Evaluation of a *Rhodobacter capsulatus nif* promoter-based system for the heterologous expression of therapeutically relevant membrane proteins

Evaluierung eines *Rhodobacter capsulatus nif* Promotor-basierten Systems für die heterologe Expression von therapeutisch relevanten Membranproteinen

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Für meine Mutter

Es gibt nur eine Landstraße der Wissenschaft, und nur diejenigen haben Aussicht ihren hellen Gipfel zu erreichen, die die Ermüdung beim Erklettern ihrer steilen Pfade nicht scheuen.

Karl Marx

List of Publications

Özgür, AY., Heck, A., Svensson, V., Pünder K., Malach, A., Gamer, M., Jaeger KE., Drepper, T. ***Rhodobacter capsulatus*: an alternative platform organism for the expression of therapeutically relevant membrane proteins** *Manuscript in preparation*

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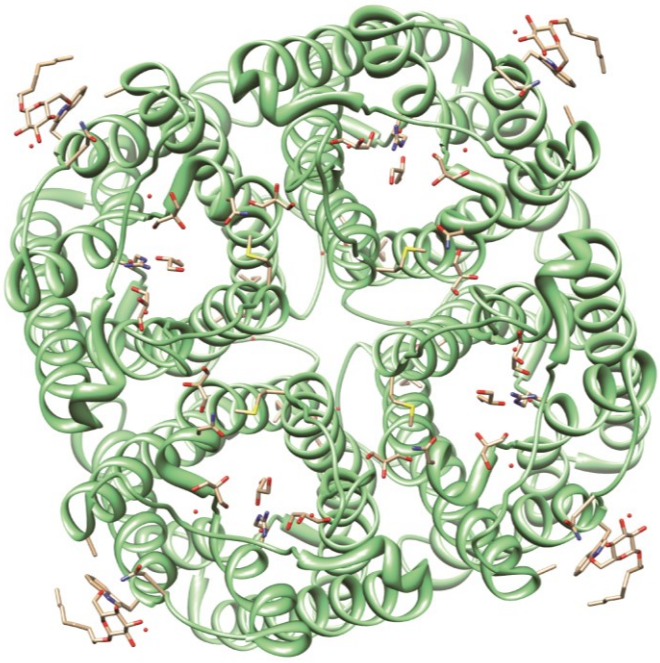
Abbreviations

λ_{max}	absorption maximum
ADP	adenosine diphosphate
α	greek letter alpha
A	ampere
ATP	adenosine triphosphate
Bp	base pair
β	greek letter beta
°C	degree Celsius
cm	centimeter
Da	Dalton
dNTPs	deoxynucleoside triphosphates
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
γ	greek letter gamma
g/l	gramm per liter
h	hour (s)
Kb	kilobase pair
kDa	kilodalton
l	liter
LB	Luria-Bertani Broth
max	maximum /maximal
mRNA	messenger RNA
µl	microliter
mg	milligram
ml	milliliter
min	minute (s)
MW	molecular weight
M	mole per liter
MCS	multiple cloning site
ng	nanogram
nm	nanometer
Nif	nitrogen fixation
NMR	nuclear magnetic resonance
P_x	promoter of gene x
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
Pfu	*Pyrococcus furiosus*
PVDF	polyvinylidene fluoride
rRNA	ribosomal ribonucleic acid
RT	room temperature
rpm	rounds per minute
SDS	sodium dodecyl sulfate
tRNA	transfer RNA
V	Volt
v/v	volume per volume
W	watt
w/v	weight per volume

CHAPTER I: INTRODUCTION



Human AQP4 (PDB file 3GD8)

All living organisms are comprised of cells, which are separated by a biological membrane from their surrounding environment. In addition to protecting the interior of cells from their exterior, biological membranes may also divide the cell internally into different compartments to facilitate biochemical reactions that would otherwise not be possible in the same physiological environment [1, 2]. The core structure of biological membranes is almost always composed of a continuous lipid bilayer: two thin sheets of lipid molecules, which point their hydrophobic tails inwards and their hydrophilic heads towards the intracellular and extracellular fluids. The major lipid classes found in biological membranes are phospholipids, sterols and glycolipids. While the presence and amount of each lipid class depends upon the individual function of the membrane, phospholipids are the most abundant in biological membranes [3].

Since interaction with the "outside world" is crucial for the survival of a living cell, the cell membrane mustn't be viewed as an impenetrable barrier, but rather as a gatekeeper who governs very strictly who may enter and leave the cell. Biological membranes are a complex blend of lipids, carbohydrates and proteins [4]. The amount of each of these compounds depends on the specific biological membrane. Major cellular functions are governed by membranes, including cell-cell interactions, energy metabolism, transport processes and signal transduction. But how does the membrane accomplish these tasks? Although all membranes share a basic similarity, the biochemical diversity is attributed primarily to the presence of membrane proteins.

1 Membrane proteins

1.1 Membrane protein types

Membrane proteins form the functional and structural framework of biological membranes. The classification of membrane proteins into different types is still challenging, because it relies mainly on interpretation. For this work, the classification described by William Stillwell [5] will be employed. As depicted in **Figure I-1**, this system divides membrane proteins into four different groups: peripheral membrane proteins, amphitropic membrane proteins, lipid-anchored membrane proteins and integral membrane proteins.

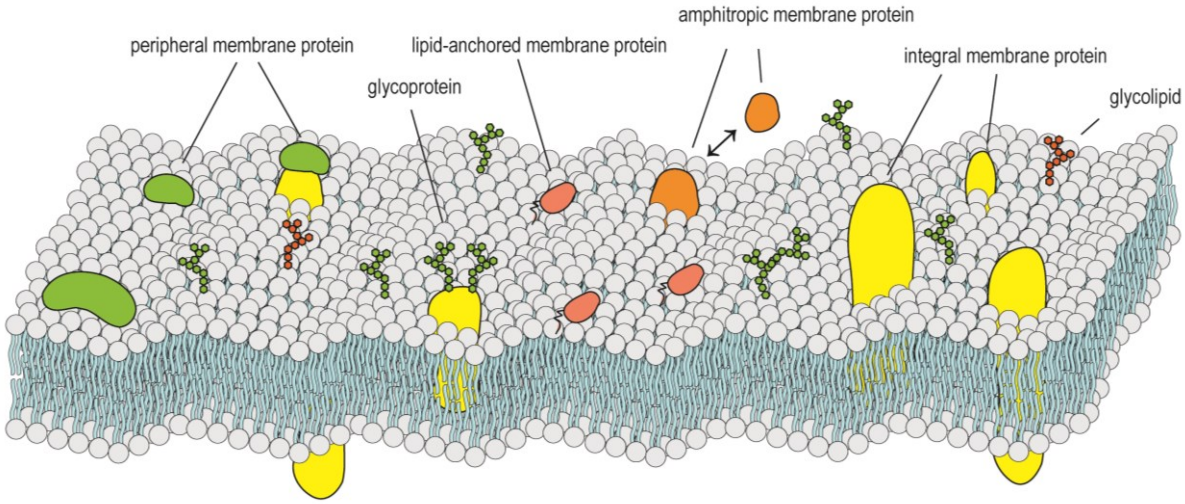


Figure I-1: Cartoon depiction of different membrane protein types
The biological membrane is composed of a continuous lipid bilayer which harbors a complex assortment of membrane proteins, carbohydrates and lipids. Proteins may interact differently with the membrane depending on their function. While peripheral membrane proteins are associated with the membrane surface, integral membrane proteins penetrate the biological membrane. Furthermore, membrane proteins can be covalently attached to fatty acids or glycolipids in which case they are classified as lipid-anchored membrane proteins. Another class of membrane proteins, the amphitropic proteins, exists in either a water soluble state or a membrane bound state. Depending on the individual protein the alteration between the two forms can be reversible or irreversible. Owing to their specific function, membrane proteins (glycoprotein) and lipids (glycolipid) can be glycosylated. Modified from [5].

1.1.1 Peripheral and amphitropic membrane proteins

As shown in **Figure I-1**, peripheral membrane proteins are basically water-soluble proteins that are non-covalently attached to the membrane by interacting with either integral membrane proteins or with lipids through electrostatic interactions and hydrogen bonds [6-8]. These interactions can be easily nullified by altering the pH or salt concentration, which is one reason why these proteins can be removed from the membrane in a lipid-free state without all the complications encountered when isolating integral membrane proteins (see also **I-2.2.5**)

Another class of membrane proteins, which cannot be easily discerned from peripheral or integral membrane proteins, is the group of amphitropic proteins [9, 10]. Proteins that belong to this class can exist in either a water soluble state or a membrane bound state. The alteration between the water soluble form and membrane bound state depends on a conformational change, which is induced by a post-translational modification or binding of a particular substrate. This conformational change reveals hidden hydrophobic regions in the protein that form the basis for the interaction with the membrane. The nature of this interaction depends on the protein and can be either reversible, as in the case of the

Escherichia coli pyruvate oxidase [11], or irreversible, as observed for many pore forming toxins, which exist as soluble monomers that can assemble into membrane integrated pore complexes [12].

1.1.2 Lipid-anchored membrane proteins

This protein class is characterized by proteins that exhibit a covalent bond to a fatty acid (e.g. myristate or palmitate), isoprene compound (e.g. farnesol, geranylgeraniol) or a glycolipid (Glycophosphatidylinositol – GPI) that anchors the protein to the membrane [13-17]. The function of the added lipid-anchor is multilayered and by no means restricted to the simple act of anchoring the protein to the membrane. Some lipid-anchors are assumed to function as a targeting signal for membrane proteins, considering that lipid-attachments seem to illustrate a preference for one side, and a specific region, of the membrane leaflet [18, 19]. For instance, GPI-anchored proteins are only found at the outer surface of eukaryotic cells, whereas lipid-anchors derived from prenylation, palmitoylation or myristolyation are found on membrane proteins localized at the cytoplasmic side [5]. Another function of lipid-anchors is assumed to be one of regulation, demonstrated by the fact that some lipid-anchors can be removed from the protein in response to a specific signal [20]. Examples include the reversible fatty acid attachment palmitoylation [21, 22] or the GPI-anchor, which can be removed by phospholipase C [18].

