnol, 2002. **3**(2): p. 77-98.

50. Poole, K., *Resistance to beta-lactam antibiotics.* Cell Mol Life Sci, 2004. **61**(17): p. 2200-23.

51. George, A.M. and S.B. Levy, *Gene in the major cotransduction gap of the Escherichia coli K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics.* J Bacteriol, 1983. **155**(2): p. 541-8.

52. Viveiros, M., et al., *Antibiotic stress, genetic response and altered permeability of E. coli.* PLoS One, 2007. **2**(4): p. e365.

53. Domenech-Sanchez, A., et al., *Identification and characterization of a new porin gene of Klebsiella pneumoniae: its role in beta-lactam antibiotic resistance.* J Bacteriol, 1999. **181**(9): p. 2726-32.

54. De, E., et al., *A new mechanism of antibiotic resistance in Enterobacteriaceae induced by a structural modification of the major porin.* Mol Microbiol, 2001. **41**(1): p. 189-98.

55. Simonet, V., M. Mallea, and J.M. Pages, *Substitutions in the eyelet region disrupt cefepime diffusion through the Escherichia coli OmpF channel.* Antimicrob Agents Chemother, 2000. **44**(2): p. 311-5.

56. Huang, H. and R.E. Hancock, *The role of specific surface loop regions in determining the function of the imipenem-specific pore protein OprD of Pseudomonas aeruginosa.* J Bacteriol, 1996. **178**(11): p. 3085-90.

57. Mueller, P., et al., *Reconstitution of cell membrane structure in vitro and its transformation into an excitable system.* Nature, 1962. **194**: p. 979-80.

58. White, S.H., *The Physical Nature of Planar Bilayer Membranes*, in *Ion Channel Reconstitution*, C. Miller, Editor. 1986, Springer US: Boston, MA. p. 3-35.

59. MacRae, J.D. and J. Smit, *Characterization of caulobacters isolated from wastewater treatment systems.* Applied and Environmental Microbiology, 1991. **57**(3): p. 751-758.

60. Inagaki, F., et al., *Distribution and phylogenetic diversity of the subsurface microbial community in a Japanese epithermal gold mine.* Extremophiles, 2003. **7**(4): p. 307-317.

61. Mannisto, M.K., et al., *Diversity of chlorophenol-degrading bacteria isolated from contaminated boreal groundwater.* Arch Microbiol, 1999. **171**(3): p. 189-97.

62. Merker, R.I. and J. Smit, *Characterization of the Adhesive Holdfast of Marine and Freshwater Caulobacters.* Applied and Environmental Microbiology, 1988. **54**(8): p. 2078-2085.

63. Cole, J.L., et al., *The HfaB and HfaD adhesion proteins of Caulobacter crescentus are localized in the stalk.* Mol Microbiol, 2003. **49**(6): p. 1671-83.

64. Curtis, P.D. and Y.V. Brun, *Getting in the loop: regulation of development in Caulobacter crescentus.* Microbiol Mol Biol Rev, 2010. **74**(1): p. 13-41.

65. McAdams, H.H. and L. Shapiro, *A bacterial cell-cycle regulatory network operating in time and space.* Science, 2003. **301**(5641): p. 1874-7.

66. Fukuda, A., H. Iba, and Y. Okada, *Stalkless mutants of Caulobacter crescentus.* J Bacteriol, 1977. **131**(1): p. 280-7.

67. Sciochetti, S.A., et al., *Protein sequences and cellular factors required for polar localization of a histidine kinase in Caulobacter crescentus.* J Bacteriol, 2002. **184**(21): p. 6037-49.

68. Jenal, U., *Signal transduction mechanisms in Caulobacter crescentus development and cell cycle control.* FEMS Microbiology Reviews, 2000. **24**(2): p. 177-191.

69. Hughes, V., C. Jiang, and Y. Brun, *Caulobacter crescentus.* Current Biology, 2012. **22**(13): p. R507-R509.

70. Nierman, W.C., et al., *Complete genome sequence of Caulobacter crescentus.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 4136-41.

71. Neugebauer, H., et al., *ExbBD-dependent transport of maltodextrins through the novel MalA protein across the outer membrane of Caulobacter crescentus.* J Bacteriol, 2005. **187**(24): p. 8300-11.