1.1.3 Integral membrane proteins

In contrast to peripheral, amphitropic or lipid-anchored membrane proteins, integral membrane proteins are permanently attached to the membrane and cannot be removed without the usage of detergents, chaotropic agents or non-polar solvents. Integral membrane proteins may be divided into two groups: proteins which traverse the whole membrane (polytopic or transmembrane proteins) and those which penetrate the hydrophobic interior of the membrane, but only from one side (monotopic proteins). Transmembrane proteins exhibit a specific amino acid distribution that correlates to their position relative to the membrane. While amino acids facing the hydrophobic core of the lipid bilayer are mostly hydrophobic, amino acids at the interface are usually composed of tryptophan or tyrosine (“aromatic belt”) and amino acids in the surrounding fluids polar [23]. Furthermore, the “positive-inside rule” proposed by von Heijne implies that amino acids present in the cytoplasmic flanking regions are almost always positively charged [24] and the majority of transmembrane proteins are characterized by a cytoplasmic C-terminus [25]. In contrast to soluble proteins, transmembrane proteins are restricted by their hydrophobic environment to assume diverse folding structures [26]. Usually, structural motives found in integral membrane proteins are limited to α-helices and β-barrels [27], which are presented in **Figure I-2**.

β-barrel proteins are commonly found in the outer membrane of Gram-negative bacteria, where they are encoded by 2 % - 3 % of the genome, but can be found in the outer membranes of organelles such as mitochondria or chloroplasts as well [28]. As can be deduced from the name of these proteins, they span the membrane by β-sheet segments that usually form a channel. One major class of β-barrel proteins is the group of porins [29]. To transport polar molecules across the hydrophobic core of the membrane, porins assume a tertiary structure, much like a cylinder, where hydrophilic amino acids line the inside of the channel, while hydrophobic amino acids face the hydrophobic core of the membrane. Thus, instead of a membrane spanning domain exclusively comprised of hydrophobic amino acids, the order of amino acids is alternating between hydrophilic and hydrophobic residues in these proteins.

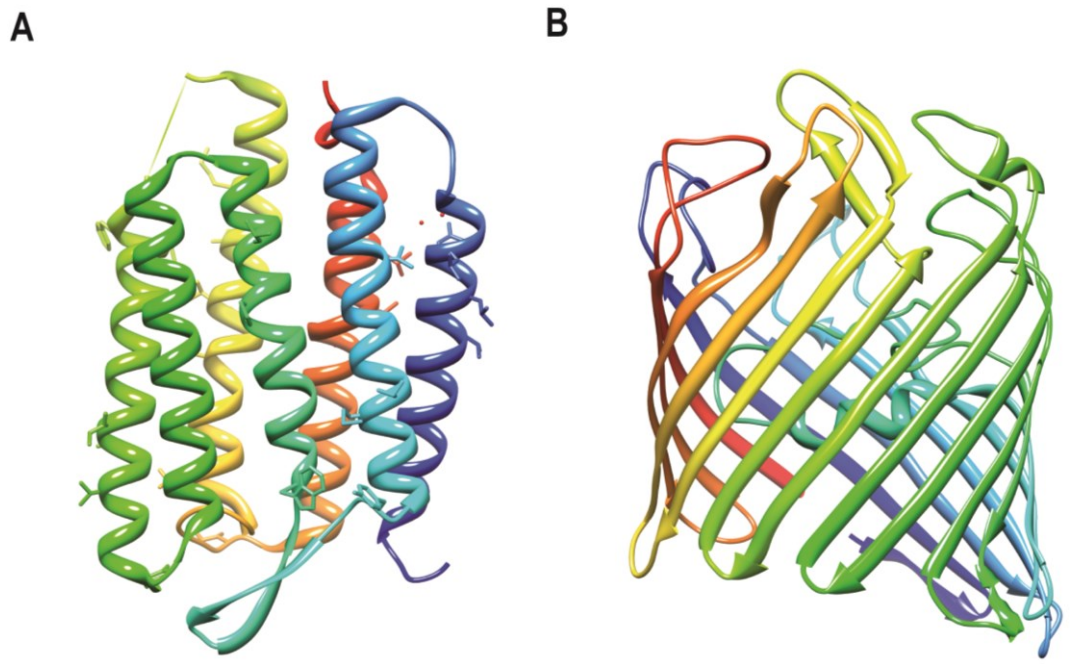


Figure I-2: Structural motives found in integral membrane proteins
Integral membrane proteins traverse the membrane by two different structural domains: α-helices and β-barrels. A representative example for a helix bundle membrane protein is depicted on the left side (A) and an example for a β-barrel protein on the right side (B). Images were visualized by using the software UCSF Chimera with PDB files 3PQR (A) and 1BT9 (B).

In contrast to β-barrel proteins, helix bundle proteins traverse the membrane by one or multiple α-helices. Typically, the α–helix present in the transmembrane domain (TM) is composed of approximately 20 hydrophobic amino acids [30]. While the TM in a single-spanning membrane protein often acts as a membrane-anchor, multi-spanning membrane proteins exhibit TMs that are an inherent part of the proteins function [31]. As opposed to β-barrel proteins helix bundle proteins can be found in nearly all biological membranes [5], which is why the majority of physiological functions governed by biological membranes are mediated by integral membrane proteins of the α –helix type.

To clarify the scientific and medical interest in this protein class, the following section will discuss the physiological relevance of integral membrane proteins. Unless stated otherwise, integral membrane proteins will hereafter be referred to simply as “membrane proteins”.

1.2 Physiological role of membrane proteins

Membrane proteins play a pivotal role for the physiology of all living cells. They take part in major cellular processes, such as cell-cell interaction, solute transport, signal transduction or energy metabolism. Thus, it is not surprising that 20 % - 30 % of all open reading frames of eukaryotic and prokaryotic organisms are predicted to encode membrane proteins [32]. The following section will give a summarized overview of four major biological processes mediated by membrane proteins.

1.2.1 Cell-cell interaction

Communication between cells forms the basis for the function and the development of certain cell types or tissues, which is why cell-cell interactions are of particular importance in multicellular organisms [33, 34]. Cell-cell interactions are highly selective and can be divided into two different groups: stable interactions and transient interactions. Stable interactions refer to interactions based on intercellular junctions, which act as gateways between neighboring cells or the cell with the extracellular matrix (EM) [35]. In addition, these proteins are responsible for adhering cells to each other and to the EM, providing structural and functional integrity for the cells [36, 37]. Cell junctions are composed of cell adhesion molecules that belong to different protein families: Selectins, Cadherins, Integrins and the Immunoglobulin superfamily [38]. With the exception of the Immunoglobulin superfamily, which includes soluble as well as membrane proteins, all listed proteins belong to the class of integral membrane proteins.

1.2.2 Solute transport

Many nutrients, including ions, amino acids and macromolecules are needed for the survival and growth of cells. These compounds are transported into the cells with the aid of membrane transport proteins, which are made out of channels, pumps and carriers. Typically, transport proteins move molecules with a high specificity but very different mechanisms across the membrane. For instance, channel proteins transport ions and water across the membrane by facilitated diffusion [39, 40]. Ion channels are very

important for the nervous system, since they maintain the resting potential of cells and coordinate the flow of ions in response to a certain stimulus. Hence, biological processes where ions are quickly released to affect physiological changes within the cell very rapidly (e.g. second messenger molecules such as Calcium) are accomplished often by means of ion channels. These proteins can open or close in response to a ligand they bind, to the membrane potential of a cell, to physical parameters such as light, temperature or pressure [41, 42].

While channel proteins simply open or close in response to a stimulus, transporters / carriers undergo a complicated conformational change to transport selected molecules across the membrane. Carrier proteins may transport molecules by facilitated diffusion down a concentration gradient (downhill) or by active transport against a concentration gradient of a transported substance (uphill). They can be classified with regard to the manner and number of molecules they transport [40]. Uniporters transport one molecule at a time by facilitated transport, while symporters or antiporters couple the transport of one molecule to the movement of another. If the coupled transport of molecules is in the same direction, the mechanism is referred to as symport, whereas the transport of molecules in opposite directions is termed antiport. When the active transport utilizes chemical energy derived from ATP hydrolysis, redox energy or photon energy, the carrier mediated transport mechanism is termed primary active transport and the protein primary active transporter. An example for one of the largest protein family of primary active transporters is the ABC transporter super family, which can be found in both prokaryotic and eukaryotic organisms. They fulfill very important functions, including uptake of nutrients (importers), pumping of toxins or drugs out of the cell or take part in transport unrelated actions such as DNA repair or translation [43-45].

In contrast to ABC transporters, which couple the transport of substrates to the hydrolysis of ATP, secondary active transporters utilize the energy derived from the transport of a molecule along a concentration gradient to move another substance against their electrochemical gradient. A very prominent example for an important family of carrier proteins, which includes many secondary active transporters, is the solute carrier family (SLC) of membrane proteins. Next to G protein-coupled receptors (GPCRs), this protein family represents the largest family of membrane proteins in humans. The term solute carrier protein is to be understood as a collective term rather than a defined protein family designation, since the classification is based on a functional relation and not a strict phylogenetic one. As such, SLC proteins include different antiporters, symporters and passive transporters, while ion channels, aquaporins or ABC transporters are not included in the SLC protein family. With a few exceptions, all SLC proteins transport molecules across the membrane by utilizing the driving force of

an ion gradient. Transported molecules include sugars, ions, drugs, nucleotides or amino acids, while the transport mechanism is limited to facilitated transport or secondary active transport [46, 47].

1.2.3 Signal transduction

Reacting to changes in the environment is of paramount importance for organisms. A physiologically important class of integral membrane proteins, responsible for sensing environmental stimuli and transducing signals across the membrane (**Figure I-3**), are G-protein coupled receptors (GPCR) [48]. GPCRs span the membrane seven times, which is why these proteins go by names such as heptahelical receptors, serpentine receptors or the "great seven" [49]. So far, GPCRs have been found only in eukaryotic cells, including animals and yeasts. In humans they are encoded by more than 800 genes and represent one of the most important biological targets of drugs [50].