72. Hong, H., et al., *The outer membrane protein OmpW forms an eight-stranded beta-barrel with a hydrophobic channel.* J Biol Chem, 2006. **281**(11): p. 7568-77.

73. Touw, D.S., D.R. Patel, and B. van den Berg, *The crystal structure of OprG from Pseudomonas aeruginosa, a potential channel for transport of hydrophobic molecules across the outer membrane.* PLoS One, 2010. **5**(11): p. e15016.

74. Jalajakumari, M.B. and P.A. Manning, *Nucleotide sequence of the gene, ompW, encoding a 22kDa immunogenic outer membrane protein of Vibrio cholerae.* Nucleic Acids Research, 1990. **18**(8): p. 2180.

75. Smajs, D., et al., *Bacteriocin synthesis in uropathogenic and commensal Escherichia coli: colicin E1 is a potential virulence factor.* BMC Microbiol, 2010. **10**: p. 288.

76. Wu, X.B., et al., *Outer membrane protein OmpW of Escherichia coli is required for resistance to phagocytosis.* Res Microbiol, 2013. **164**(8): p. 848-55.

77. Steinle, A., et al., *Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family.* Immunogenetics, 2001. **53**(4): p. 279-87.

78. O'Callaghan, C.A., et al., *Production, crystallization, and preliminary X-ray analysis of the human MHC class Ib molecule HLA-E.* Protein Sci, 1998. **7**(5): p. 1264-6.

79. Laemmli, U.K., *Cleavage of structural proteins during the assembly of the head of bacteriophage T4.* Nature, 1970. **227**(5259): p. 680-5.

80. Ford, M.J., J.F. Nomellini, and J. Smit, *S-Layer Anchoring and Localization of an S-Layer-Associated Protease in Caulobacter crescentus.* Journal of Bacteriology, 2007. **189**(6): p. 2226-2237.

81. Poindexter, J.S., *Biological properties and classification of the Caulobacter group.* Bacteriol Rev, 1964. **28**: p. 231-95.

82. Smit, J., M. Hermodson, and N. Agabian, *Caulobacter crescentus pilin. Purification, chemical characterization, and NH2-terminal amino acid sequence of a structural protein regulated during development.* J Biol Chem, 1981. **256**(6): p. 3092-7.

83. Altschul, S.F., et al., *Basic local alignment search tool.* J Mol Biol, 1990. **215**(3): p. 403-10.

84. Zhang, J. and T.L. Madden, *PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation.* Genome Res, 1997. **7**(6): p. 649-56.

85. Eswar, N., et al., *Comparative protein structure modeling using Modeller.* Curr Protoc Bioinformatics, 2006. **Chapter 5**: p. Unit 5.6.

86. Smart, O.S., et al., *HOLE: a program for the analysis of the pore dimensions of ion channel structural models.* J Mol Graph, 1996. **14**(6): p. 354-60, 376.

87. Fliermans, C.B., et al., *Ecological distribution of Legionella pneumophila.* Applied and Environmental Microbiology, 1981. **41**(1): p. 9-16.

88. Fields, B.S., R.F. Benson, and R.E. Besser, *Legionella and Legionnaires' disease: 25 years of investigation.* Clin Microbiol Rev, 2002. **15**(3): p. 506-26.

89. Abu Kwaik, Y., et al., *Invasion of protozoa by Legionella pneumophila and its role in bacterial ecology and pathogenesis.* Appl Environ Microbiol, 1998. **64**(9): p. 3127-33.

90. Fraser, D.W., et al., *Legionnaires' disease: description of an epidemic of pneumonia.* N Engl J Med, 1977. **297**(22): p. 1189-97.

91. Doleans, A., et al., *Clinical and environmental distributions of Legionella strains in France are different.* J Clin Microbiol, 2004. **42**(1): p. 458-60.

92. Kroemer, G., et al., *The biochemistry of programmed cell death.* Faseb j, 1995. **9**(13): p. 1277-87.

93. Scorrano, L., et al., *A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis.* Dev Cell, 2002. **2**(1): p. 55-67.

94. Miyake, M., et al., *Characterization of Legionella pneumophila pmiA, a Gene Essential for Infectivity of Protozoa and Macrophages.* Infection and Immunity, 2005. **73**(10): p. 6272-6282.

95. Gao, L.-Y. and Y. Abu Kwaik, *Activation of Caspase 3 during Legionella pneumophila-Induced Apoptosis.* Infection and Immunity, 1999. **67**(9): p. 4886-4894.

96. Walz, J.M., et al., *Legionella species of different human prevalence induce different rates of apoptosis in human monocytic cells.* APMIS, 2000. **108**(6): p. 398-408.

97. Abu-Zant, A., et al., *Incomplete activation of macrophage apoptosis during intracellular replication of Legionella pneumophila.* Infect Immun, 2005. **73**(9): p. 5339-49.

98. Horwitz, M.A., *The Legionnaires' disease bacterium (Legionella pneumophila) inhibits phagosome-lysosome fusion in human monocytes.* J Exp Med, 1983. **158**(6): p. 2108-26.

99. Molofsky, A.B. and M.S. Swanson, *Differentiate to thrive: lessons from the Legionella pneumophila life cycle.* Mol Microbiol, 2004. **53**(1): p. 29-40.

100. Muller, A., J. Hacker, and B.C. Brand, *Evidence for apoptosis of human macrophage-like HL-60 cells by Legionella pneumophila infection.* Infect Immun, 1996. **64**(12): p. 4900-6.

101. Lurie-Weinberger, M.N., et al., *The origins of eukaryotic-like proteins in Legionella pneumophila.* Int J Med Microbiol, 2010. **300**(7): p. 470-81.

102. de Felipe, K.S., et al., *Evidence for acquisition of Legionella type IV secretion substrates via interdomain horizontal gene transfer.* J Bacteriol, 2005. **187**(22): p. 7716-26.

103. Cazalet, C., et al., *Evidence in the Legionella pneumophila genome for exploitation of host cell functions and high genome plasticity.* Nat Genet, 2004. **36**(11): p. 1165-73.

104. Massari, P., Y. Ho, and L.M. Wetzler, *Neisseria meningitidis porin PorB interacts with mitochondria and protects cells from apoptosis.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9070-5.

105. Müller, A., et al., *Targeting of the pro-apoptotic hVDAC-1 like porin (PorB) of Neisseria gonorrhoeae to mitochondria of infected cells.* The EMBO Journal, 2000. **19**(20): p. 5332-5343.

106. Khemiri, A., T. Jouenne, and P. Cosette, *Presence in Legionella pneumophila of a mammalian-like mitochondrial permeability transition pore?* FEMS Microbiology Letters, 2008. **278**(2): p. 171-176.

107. Schulein, K., C. Andersen, and R. Benz, *The deletion of 70 amino acids near the N-terminal end of the sucrose-specific porin ScrY causes its functional similarity to LamB in vivo and in vitro.* Mol Microbiol, 1995. **17**(4): p. 757-67.

108. Petersen, T.N., et al., *SignalP 4.0: discriminating signal peptides from transmembrane regions.* Nat Methods, 2011. **8**(10): p. 785-6.

109. Ludwig, O., et al., *Pore formation by the mitochondrial porin of rat brain in lipid bilayer membranes.* Biochim Biophys Acta, 1986. **860**(2): p. 268-76.

110. Benz, R., A. Schmid, and R.E. Hancock, *Ion selectivity of gram-negative bacterial porins.* J Bacteriol, 1985. **162**(2): p. 722-7.

111. Benz, R., K. Janko, and P. Lauger, *Ionic selectivity of pores formed by the matrix protein (porin) of Escherichia coli.* Biochim Biophys Acta, 1979. **551**(2): p. 238-47.

112. Shoshan-Barmatz, V., et al., *Apoptosis is regulated by the hVDAC-1 N-terminal region and by hVDAC-1 oligomerization: release of cytochrome c, AIF and Smac/Diablo.* Biochim Biophys Acta, 2010. **1797**(6-7): p. 1281-91.

113. De Pinto, V., et al., *New functions of an old protein: the eukaryotic porin or voltage dependent anion selective channel (hVDAC-1).* Ital J Biochem, 2003. **52**(1): p. 17-24.