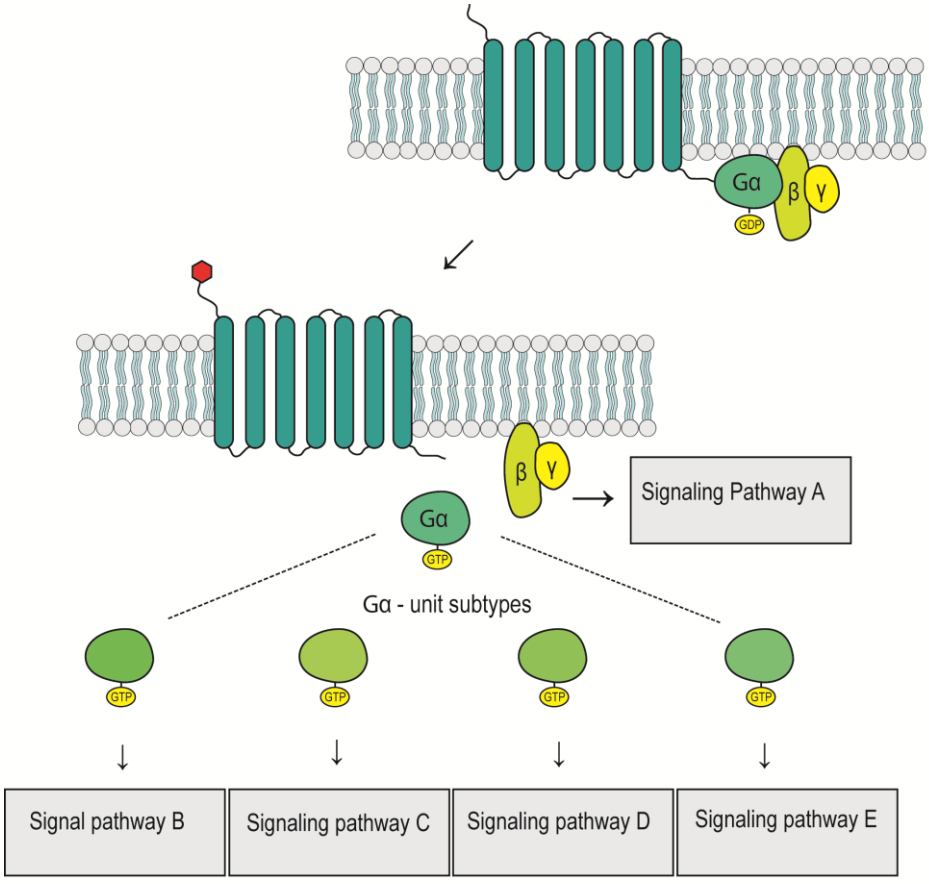


Figure I-3 Common mechanism of GPCR mediated signal transduction
Schematic depiction of a GPCR mediated signal transduction through activation of G-proteins. Upon binding of a ligand (red) the GPCR undergoes a conformational change that leads to the exchange of GDP through GTP at the subunit Gα of the heterotrimeric G-protein. GTP bound Gα is able to dissociate from the βγ- complex of the G-protein in order to interact with an effector target. Since each G-protein activates different signaling pathways within the cell, GPCRs are able to activate different signaling pathways through different Gα-unit subtypes. Although the Gα subunit functions as the main activator of signaling pathways, depending on the G-protein the βγ-complex may activate signaling pathways as well.

The physiological function of GPCRs is manifold, owing this to the fact that these receptors are involved in perceiving information from the “outside” and preparing a response from the “inside” of a cell. These proteins are able to bind a very broad spectrum of ligands that differ greatly in their size, their complexity and their chemical nature. Examples include: photons, neurotransmitters, hormones, cytokines or odors [51]. Structurally, the proteins are characterized by an extracellular N-terminal tail that varies in its length with respect to the particular GPCR, seven transmembrane α-helices, which are connected by three extracellular and three intracellular loops, and a cytoplasmic C-terminal tail. Usually, the α-helices of the protein assume a conformation which forms a cavity within the membrane to bind the ligand. Typically, upon binding of the ligand the receptor undergoes a conformational change that leads to the activation of a specific heterotrimeric G-protein complex [52]. The heterotrimeric G-protein complex consists of the subunits G_{α} and $G_{\beta\gamma}$ [53], which can act independently to transduce a signal from the outside to affect a response from the inside of the cell, by activating different signaling pathways. Depending on the type of bound G-protein different signaling pathways are activated [54]. However, there is also evidence that suggests G-protein independent signaling by GPCRs [55, 56], which demonstrates the complexity involved in the mechanisms governing GPCR related signal transduction.

1.2.4 Energy metabolism

Cells routinely utilize the energy carrier adenosine triphosphate (ATP) as a currency to power various cellular functions such as mechanical work, transport work or chemical work. One of the most important biochemical reactions to synthesize ATP is accomplished by an integral membrane protein identified as F_OF_1 - ATP synthase [57, 58]. This enzyme is comprised of several subunits, which vary with respect to the originating organism, but can be divided into two different domains with regard to their function and localization. The F_O-domain is a proton pore and localized in the membrane, whereas the F_1-domain is water-soluble and the portion of the protein responsible for the ATPase activity [59]. The activity of the enzyme is reversible with regard to the physiological conditions and originating organism, thus the enzyme can either form or hydrolyze ATP. ATP synthesis is driven by harnessing energy derived from an electrochemical concentration gradient of H^+ or Na^+ across the membrane [60]. According to the primary energy source utilized, and the manner by which the electrochemical gradient is built, two metabolic pathways of ATP formation are differentiated: oxidative phosphorylation and photophosphorylation [61]. While both pathways rely on building a proton gradient by coupling the transfer of electrons through a series of electron carriers (electron transport chain), oxidative phosphorylation uses chemical energy inherent in organic or inorganic molecules as an energy source, whereas photophosphorylation utilizes light energy. However, not all organisms build a proton gradient

through an electron transport chain for chemiosmosis. For instance, the archaeal bacterium *Halobacterium salinarum* (*H. salinarum*) employs the very remarkable integral membrane protein bacteriorhodopsin (BR) to build a proton gradient across the membrane [62]. BR is characterized by seven α-helical transmembrane domains and can make up to 50 % of the membrane surface of *H. salinarum*. It belongs to the class of retinylidene proteins and contains one molecule of retinal as a chromophore. Retinal absorbs a photon and changes its conformation, which leads to the transfer of protons across the membrane [63-65]. In this manner, BR can act as a light-driven proton pump that builds an electrochemical gradient across the membrane, which can be utilized by an ATP synthase to form ATP.

Considering the elucidated functions mediated by membrane proteins, it is not surprising that dysfunctions of these proteins are also implicated in the pathogenesis of several diseases. The following section will focus on the pathological implications and consequently on the therapeutic relevance of membrane proteins.

1.3 Pathological implications and therapeutic relevance of membrane proteins

The list of diseases associated with the dysfunction of membrane proteins is continuously expanding. Typically, the dysfunction of the membrane protein is caused by external factors such as drugs and toxins or by something inherent in the protein (mutation), which leads to a loss of function or to a gain of function. For instance, mutations in the human GPCR Rhodopsin account for 30 % - 40 % [66] of a certain type of retinitis pigmentosa: a progressive neurodegenerative disease causing the loss of visual field that can degenerate to blindness [67]. Of particular importance are disorders caused by dysfunctions of ion channels (channelopathies). These include diseases such as epilepsy, migraine, blindness, hypertension, cardiac arrhythmia, asthma, Bartter syndrome or cancer [68]. Furthermore, several diseases such as Alzheimer, Parkinson or Cystic fibrosis have been ascribed to mutated or misfolded membrane proteins [69-72].

Naturally, this physiological and pathological importance of membrane proteins encourages not only fundamental biomedical research but also research directed towards therapeutic and diagnostic applications. Since many diseases are connected to the dysfunction of membrane proteins, the possibility of those proteins to act as an indicator for a particular disease can be very high. Diagnostic biomarkers can help with early diagnosis, monitoring and possible prevention of a disease [73]. Apart from their value as diagnostic biomarkers, membrane proteins play a pivotal role as drug targets and

key factors for developing new drugs [74]. Indeed, many people are using drugs in their everyday life which target directly or indirectly membrane proteins. For instance, problems of the digestive tract caused by peptic ulcers or the Gastroesophageal reflux disease (GERD) are treated commonly with Omeprazole. Omeprazole is a proton pump inhibitor, which inhibits the membrane integrated gastric H^+, K^+- ATPase and thereby stops the acidification of the stomach [75, 76]. During July 2013 – June 2014, the *S*-enantiomer of omeprazole (brand name Nexium®) ranked third on the list of most prescribed (18.6 million) and most sold drugs ($6.3 billion) in the United States of America (http://www.webmd.com/news/20140805/top-10-drugs). Other prominent drugs targeting membrane proteins include Beta-Blockers [77], employed for hypertension or cardiac arrhythmias (targeting β-adrenergic receptors), and β_2-adrenergic receptor agonists [78], marketed as inhalers for treatment of asthma or other pulmonary disorders. The impact of membrane proteins as drug targets is best illustrated by a list of selected drugs with their respective global sales in the years 2012 and 2013 depicted in **Table I-1**.

Table I-1: Selective assortment of drugs targeting membrane proteins in humans

Brand name	Generic name	Company	Disease	Target protein	million $ (2012)	million $ (2013)
Advair	Fluticasone/ Salmeterol	GlaxoSmithKline	Asthma, chronic obstructive pulmonary disease	Salmeterol targets the GPCR beta$_2$-adrenergic receptor	7995	8356
Abilify	Aripiprazole	Otsuka America Bristol-Myers Squibb	Schizophrenia and bipolar disorder	Several GPCRs including Dopamine receptor D_2, 5-HT_{1A} receptor	8321	8031
MabThera (EU)	Chimeric monoclonal antibody	Roche	Leukemias, lymphomas, rheumatoid arthritis, transplant rejection	CD20	7150	7410
Crestor	Rosuvastatin	AstraZeneca	Dyslipidemia	HMG-CoA reductase	7520	6871
Herceptin	Trastuzumab	Roche	Breast cancers	Receptor tyrosine-protein kinase erbB-2 (HER_2/neu)	6278	6481
Spiriva	Tiotropium bromide	Pfizer Boehringer-Ingelheim	Chronic obstructive pulmonary disease	Muscarinic receptors	4577	4922
Lyrica	Pregabalin	Pfizer	Neuropathic pain, partial seizures	Voltage dependent calcium channels	4332	4815
Nexium	Esomeprazole	AstraZeneca	Peptic ulcer disease, gastroesophageal reflux disease	H+ / K+ ATPase in the parietal cells of the stomach	4215	4218
Diovan	Valsartan	Novartis	High blood pressure, congestive heart failure	GPCR Angiotensin II receptor	4417	3524

Source: http://www.pmlive.com/top_pharma_list/Top_50_pharmaceutical_products_by_global_sales

Around 50 % to 70 % of all available drugs are either directly or indirectly targeting membrane proteins [79, 80]. Among membrane proteins, GPCRs constitute the largest class of drug targets, closely followed by nuclear receptors and ion channel receptors [81].