114. Guo, X.W., et al., *Molecular design of the voltage-dependent, anion-selective channel in the mitochondrial outer membrane.* J Struct Biol, 1995. **114**(1): p. 41-59.

115. Ujwal, R., et al., *The crystal structure of mouse hVDAC-1 at 2.3 A resolution reveals mechanistic insights into metabolite gating.* Proc Natl Acad Sci U S A, 2008. **105**(46): p. 17742-7.

116. Teijido, O., et al., *Affixing N-terminal alpha-helix to the wall of the voltage-dependent anion channel does not prevent its voltage gating.* J Biol Chem, 2012. **287**(14): p. 11437-45.

117. Hiller, S. and G. Wagner, *The role of solution NMR in the structure determinations of hVDAC-1 and other membrane proteins.* Curr Opin Struct Biol, 2009. **19**(4): p. 396-401.

118. Schneider, R., et al., *The native conformation of the human hVDAC-1 N terminus.* Angew Chem Int Ed Engl, 2010. **49**(10): p. 1882-5.

119. Popp, B., et al., *The role of the N and C termini of recombinant Neurospora mitochondrial porin in channel formation and voltage-dependent gating.* J Biol Chem, 1996. **271**(23): p. 13593-9.

List of Figures:

Figure 1. A Schematic overview of all the potential roles of porins in acquisition of multidrug resistance in bacteria [43]. OM: outer membrane, PP: periplasmic space, IM: inner membrane. Blue circles indicate antibiotic molecules, blue arrow shows transport through the porin and the red cross shows inhibition of the drug transport through the porin..... 4
Figure 2. Life cycle of *Caulobacter crescentus.* The stalked cell in S phase is ready for DNA replication. As the cell grows and replicates its DNA, it becomes a pre-divisional cell. During this stage the cells cannot perform DNA replication, entering the G2 phase. In the late pre-divisional stage, a flagellum develops at the swarmer cell pole. After compartmentalization pili are extruded. One cell is a stalked cell which reenters the cyclic developmental program and S phase, completing the circle. The other cell is a swarmer cell. The swarmer cell cannot replicate, that's why they have been assigned to a separate phase G1 [68]..... 12
Figure 3. A phylogenetic tree pointing out the existence of dimorphic cycle, holdfast and stalk secretion in different bacteria [69]. 13
Figure 4. Schematic overview of OmpW from *E. coli.* L1-L4: the loops, S1-S8: Eight beta strands. N: N terminus, C: C terminus [72]..... 14
Figure 5. Plasmid map of the expression vector pAraJS2. The map indicates all the significant features of the plasmid, including signal sequence for OmpT of *E. coli*, arabinose inducible promoter (araBAD) and 10x histidine tag. The map was designed with SnapGene® software (from GSL Biotech; available at snapgene.com). 16
Figure 6. Map of the plasmid designed to express OmpW *C. crescentus* with an N-terminally his-tagged in the outer membrane of *E. coli* host..... 16
Figure 7. Schematic diagram of the TALON® IMAC System. Part A: TALON® Metal Affinity Resin; a Sepharose bead bearing the tetradentate chelator of the Co2+ metal ion. Part B: The polyhistidine-tagged recombinant protein binds to the resin. Source (http://wolfson.huji.ac.il/expression/local/Talon_Products.pdf)..... 18
Figure 8. The parent plasmid provided by our collaborators harboring the *ompW C. crescentus* with its native signal peptide..... 19
Figure 9. Map of the plasmid designed to express OmpW *C. crescentus* in the inclusion bodies of the host cell. There is no signal peptide with the gene sequence of the OmpW. The primers used to amplify the gene are also mentioned as *ompW Nde1* and OmpW *Xho1*. RBS: ribosome binding site. 20

Figure 10. Plasmid map destined to express the *ompW* gene in the outer membrane of the host cell without any tag. *E. coli* OmpT signal sequence is attached to *ompW* to export the protein to the outer membrane. Fwd *SgrD1* and rev *Sac1* are the primers which were used to amplify the gene from its parent plasmid. 21
Figure 11. A schematic representation of the setup we use for biophysical characterization of membrane proteins using black lipid bilayer assay. Courtesy of Hashem Al Ghaili. Schematic representation of bilayer formation and the electrical circuit..... 24
Figure 12. Single channel recording for enriched outer membrane extracts of *C. crescentus*. A: Single channel recording of a membrane formed of PC/*n*-decane after addition of enriched outer membranes of *C. crescentus* to 1 M KCl solution bathing the membrane. The applied membrane potential was 20 mV and temperature 20 °C. B: P(G) is the probability of occurrence for a specific conductance increment. It was obtained by the division of number of channels for the specific conductance and the total number of channels. Applied membrane potential was 20 mV and T= 20 °C. 25
Figure 13. Coomassie stained 12 % SDS-PAGE gel with different extracts of *C. crescentus*. Lane1: Crude membrane preparations, Lane2: un-boiled PBS/EDTA cell wall extracts, Lane3: Boiled PBS/EDTA extracts. 26
Figure 14. Coomassie stained 15 % SDS-PAGE gel of OmpW *C. crescentus* purified by preparative SDS-PAGE gel. Lane 1: Protein marker, 2: un-boiled purified OmpW, 3: Boiled purified OmpW. 27
Figure 15. Single-channel recording of a PC/*n*-decane membrane caused by pure OmpW *C. crescentus*. A: The protein was added at 20 ng/ml dissolved in 1 % Genapol to the KCl solution bathing the membrane. The recording was performed at applied membrane potential of 20 mV at 20 °C. B: A histogram showing the probability of occurrence of a channel of specific conductance. The probability was calculated by dividing the number of insertions with a specific conductance by the total number of insertions observed. The aqueous phase was 1 M KCl, applied membrane potential of 20 mV and T of 20 °C. 28
Figure 16. Amino acids sequence alignment of OmpW *C. crescentus* with the two well-known members of the OmpW family i.e. OmpW *E. coli* and OprG *P. aeruginosa*. Red color shows identical amino acids in all the three proteins (*), green colored are strongly similar residues (:), and blue colored are the weekly similar ones (.), The blue bars indicate the beta strands in OmpW *E. coli* and OprG *P. aeruginosa* as has been shown in their crystal structures [72], [73]. The yellow highlighted residues were identified after tryptic digestion of the protein and analysis of the resulting peptides by mass spectrometry. For alignment tools from “Pole

Bioinformatique Lyonnaise Network Protein Sequence Analysis" (http://npsa-pbil.ibcp.fr) were used. The black arrow indicates the presence of lysine in OmpW *C. crescentus*, as compared to tryptophan in OmpW *E. coli* and OprG *P. aeruginosa*. 29
Figure 17. A:Structural comparison of a) OmpW *C. crescentus*, b) OmpW *E. coli* and c) OprG *P. aeruginosa*. Putative hydrophobic gate comprised of W155 and L56 in *E. coli*, W170 and V65 and the corresponding residues in *C. crescentus* K159 and K165 are shown as spheres. The representation of amino acids is color coded i.e. Green: hydrophilic, white: hydrophobic, red: acidic, blue: basic. The black box in the middle of the proteins represents the putative passage for the transport. EC: Extracellular side, PC: periplasmic side. The structures were modeled by homology modeling, taking OmpW *E. coli* as bait, through Modeller [85] B: Graphical representation of the channel radii of OmpW *E. coli* (red), OmpW *C. crescentus* (blue) and OprG *P. aeruginosa* (green) along the channel axis. The data is obtained using HOLE [86]..... 30
Figure 18. Purification of his-tagged OmpW *C. crescentus*. A: Silver stained 15 % SDS-PAGE gel with purified his-tagged OmpW. 1) pre stained protein ladder, 2) OmpW eluted from the beads using 500 mM imidazole, 3) OmpW eluted from the beads with 1 M imidazole. The samples were unboiled, so some of the protein can be seen in denatured (upper band) and a fraction as refolded form (lower). B: Dot blot of the purified his-tagged OmpW. 1) OmpW eluted with 500 mM imidazole, 2) OmpW eluted with 1 M imidazole, 3) A his-tagged protein as positive control. Mouse anti his antibody at 1:3000 dilution was used as a primary antibody and anti-mouse IgG alkaline phosphatase at 1:30,000 dilution was used as secondary antibody..... 31
Figure 19. Signal peptide prediction for OmpW *C. crescentus*. The prediction was performed using the SignalP 4.0 prediction servers. The data suggests that the signal peptide will be cleaved between residues 21 and 22..... 32
Figure 20. Silver stained 15% SDS-PAGE gels showing the proteins from purified inclusion bodies and purified OmpW. A: The proteins from inclusion bodies were mixed with sample buffer and boiled at 95 °C for 10 mins. Lane1: prestained protein ladder, Lane2: proteins from purified inclusion bodies. B: The gel shows the heat modifiability of OmpW extracted from inclusion bodies, as can be seen in un-boiled sample (Lane2) and boiled (Lane3). 1: prestained protein ladder 33
Figure 21. Silver stained 15% SDS-PAGE gel showing proteins from the outer membrane of the expression host harboring over expression plasmid for OmpW. Lane1: pre stained protein ladder, Lane 2: cell wall extracts of the expression host, with overexpressed OmpW shown. 34