2 Recombinant membrane protein production

Despite their medical and pharmaceutical importance, fundamental knowledge is still lacking about the majority of membrane proteins with regard to their function and structure. Although there has been a steady increase in the number of solved crystal structures of integral membrane proteins over the last decades [82], they still constitute for only 1 % of known protein structures, while human integral membrane proteins correspond to less than 50 of these [83]. Most challenges in working with membrane proteins arise from their hydrophobic nature and their cellular localization, seeing that many techniques like crystallization or NMR spectroscopy are primarily suited for proteins in aqueous solutions [84-87]. One of the main bottlenecks in obtaining crystal structures of membrane proteins remains the isolation of sufficient amounts of pure protein [88]. Yet, understanding the structure of a protein holds the key to understanding the corresponding function. Structural information is especially important for drug designing, since comprehending the mechanisms of known drug-protein interactions facilitates the prediction of new interaction sites and thereby designing of new drugs [89-93]. Although the first structural information of membrane proteins was acquired from natural sources [94-96], scientist realized that this strategy was not feasible in the long run. Natural sources provide in most cases only low amounts of target protein, prevent protein modifications for function-structure relationship studies and impair isotopic labeling of proteins for NMR spectroscopy. Hence, in recent years the trend in obtaining crystal structures has changed towards structural information acquired from proteins produced by recombinant protein production [97-99].

The production of proteins in their original host is often hampered by their complicated and expensive cultivation or their inability for easy upscaling. For this reason, the production of proteins in organisms other than their originating one is a practice which takes a pivotal role for biomedical research and industrial biotechnology. Heterologous expression of genes is usually accomplished by cloning the gene encoding the protein of interest (target gene) into a DNA vehicle (expression plasmid) that mediates the transfer into the host (expression host) and provides the genetic instructions to produce the protein of interest [100-102]. Although the procedure seems trivial, there can be bottlenecks arising from

incompatibilities of the employed expression host, the target gene or the expression plasmid which prevent efficient production of the protein. The following section will discuss bottlenecks and considerations for expression plasmids employed in the production of membrane proteins.

2.1 Expression plasmids

Next to the target gene and the expression host, the choice of the expression plasmid represents one of the most important steps in successful production of recombinant proteins in general and membrane proteins in particular. Since the main objective is the maximized production of target protein, an expression plasmid must be chosen under consideration of your expression host and your target protein. Genetic elements present in expression plasmids can affect the transcription and the translation of a target gene [103]. Considerations with respect to the promotor choice, problems regarding mRNA stability and translation initiation, as processes affected by elements present in the expression plasmid, will be discussed below.

2.1.1 Promoter

Considering industrial applications of recombinant protein production, promoters selected for expression of target genes should be strong and inducible in a simple and cost-efficient manner. While promoters mediating a constitutive expression of a target gene can be employed, stress inducing or toxic proteins are more efficiently expressed with promoters facilitating a regulated expression. This quality is especially important for proteins that prevent obtaining high biomasses, because utilizing an inducible promoter enables reaching high cell densities before protein production is triggered. Furthermore, an optimal promoter should be characterized by a tightly controlled expression of target gene, since basal expression of membrane proteins can have effects on the growth or the survival of the cell (**I.2.1.2**). Another factor that needs to be considered for the promoter choice is the manner in which the promoter is induced. While promoters involved in sugar uptake (e.g. P_{lac}, P_{araBAD}) are routinely employed, inducers such as IPTG can be toxic or very expensive for large-scale production [104, 105]. To bypass these bottlenecks, alternative promoters regulated by physical stimuli such as temperature, osmolarity and the pH have been implemented in different expression plasmids [106-108]. Although these promoters seem an attractive alternative, inducing the expression of genes by environmental factors can have repercussion for the physiology of the expression host. Especially thermally induced promoters are known to induce the expression of host specific genes [109]. The strength of the promoter is a factor

which needs to be considered as well. Although a very strong promoter is desired for maximal production of proteins, a modest promoter may sometimes be the better alternative to alleviate toxic effects associated with the production of the protein [31]. However, this bottleneck can be bypassed by choosing a strong promoter that can be effectively modulated by the cultivation conditions. This way, one has a versatile promoter which can be utilized for a set of different proteins.

2.1.2 mRNA stability

The transcription of a gene usually results in mRNA molecules that are not translated completely. Portions of the mRNA transcript that are not translated are referred to as untranslated regions (UTR), more specifically the 5´ end (5´UTR) or the 3´ end (3´UTR) of the coding sequence. Both regions are of paramount importance for mRNA structure and consequently for the stability of mRNA molecules [110]. The mRNA stability can be a major bottleneck for heterologous expression of membrane proteins, considering that mRNA molecules have a natural half-life, rapid degradation of mRNAs can therefore compromise translation. The mRNA stability is mainly influenced by the secondary structure of the mRNA and therefore affected by both the expression plasmid and the target gene. Accordingly, there have been cases where the addition of protective structure elements (e.g. hairpin or stem-loop) to the 5´UTR or 3´UTR of mRNAs resulted in a prolonged half-life or an increased stability [111, 112]. Another factor that influences mRNA stability can be the transcription terminator signal utilized in the expression plasmid. A read-through transcription into the vector can lead to unwanted production of proteins encoded by the remaining genes on the plasmid, which may result in plasmid instability or reduced protein production.

2.1.3 Translation initiation

The translation efficiency of mRNA is majorly affected by structural elements present at the 5´ end of the mRNA [113]. This portion of the mRNA includes the ribosome binding site (RBS), which in prokaryotes contains the Shine-Dalgarno sequence (SD) and represents the complementary region to the 16srRNA of the ribosome [114, 115]. In addition to the SD sequence, the 5´ end of the mRNA as a whole can have a severe impact on translation. This could be illustrated by mRNA molecules that were genetically altered by addition of foreign 5´-UTRs that lead to an enhanced translation of the particular mRNA [116]. Furthermore, the start codon as well as the stop codons implemented in the expression plasmid may be incompatible in foreign expression hosts. Although the most common start codon is AUG, there are genes which utilize alternate start codons such as GUG or UUG [117]. The presence of those codons

can be of significance for the particular gene and must therefore be considered. As with the transcription termination, the translation termination is a major factor for protein production. To alleviate ribosome stagnation or skipping, expression plasmids should contain multiple stop-codons compatible with the respective expression host.

2.2 Bottlenecks associated with the heterologous expression and isolation of membrane proteins

In addition to common bottlenecks such as a differing codon usage bias, the requirement for cofactors, degradation by proteases or the formation of inclusion bodies [118-124], the following section will give a short overview of bottlenecks associated more commonly with the heterologous expression and isolation of membrane proteins.

2.2.1 Membrane protein biogenesis

In contrast to cytoplasmic soluble proteins membrane proteins need to be targeted, inserted and also folded at/in the membrane. The translocation of proteins can be partial, so that the proteins are integrated into the membrane, or complete, in which case the protein is transferred across the membrane into another compartment or out of the cell. Although there are differences between the translocation in pro- and eukaryotic cells, some steps of the translocation are very similar. Many α-helical membrane proteins are inserted co-translationally into the membrane by the protein-conducting machinery SecYEG (prokaryotes) or Sec61 translocation complex (eukaryotes) [30, 125, 126]. Membrane protein targeting is usually mediated by a specific sequence at the N-terminal region of the protein (signal peptide) or the first hydrophobic transmembrane helix, which is recognized by a signal recognition particle (SRP). This protein complex, in concert with its respective receptor (SRP receptor), mediates the transfer and insertion of the nascent polypeptide chain into the membrane integrated Sec-translocon that is localized at the cytoplasmic membrane (prokaryotes) or the endoplasmatic reticulum membrane (eukaryotes) [126]. During translation, the translocon inserts the transmembrane domains laterally into the lipid bilayer, while soluble portions are translocated across the membrane [127-129]. Several chaperones as well as proteases are assumed to assist the folding and the quality control of membrane proteins. The translocation of membrane proteins is an immensely complicated and still not fully comprehended process, which is influenced by many factors including the hydrophobicity and

amino acid sequence of the transmembrane helix, the signal peptide, the N- and C-terminal domains and the need for specific chaperones or other auxiliary proteins [130]. Considering that organisms are likely to exhibit differences in the described processes, it is easy to see that incompatibilities can have a major impact on the membrane protein biogenesis and consequently on the production of recombinant membrane proteins in a foreign expression host. Furthermore, there is increasing evidence that some membrane proteins need very specific chaperones for proper folding (e.g. Rhodopsin [131]), for those proteins, expression in a host lacking these factors can be a major bottleneck. Apart from the protein folding, there can be a generic problem with "over"-producing membrane proteins. The membrane space as well as the membrane biogenesis factors needed to translocate the membrane protein may be insufficient to accommodate and produce membrane proteins in such quantities. Indeed, in *E. coli* it could be shown that overproduction of membrane proteins resulted in the saturation of the translocation machinery, leading to an increased formation of inclusion bodies and toxic effects [132, 133].