Figure 22. Silver stained 15% SDS-PAGE gel showing OmpW from the outer membrane of the expression host purified by cutting the protein band from a preparative SDS-PAGE gel. Lane1: pre stained protein ladder, Lane2: unboiled purified OmpW, Lane3: boiled OmpW.. 35
Figure 23. OmpW protein confirmation using MALDI TOF. Peptides resulting from the tryptic digestion of the OmpW were checked with MALDI TOF and aligned against the databases of mascot servers (http://matrixscience.com). All the peptides that matched the OmpW from *C. crescentus* are marked in red. Over all match to OmpW was about 60 %..... 35
Figure 24. Coomassie stained 12 % SDS-PAGE gel with the extracts from wildtype and Δ*ompW C. crescentus*. Lane1: PBS/EDTA extracts from wildtype *C. crescentus*, Lane2: PBS/EDTA extracts from Δ*ompW C. crescentus*. Presence of OmpW is marked by the arrow. 36
Figure 25. Single channel recording of PC/*n*-decane membrane in the presence of PBS/EDTA extracts from Δ*ompW C. crescentus*. The protein solubilized in 0.5 % LDAO was added to the 1 M KCl bathing solution at 100 ng/ml concentration. Applied membrane potential was 50 mV and T=20 °C. 37
Figure 26. *Legionella pneumophila* intracellular replication cycle. *L. pneumophila* uses the Dot/Icm T4SS (pink) to deliver a multitude of effector proteins (blue) into the cytosol of an infected macrophage. The pathogen recruits host cell transport vesicles to establish a replication vacuole. (National Institute of Health, 2009). 44
Figure 27. Schematic view of the selected components comprising the MPTP complex (left) and the corresponding components identified in the genome of *L. pneumophila*. OMM: outer mitochondrial membrane, IMM: inner mitochondrial membrane, PBR: peripheral benzodiazepine receptor, PPID, cyclophilin D (peptidyl prolyl isomerase D), ANT: adenine nucleotide translocator, IM: inner membrane, OM: outer membrane. Counter part of ANT in *L. pneumophila* is still to be known. Adapted from [106]. 45
Figure 28. Protein sequence of hVDAC-1 as obtained from www.rcsb.org with PDB ID 2JK4. 47
Figure 29. Primary amino acids sequence of Lpg1974. The sequence was retrieved from NCBI database with accession number AAU28043.1. The protein is composed of 297 residues. 47
Figure 30. Plasmid map of pAraJS2 harboring the mutant gene coding for Lpg1974Δ1-21. The map shows the primers which were used to amplify the gene from the parent plasmid and also the restriction sites for *Nde1* and *Xho1* which were used to clone the gene into the plasmid. araBAD is an arabinose inducible promoter. 50