2.2.2 Toxic effects

The production of membrane proteins is usually accompanied by toxic effects, resulting in a growth arrest and in some instances in the death of the cell [133, 134]. The observed effect mediated by the protein is not always a product of the protein´s inherent toxicity, but rather a consequence of affected metabolic pathways. Heterologous expression resorts to the host´s own cell resources and energy supply for maintaining the expression plasmid and for production of the target protein [135]. Particularly for membrane protein production toxic effects in bacteria have been attributed primarily to the saturation of the Sec-translocon capacity of the cell [136]. As mentioned before, saturation of the translocation machinery in *E. coli* results in the formation of inclusion bodies, but also in an impaired respiration and the activation of the Arc response, which leads to inefficient ATP production and acetate production [133]. Furthermore, the membrane integrity of the organism can be compromised as well if it accommodates too many, and structurally incompatible, membrane proteins. All of these factors can induce stress responses in the cell, which may result in proteolytic degradation by proteases [137, 138] or plasmid instability [139]. To alleviate toxic effects mediated by membrane protein expression, different strategies have been applied. To avoid saturation of the translocation capacity of the cell, moderate production of protein is recommended [140], while the employment of tight promoters prevents unwanted expression of the membrane protein [141]. To improve targeting and folding of membrane proteins, co-expression of chaperones has been utilized with varying degrees of success [142]. Furthermore, lowering the cultivation temperature resulted in a reduced formation of inclusion

bodies and a higher percentage of membrane inserted proteins. These positive effects are attributed to a combination of the activation of chaperones and a decrease in the transcription and translation rate of the organism [143, 144].

2.2.3 Lipids

New insights about the interaction between membrane and proteins underline the importance of the lipid environment for the insertion, correct folding and the activity of membrane proteins [143, 145]. There is increasing evidence that membrane proteins bind lipids selectively to modulate their structure and their function [146]. This is also reflected in the observation that many X-ray structures of membrane proteins contain tightly bound endogenous lipids, which are usually co-purified with the membrane protein. For instance, it could be shown that the secondary active transporter lactose permease (LacY) of *E. coli* requires the lipid phosphatidylethanolamine for *in vivo* function [147]. Considering that membranes of organisms may differ greatly with regard to their lipid composition, it is not surprising that failure to produce membrane proteins by heterologous expression is often ascribed to the fact that the lipid environment of the expression host is not suited for proper folding or activity of a particular protein. Bacterial membranes for example are devoid of cholesterol and sphingolipids [148], both of which are assumed to be important for the activity of some mammalian receptors, including the serotonin transporter [149]. However, the lipid composition is not always an insuperable bottleneck. Depending on the target protein, addition of lipids during expression or purification of the membrane protein may restore functional activity, as could be shown for the LacY protein [150] or the human Adenosine A_{2A} receptor [151], respectively. A more complicated but also successful approach deals with changing the lipid composition of the organism altogether, this could be demonstrated for the production of the F_1F_O ATP synthase in *E. coli* [152].

2.2.4 Post-translational modifications

Another bottleneck for the heterologous expression of membrane proteins can be the post-translational modification of proteins. Eukaryotic membrane proteins may require post-translational modifications such as acylation, prenylation, phosphorylation, disulfide-bond formation or glycosylation [153]. Most prokaryotic expression platforms cannot carry out post-translational modifications, so that these can pose a serious bottleneck for recombinant protein production [153]. Of particular importance is the N-linked glycosylation of membrane proteins. For this modification, sugar molecules (N-glycans) are attached at a very specific sequence to the nitrogen atom of the amino acid asparagine [154]. The

attached glycan is usually further processed and further specifically modified depending on the protein. Common expression hosts such as *Escherichia coli* or *Lactococcus lactis* are not able to glycosylate proteins [155], whereas eukaryotic expression platforms including yeast or insect cells are able to perform glycosylation, but differ with respect to the glycosylation patterns performed on proteins [140]. However, for membrane proteins that are usually modified, not all predicted post-translational modifications are always crucial for functional expression in a foreign host. This is best illustrated by the example of the adenosine receptor A_{2A}. This GPCR has a potential glycosylation site for N-glycosylation [156] but can be functionally expressed in a host lacking N-type glycosylation [157].

2.2.5 Solubilization

When membrane proteins are extracted from their natural environment, the hydrophobic domains need to be surrounded by a hydrophobic environment replacing the membrane. For membrane protein purification this task is usually accomplished by detergents in a process termed solubilization (**Figure I-4**).

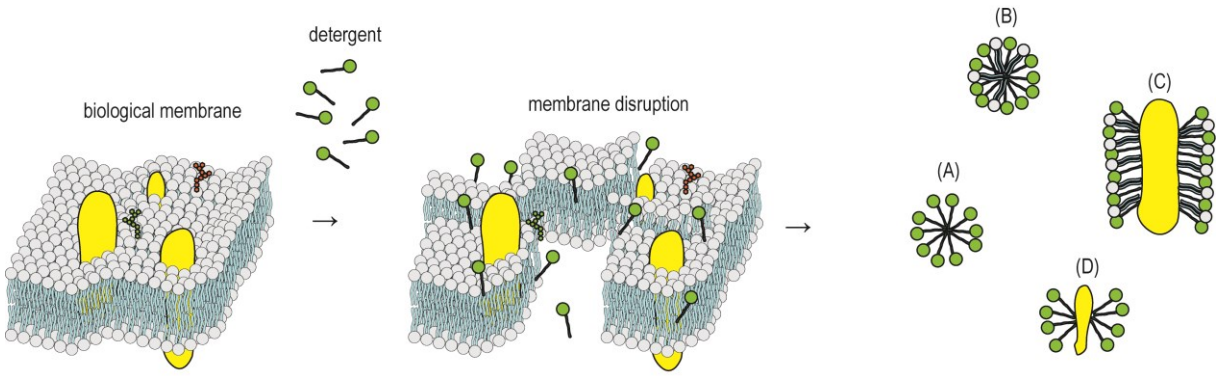


Figure I-4: Cartoon depiction of membrane protein solubilization by detergents
Schematic depiction illustrating various stages of membrane protein solubilization by detergents. Addition of increasing concentration of detergents leads at first to penetration of the membrane, followed by membrane disruption. Further increasing of detergent concentration (above the critical micellar concentration) results in the formation of mixed micelles: micelles comprised of detergents (A), phospholipid - detergent micelles (B), integral membrane protein - phospholipid - detergent micelles (C) and micelles composed of protein - detergent micelles (D). Modified from [158].

Detergents are amphipathic molecules which exhibit a polar head group and a hydrophobic tail, that spontaneously form micellar structures in aqueous solutions [158]. These structures can mimic the

biological membrane in that they are able to incorporate membrane proteins into these micelles. The difficult task of detergents is not restricted to extracting and maintaining the membrane proteins in solution, but to keep the proteins in a functionally active state. As a very diverse class of molecules, detergents are characterized by different biophysical attributes, which are of paramount importance depending on the application. Apart from the solubilization efficiency and the ability to maintain the protein in an active state, certain characteristics of detergents need to be considered for membrane protein purification. One of these characteristics is the critical micelle concentration (cmc). This parameter describes the minimal concentration of detergent above which micelles are formed in aqueous solutions [159]. Since the ability to form micelles is what makes detergents attractive, the concentration of employed detergents in buffers must always be above the cmc, in order to maintain protein-micelle complexes at all times. The size of the micelles is determined by the number of monomers (aggregation number) and the length of the detergent molecules. This information can be important when deciding which protein to solubilize, since small sized micelles may not be suited to solubilize large sized proteins. Moreover, one of the most important practical aspects determining the choice of detergent is the charge of the polar head group [159].

While anionic detergents contain a negatively charged head group, cationic detergents exhibit a positive one. Ionic detergents are typically very efficient at extracting proteins from the membrane, but oftentimes at the cost of denaturing the protein or affecting the protein´s folding state, which is why ionic detergents are often called “harsh”. In contrast, non-ionic detergents are typically referred to as “mild”, since they often maintain the native structure of the protein and are less likely to affect the activity of the protein. Many crystal structures of membrane proteins have been obtained by utilizing non-ionic detergents such as n-Dodecyl β–D-maltoside (DDM) or Octyl-glucoside (OG). However, mild detergents exhibiting a shorter hydrophobic chain length can also deactivate proteins. Another class of detergents, which combines properties of both ionic and non-ionic detergents, is the class of zwitterionic detergents. These molecules are not as “harsh” as ionic detergents, but demonstrate a superior ability over mild detergents with regard to extracting proteins from the membrane [159-161].

Although detergents are applied to mimic the biological membrane, they create a fully artificial set-up that can destabilize membrane proteins and consequently lead to aggregation. Further considerations need to be made depending on the particular downstream processes, since techniques such as crystallography or NMR can have different demands than the purification itself. For this reason, different detergents are typically used at different steps of membrane protein characterization. For instance,

sometimes a protein is solubilized and purified with a harsh detergent but refolded in a mild detergent in order to regain an active form at a later step.
The next section will give a short overview of frequently employed expression hosts for membrane protein production, followed by a detailed presentation of the bacterium *E. coli* as an expression platform.

2.3 Expression hosts

The biotechnological progress with regard to membrane protein (over-) production has resulted in frequently used platform organisms. These include the bacterial strains *E. coli* and *L. lactis*, the yeast strains *Pichia pastoris* and *Saccharomyces cerevisiae*, as well as different insect and mammalian cell lines, respectively [162-165]. Although eukaryotic expression platforms are widely employed, there is a major interest in replacing them with prokaryotic expression systems. The main advantages in utilizing prokaryotic organisms are their genetic accessibility, their cost-efficient culturing, their high biosynthetic capacities, and their straightforward up-scaling as well as their applicability for (ultra)high-throughput approaches [102, 166, 167]. Due to its relevance for this work, special attention shall be given to the prokaryotic expression platform *E. coli* in the following section.

2.3.1 *Escherichia coli*

E. coli is one of the most widely employed prokaryotic hosts for the heterologous expression of membrane proteins [168]. Although many different expression plasmids and new expression strains have been developed in the last decades, one of the most popular *E. coli* strain remains BL21 (DE3). This strain was developed by Studier & Moffatt in the year 1986 [169] and revolutionized the recombinant protein production in bacteria. The strain harbors the chromosomally integrated λ DE3 lysogen, which enables expression of the T7 RNA polymerase (T7 RNAP) under the control of a *lac*UV5 promoter. The expression of the gene encoding the T7 RNAP is repressed by binding of the *lac* repressor LacI to the operator region of the *lac*UV5 promoter and induced by allolactose or its non-hydrolyzable analog IPTG. These compounds are able to bind the LacI, causing an allosteric change of the protein that facilitates the dissociation of the repressor from the promoter. This in turn enables the expression of the T7 RNAP and consequently the transcription of target genes placed under the control of the T7 promoter. The T7 RNAP driven transcription of genes is characterized by a number of advantages: i) very high specificity of the T7 RNAP towards the T7 promoter ii) high processivity iii) no need for external transcription factors iv) transcript termination signals that differ distinctly from that of

bacterial based termination signals [170-173]. These characteristics make the T7 RNAP driven transcription a powerful tool for heterologous expression of genes [174]. Furthermore, to optimize the production of recombinant proteins, the *E. coli* BL21 (DE3) strain is deficient in the proteases lon and OmpT, which together with the high processivity of the T7 RNAP can result in the production of target proteins corresponding to 50 % of total cell protein in *E. coli* [175].

To overcome toxic effects connected to the overproduction of membrane proteins, a screening by Miroux & Walker led to the development of the BL21 (DE3) derivative *E. coli* C43 (DE3) [176]. This mutant host is characterized by a superior ability to cope with toxic effects caused by the production of membrane and soluble proteins [139]. Positive effects observed in this strain are attributed to a reduced transcription of target genes. The reduced transcription of genes is the result of a lower production of the T7 RNAP, which is caused by mutations in the *lac*UV5 promoter region and the lac operator region, respectively [177]. Furthermore, an altered membrane morphology could be observed upon expression of some proteins in this strain. This enlargement of membrane space led to the reduction of inclusion body formation and higher protein yields [152]. Although this effect was not observed for many proteins, it underlines the importance of the membrane space for heterologous production of membrane proteins. Indeed, enhancing the proliferation of the endoplasmic reticulum membrane in the yeast *Yarrowia lipolytica* resulted in higher accumulation of recombinant membrane proteins [178].

Apart from genetically altering expression platforms for the optimized production of membrane proteins, it can be useful to exploit organisms that are naturally characterized by a higher membrane protein biogenesis capacity and a greater membrane space. One of those organisms is the phototrophic Gram-negative bacterium *Rhodobacter capsulatus.*

3 *Rhodobacter capsulatus*

R. capsulatus is a non-sulfur purple bacterium, which belongs to the class of α-proteobacteria and the family of Rhodobacteraceae. The rod-shaped bacterium is usually found in aquatic environments suffused with light, including mud, stagnant or fresh water habitats, but can be found in paddy fields as well as clarification plants [179]. Under specified growth conditions the organism is identified by a purple coloration and an eponymous polysaccharide capsule. The complete genome of *R. capsulatus* encompasses a 3.7 Mb chromosome and a 133 kb plasmid [180]. The organism is highly adaptable to environmental changes owing to a high metabolic versatility [181]. In contrast to many other life forms,

the bacterium can utilize two different sources of energy: light energy (phototrophy) and energy inherent in preformed molecules (chemotrophy) [182, 183]. As a phototrophic organism *R. capsulatus* can harness light energy by anoxygenic photosynthesis, when the oxygen tension is lowered or completely absent [184, 185]. In contrast to the photosynthesis performed in plants, *R. capsulatus* cannot use water as an electron donor. Consequently there is no production of oxygen or reducing equivalents during photosynthesis. However, when oxygen tension is over a particular threshold the organism can perform respiration in the dark to harness energy derived from oxidation of nutrients. In addition to aerobic respiration, *R. capsulatus* can also perform anaerobic respiration and utilize organic (heterotrophy) as well as inorganic sources of carbon (autotrophy) [186, 187]. Furthermore, *R. capsulatus* can utilize a variety of different nitrogen sources, including amino acids, urea, polyamines, ammonium and atmospheric dinitrogen (N_2) by nitrogen fixation [188].

3.1 Nitrogen fixation

Nitrogen fixation is a key process of the global nitrogen cycle and can be performed only by diazotrophic bacteria and archaea. The biochemical reaction to fix dinitrogen is catalyzed by metalloenzymes identified as nitrogenases [189]. *R. capsulatus* exhibits two nitrogenase complexes that differ with respect to the bound iron-sulfur co-factor: the molybdenum dependent nitrogenase (Mo-nitrogenase), encoded by the *nif*-genes (nitrogen fixation), and the iron-only nitrogenase (Fe-nitrogenase), encoded by the *anf*-genes (alternative nitrogen fixation) [190, 191]. The Mo-nitrogenase complex is encoded by the *nifHDK* operon [192], and composed of two dissociable metalloproteins: the dinitrogenase reductase (NifH) and the dinitrogenase (NifDK). The dinitrogenase is the component of the enzyme complex that catalyzes the actual reduction of N_2 to ammonia, while the dinitrogenase reductase serves as a physiological electron donor [193].

As can be deduced from the reaction equation: $N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \rightarrow 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i$, reduction of dinitrogen is a highly ATP demanding process, which is tightly coupled to the anoxygenic photosynthesis of the organism [194]. The expression and activity of the enzyme complex is strictly regulated with regard to molybdenum, light, oxygen and ammonium [188]. Of particular importance is the ammonium control of the nitrogenase expression, which encompasses three different levels of regulation, illustrated in **Figure I-5**.

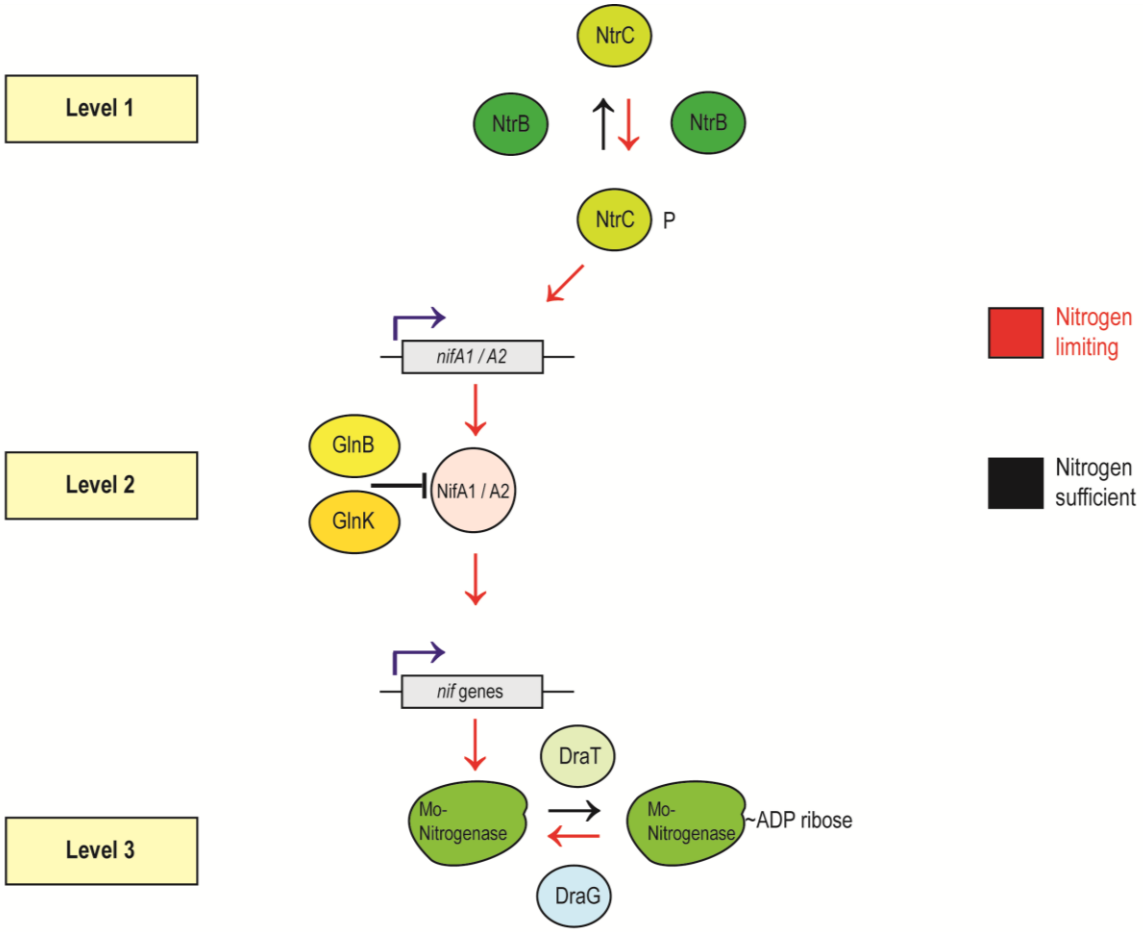


Figure I-5 Ammonium control of nitrogenase expression and activity in *R. capsulatus*
As a diazotrophic organism *R. capsulatus* is capable of reducing N_2 to ammonium by employing a conventional Mo-nitrogenase (encoded by *nif*-genes) or an alternative Fe-nitrogenase (encoded by *anf*-genes). Here depicted is the regulatory cascade controlling the expression and activity of the Mo-nitrogenase at three levels with respect to nitrogen-limiting conditions. At the first level, the bacterium senses the fixed nitrogen status and according to the presence (black arrows) or absence (red arrows) of ammonium activates or prevents the transcription of the transcription regulator NifA1 / NifA2. At the second level, the activtiy of NifA is affected indirectly by the nitrogen status through GlnB and GlnK. At the last level, nitrogenase activtiy is influenced by the presence or absence of ammonium through the DraT/DraG system. Modified from Masepohl *et al*. [188]. Further details are described in the text.

At the first level of regulation, the "fixed" nitrogen status is determined by a Nitrogen regulation (Ntr) system composed of the PII-like signal transduction proteins GlnB /GlnK and the two-component system NtrB and NtrC. Since ammonium (NH_4^+) is the preferred fixed nitrogen source for the organism, expression of either nitrogenase is inhibited by NH_4^+. However, the Ntr system does not sense NH_4^+ directly, but rather the intracellular ratio of metabolites glutamine / 2-ketoglutarate. These amino acids are indicative of the nitrogen assimilation status of the organism, because NH_4^+ is ultimately incorporated through enzymatic reactions, catalyzed by the glutamine synthetase (GS) and glutamate

synthase (GOGAT), into amino acids [195]. Under ammonium-depleting conditions, the sensor kinase NtrB phosphorylates the response regulator NtrC, NtrC~P in turn activates the transcription of the *nifA1*, *nifA2* and *anfA* genes, respectively [196]. Since the Mo-nitrogenase takes precedence over the Fe-nitrogenase, even traces of molybdenum lead to the repression of *anfA* transcription by the repressor protein MopA, irrespective of NtrC~P [197].