Figure 31. Amino acid sequence alignment of Lpg1974 and hVDAC-1. The alignment was performed using Clustal Omega. (*) shows amino acids identical in both the proteins. Strongly similar amino acids are shown by (:), and weakly similar ones are represented by (.). 51
Figure 32. Homology structure of Lpg1974 and structural alignment with hVDAC-1. A: Views of homology structure of Lpg1974 using hVDAC-1 as bait. The structure was developed using Modeller. M1 is the first amino acid methionine, and V297 is the last amino acid valine. B: Structural alignment of Lpg1974 with hVDAC-1. Red colored structure is of Lpg1974 and blue colored is hVDAC-1. The structures were developed by Niraj Modi using Modeller. 52
Figure 33. SDS-PAGE gel for purified Lpg1974-GST. A silver stained 12 % SDS-PAGE gel which shows the gel extracted pure Lpg1974-GST. 53
Figure 34. GST tag cleavage of Lpg1974-GST. A silver stained 15 % SDS-PAGE PAGE gel with the cleavage mixture of GST tag cleavage experiment. The upper band at about 60 kDa shows uncleaved protein. The band at about 33 kDa is the protein without GST tag, and the protein band at about 26 kDa is the cleaved GST tag. 54
Figure 35. SDS-PAGE PAGE gel for purified Lpg1974 after GST tag cleavage. A silver stained 15 % SDS-PAGE gel showing the purified Lpg1974 after the cleavage of GST tag. 1: Pre-stained protein ladder, 2: purified Lpg1974, 3: Cleaved off GST tag. 55
Figure 36. Single-channel recordings for Lpg1974. (A) Single-channel recording for a PC/*n*-decane membrane after addition of 100 ng/ml of the purified Lpg1974 to the 1 M KCl pH 7 bathing the membrane. Applied voltage was 20 mV and temperature 21 °C. (B) A histogram showing the probability of occurrence of channels with a specific conductance value. The most frequent channels are having a conductance of 5.5 nS. Adapted from the PhD thesis of Nafiseh Soltanmohammadi. 56
Figure 37. Graphical representation of voltage dependent behavior of Lpg1974. The aqueous phase contained 1 M KCl, 100 ng/ml of Lpg1974 refolded in 0.4 % LDAO. Lipid bilayer membrane was formed from PC/*n*-decane at a temperature of 21 °C. Conductance (G) at a specific membrane potential (V_m) divided by the conductance at 20 mV (G_0) as a function of the membrane potential. Figure made from data adapted from the PhD thesis of Nafiseh Soltanmohammadi. 57
Figure 38. Signal peptide prediction for Lpg1974. Graphical representation of the predicted signal peptide for Lpg1974 using SignalP4.0 server. The cleavage position of the signal peptide is after amino acid no 21 which is alanine. 58

Figure 39. Signal peptide prediction for hVDAC-1. No signal peptide is predicted for the amino acid sequence of the protein. 58
Figure 40. Silver stained 15 % SDS-PAGE gels showing the expressed and purified Lpg1974Δ1-21. A: Proteins from the inclusion bodies showing the over expressed Lpg1974Δ1-21 at about 33 kDa. B: Purified Lpg1974Δ1-21 after gel extraction and refolding. 59
Figure 41. Single-channel recordings for Lpg1974Δ1-21. A: Single channel recording of a PC/*n*-decane membrane after addition of 100 ng/ml of the purified truncated Lpg1974 to the bath solution of 1 M KCl pH 7. B: A histogram showing the probability of occurrence of channels of a specific conductance. The most frequent conductance value is 7.5 nS..... 60
Figure 42. Chart recording of voltage gating for Lpg1974Δ1-21. Increasing positive and negative voltages were applied across a lipid membrane in which several channels were inserted and the recordings were followed for up to 2-3 mins for each voltage increment. 61
Figure 43. Graphical representation of voltage dependent closure of Lpg1974Δ1-21. Conductance (G) at a given membrane potential (V_m) divided by the conductance at 20 mV (G_0) as a function of the membrane potential. The aqueous phase contained 1 M KCl, and 100 ng/ml of truncated Lpg1974 refolded in 0.4% LDAO (*cis* side). The membranes were formed from PC/*n*-decane at a temperature of 21°C. 62
Figure 44. Secondary structure prediction for Lpg1974. Beta sheets and alpha helices in the amino acids sequence of the protein were predicted using http://bioinf.cs.ucl.ac.uk/psipred. The sequence has 19 beta strands predicted as shown by the yellow arrows, which is the same number of beta sheets as in hVDAC-1. 65