The principal transcription activator of *nif*-genes in *R. capsulatus* is the NifA protein. This protein is present in two copies (NifA1 and NifA2) that share a high sequence homology and can functionally substitute for each other [198]. The NifA proteins, in concert with an RNA polymerase containing the alternative sigma factor RpoN, activate the transcription of all *nif*-operons by binding to a specific gene region identified as the upstream activator sequence (UAS) [199]. The second level of regulation is concerned with the activity of the NifA protein itself, which can be modulated posttranslationally in response to changes in the nitrogen status (NH_4^+ inhibition) by the proteins GlnK and GlnB [200]. So, while the first and second level of regulation affect directly or indirectly the transcription of the *nif* and *anf* genes, the third level of regulation is concerned with the posttranslational control of the nitrogenase activity. In the presence of NH_4^+, DraT (dinitrogenase reductase ADP-ribosyl transferase) reversibly ADP-ribosylates the dinitrogenase reductase component of the nitrogenase, resulting in the inactivation of the enzyme. However, with respect to the nitrogen status the activity of the nitrogenase can be restored by removal of the added ribose through the enzyme DraG (dinitrogenase reductase activating glycohydrolase) [201].

R. capsulatus is characterized by a diverse physiology that makes it an interesting microbial host for the production of biotechnologically relevant proteins and metabolites. The following section will discuss the potential relevance of *R. capsulatus* as a microbial platform for biotechnology.

3.2 Relevance for biotechnology

R. capsulatus is known to produce high levels of polyhydroxyalkanoates (PHAs) under specific growth conditions (suitable carbon source and nutrient limiting conditions) [202]. A prominent representative of this polymer type is poly-3-hydroxybutyrate (PHB), which is of particular interest for the plastic industry. Compared to commercial plastics derived from synthetics, plastic produced from PHB has the advantage of being derived from renewable sources and being biodegradable [203, 204]. In addition to bioplastic, *R. capsulatus* is also able to produce molecular hydrogen under photoheterotrophic conditions [205]. Considering the huge demand for hydrogen in our society, and that nearly all of it is

derived from fossil fuels, producing hydrogen in microorganisms such as *R. capsulatus* holds an attractive alternative pathway for cost-efficient production of biofuels with renewable sources [206].

Apart from employing *R. capsulatus* as a microbial host for producing biofuel or bioplastic, *R. capsulatus* has in recent years been explored as an expression platform for production of recombinant proteins and secondary metabolites. Examples include: cytochromes, cellulases, hydrogenases but also secondary metabolites such as carotenoids [207-210]. Heterologous production of carotenoids can be hampered by the supply of precursor molecules in the foreign expression host [211]. As a phototrophic bacterium, *R. capsulatus* produces carotenoids and terpenoids naturally [212], consequently precursor molecules are present in abundance in this host or can be metabolically engineered to accumulate within the cell at high quantities. A work by Loeschcke *et al.* [210] could illustrate that *R. capsulatus* can indeed be utilized for the heterologous production of carotenoids. By implementing the biosynthetic pathway for the carotenoid zeaxanthin from *Pantoea ananatis,* Loeschcke *et al.* could achieve yields of 4 mg/DCW in *R. capsulatus*. However, this endeavor was only possible by introducing a new molecular biological tool (TREX) for the efficient expression of gene clusters encoding whole biosynthetic pathways.

Following, an introduction into characteristics of the expression vector series utilized for the heterologous expression of genes in *R. capsulatus* and *E.coli* are discussed.

3.3 Expression tools - pRho expression vector series

As discussed in the previous section choosing the appropriate expression plasmid can be critical for the success of producing recombinant proteins, before selecting a respective expression plasmid one needs to ensure that expression plasmids are available for the organism employed as an expression platform. Owing to their properties, plasmids of the pRho series [213] have been employed routinely for expression of genes in the hosts *R. capsulatus* and *E. coli* in this work. By now, the pRho vector series numbers a set of vectors with different promoters facilitating different mechanisms of target gene expression. Due to their relevance for this work two representative examples of pRho vectors will be discussed in detail.

As illustrated in **Figure I-6**, pRho vectors are based on the vector pBBR22b, which is derived from the vectors pBBR1MCS [214] and pET22b (Novagen). A broad-host range replicon of the plasmid facilitates autonomous replication not only in *R. capsulatus* but also in other Gram-negative bacteria such as

E. coli or *Pseudomonas putida*. A MOB site allows the conjugational transfer of pRho vectors from donor strains into acceptor strains such as *R. capsulatus*. Two antibiotic resistance genes conferring resistance against kanamycin and chloramphenicol or spectinomycin enable plasmid maintenance by cultivating transformed cells in selective medium. Furthermore, detection as well as purification of proteins is facilitated by either a hexahistidine-tag (His_6-tag) or a decahistidine- tag (His_{10}-tag) encoding region.

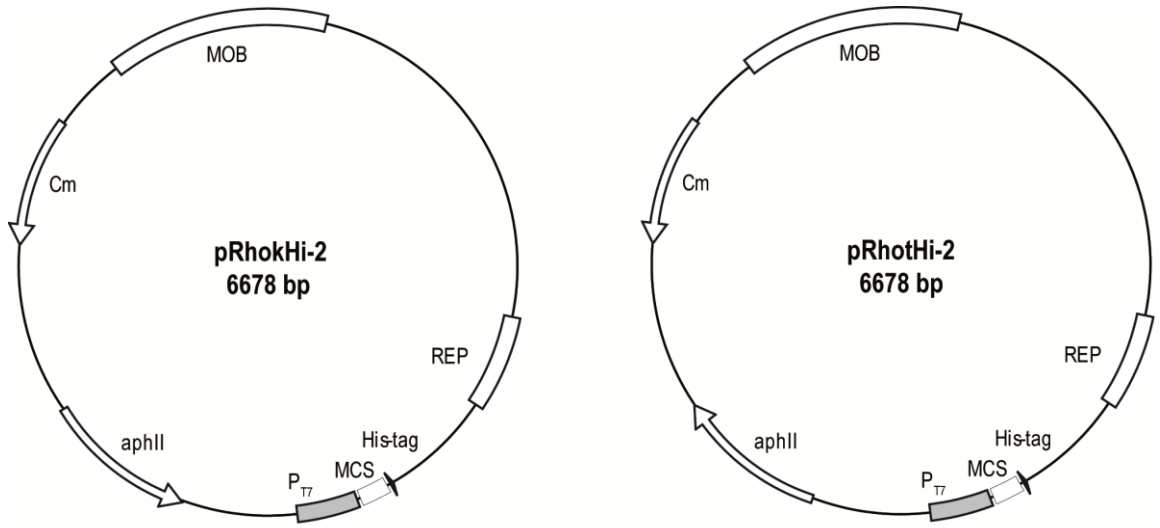


Figure I-6: Characteristics of the pRho expression vector series
Depicted are two representative examples of pRho expression vectors. The pRho vectors are characterized by two antibiotic resistance genes (either chloramphenicol (Cm) or spectinomycin and kanamycin), a broad host range origin of replication (REP) and an origin of transfer (MOB) for conjugational transfer. Target genes can be integrated into the multiple cloning site (mcs) and fused to a hexahistidine-tag (His_6-tag) encoding sequence, allowing affinity purification and immunological detection of the target protein. While the pRhokHi-2 vector mediates a constitutive gene expression based on the promoter of the *aphII* gene, the pRhotHi-2 vector facilitates an inducible expression of target genes by T7 RNA polymerase dependent expression. Further details are found in the text. P_{T7} = T7 promoter. Source: Katzke *et al.* [213]

Target gene expression can be regulated by two different mechanisms based on either the *aphII* promoter (pRhokHi-2) or the T7 promoter (pRhotHi-2). While the pRhokHi-2 vector mediates constitutive expression of target genes, the T7 promoter facilitates inducible expression of genes by T7 RNAP. In *R. capsulatus*, the pRhotHi-2 vector is used in the mutant strain B10S-T7, which carries a chromosomally integrated copy of the T7 RNAP gene under the control of a host specific fructose promoter (P_{fru}) [213]. While addition of fructose in the culture medium induces target gene expression in the mutant strain *R. capsulatus* B10S-T7, protein production in *E. coli* strains BL21 (DE3) or C43 (DE3) can be induced by addition of IPTG or Allolactose as described before (**I.2.2.1**).

The special membrane physiology of *R. capsulatus* makes it a potential platform organism for the production of membrane proteins. The following section discusses physiological characteristics of *R. capsulatus,* which may prove beneficial for the expression of membrane proteins.

3.4 Physiological characteristics relevant for heterologous expression of membrane proteins

Like other bacteria of the genus *Rhodobacter, R. capsulatus* is characterized by a specialized membrane system. In order to harness sunlight efficiently for photosynthesis, the organism enlarges its membrane surface by undergoing a morphological transformation of the cytoplasmic membrane [215-217]. This leads to distinct membrane morphologies depending on the deployed energy metabolism, as depicted in **Figure I-7**.

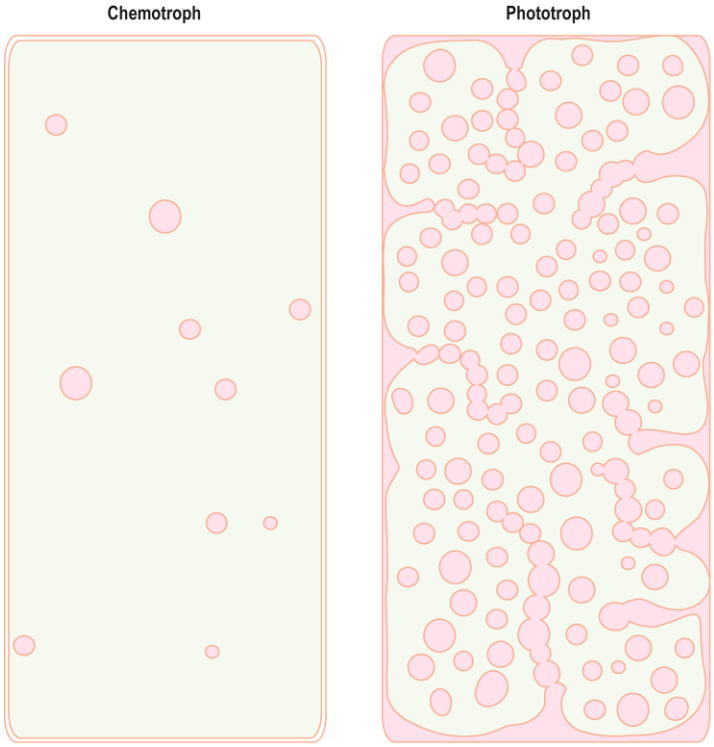


Figure I-7: Cartoon depiction of the special *R. capsulatus* membrane morphology
R. capsulatus can utilize two different sources of energy: light energy (phototrophy) and energy inherent in preformed molecules (chemotrophy). With respect to the utilized energy source, the bacterium is characterized by distinctly different membrane morphologies. Under phototrophic growth conditions the cytoplasmic membrane of the organism forms invaginations, which lead to continuous membrane structures as well as fully detached vesicles that contain proteins and pigments needed to convert light energy into chemical energy (ATP). With regard to environmental factors such as light or oxygen, a cell can harbor up to 1150 vesicles under phototrophic conditions.

Lowering oxygen tension or light intensity induces the formation of continuous invaginations of the cytoplasmic membrane as well as fully detached vesicles, both of which are identified as intracytoplasmic membranes (ICM) [218]. The ICM houses integral membrane proteins and pigment-complexes needed to convert light energy into chemical energy (ATP), which renders it distinct from the cytoplasmic membrane that lacks these compounds [219]. In response to changes in light condition and oxygen tension, the ICM can result in the enlargement of the membrane surface by a factor of three to nine, while a single cell can contain up to 1150 vesicles [215]. These vesicles exhibit a periplasmic lumen (inside-out) and a protein composition sufficient to conduct photophosphorylation autonomously. This self-governed quality of ICM vesicles resembles that of eukaryotic organelles and like those the vesicles can be extracted from the cells by sucrose-density gradient centrifugation. Isolated vesicles can be biochemically characterized and may therefore form an ideal basis for the study of membrane protein receptors or protein-protein interactions [220, 221]. Considering that under defined culture conditions the whole cell can be densely packed with ICM structures, it seems only natural to assume that the bacterium must be equipped with enough membrane protein biogenesis factors to translocate membrane proteins in such quantities. Indeed, in the related organism *Rhodobacter sphaeroides*, proteome analysis of ICM vesicles revealed the membrane to be particularly enriched in membrane assembly factors, including translocases YidC, YajC and SecY and the chaperonin GroEL, as well as proteins of unknown function [222-224]. This quality can be of paramount importance for the “over”-production of membrane proteins.

Apart from the membrane space and the membrane protein biogenesis factors, *R. capsulatus* is characterized by a special lipid composition. In addition to lipids found commonly in bacteria such as phosphatidylethanolamine, cardiolipin or phosphatidylglycerol, the lipid phosphatidylcholine is produced in this organism as well [225]. Phosphatidylcholine can be found commonly in eukaryotic organisms [226] but rarely in bacteria [227]. Considering the elucidated function of the lipid environment for the proper folding and activity of membrane proteins (**I.2.1.3**), this quality of *R. capsulatus* could prove pivotal for the expression of membrane proteins originating from eukaryotic organisms. Another remarkable characteristic of *R. capsulatus* membranes is the observation that it exhibits non-pyrogenic LPS compared to common expression host such as *E. coli* [228]. This feature makes *R. capsulatus* a potential candidate for producing therapeutic proteins.

Another physiological characteristic of *R. capsulatus* which may be relevant for the expression of membrane proteins is the ability to produce several cofactors, including molybdenum cofactors, FAD, iron-sulfur clusters and electron donors such as ferredoxins [229-233]. The importance of these

cofactors on heterologous expression of cofactor dependent proteins could be shown for several c-type cytochromes, which failed to be functionally expressed in *E. coli* but could be successfully produced in *R. capsulatus* [234, 235].

4 Aim of the thesis

Membrane proteins are amongst the most important class of proteins in living cells and represent one of the most important classes of drug targets. Obtaining sufficient amounts of membrane proteins for structural and functional studies remains a challenging task, because common expression hosts are not suited to functionally produce these proteins in high quantities. For this reason, novel expression strategies that exploit alternative organisms need to be developed. The photosynthetic bacterium *R. capsulatus* is characterized by a specialized membrane physiology that may prove beneficial for the expression of membrane proteins. Therefore, the aim of this study will be to evaluate *R. capsulatus* for the heterologous expression of therapeutically relevant membrane proteins based on a new expression plasmid. To this end, the following objectives will be processed:

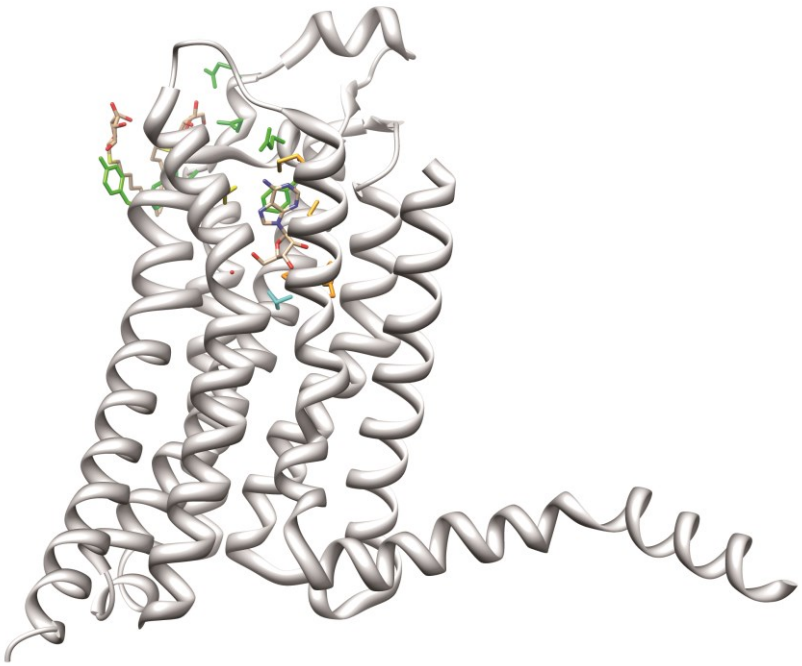
i) In order to evaluate the organism for the heterologous expression of membrane proteins, a new expression plasmid, considerate of the special needs of membrane protein production, will be constructed. This plasmid will be based on the *R. capsulatus nifHDK* promoter and evaluated with regard to its modulation of gene expression and its promoter strength by expression studies employing the reporter protein YFP. The new expression vector will be further characterized with respect to the employed *R. capsulatus* strain and the utilized culture vessels

ii) A diverse set of predominantly human membrane proteins will be comparatively expressed in bacterial strains *E. coli* and *R. capsulatus*. *R. capsulatus* will be evaluated for its ability to insert therapeutically relevant membrane proteins into the membrane, investigating the influence of the following parameters: i) promoter ii) growth phase iii) *R. capsulatus* strain. These results shall give insights to conditions relevant for the optimal accumulation of membrane proteins in *R. capsulatus*.

iii) In the third section of this work *R. capsulatus* driven accumulation of therapeutically relevant membrane proteins will be further optimized. For this, the impact of light condition, the medium composition as well as the codon usage bias as factors affecting membrane insertion will be analyzed.

iv) The last part of the thesis will be concerned with the development of protocols for the purification of integral membrane proteins produced in *R. capsulatus*. A detergent screening shall reveal suitable detergents for extracting membrane proteins from *R. capsulatus*. Based on this information, the applicability of the new expression platform for the production of correctly folded proteins will be surveyed, using the example of the proton pump bacteriorhodopsin from *Halobacterium salinarum*.

CHAPTER II: MATERIAL & METHODS



Thermostabilised human A2A Receptor with Adenosine bound (PDB file 2YDO)

1 Chemicals, antibiotics and enzymes

Unless stated otherwise, all chemicals and antibiotics were purchased from the companies Anatrace, Roth, Invitrogen, Sigma, Serva, Gerbu Biotechnik GmbH, Fluka, Merck, Calbiochem, Gibco BRL and Biomol. With the exception of a homemade Pfu polymerase, all enzymes used in this work were obtained from Fermentas.

2 Bacterial strains

Table II-1: *Escherichia coli* strains used in this work

Bacterial strain	Genotype	Reference
DH5α	*F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 deoR recA1 endA1hsdR17 (rk-, mk+) phoA supE44λ- thi-1 gyrA96 relA1*	[236]
S17-I	*Ec294::[RP4(Tc::Mu)(Km::Tn7)], pro, res, recA, Tp^R, Sm^R*	[237]
BL21 (DE3)	*F– ompT gal dcm lon hsdSB(rB- mB-)λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*	[169]
C43 (DE3)	BL21 (DE3) derivative with mutations in the *lacUV* promoter	[176]

Table II-2: *Rhodobacter capsulatus* strains used in this work

Bacterial strain	Genotype	Reference
B10S	Spontaneous *Rhodobacter capsulatus* B10 mutant strain which exhibits a resistance to streptomycine	[238]
B10S-T7	*R. capsulatus* B10S *recA*:: (P_{Fru}>*T7Pol*Gm^R)	[213]
TD22	*R. capsulatus* deletion mutant strain *nifHDK*::Gm^R	[239]

3 Expression vectors

Table II-3: Expression vectors used in this work

Vector	Relevant features	Reference
pRhotHi-2	pBBR1mcs (rep mob Cm^R), pET22b (MCS, *pelB*), pBSL15 (*aphII*) orientation II, P_{T7}	[213]
pRhon$_5$Hi-2	pBBR1MCS (rep mob Cm^r), pET22b (MCS), pBS215 (aphII) orientation II, P_{nifH5}	This work

pBlueScriptKS	Ampr, *lacZα*	Stratagene
pDONR223-AGT1	Gateway®-adapted donor vector containing human *AGT1*	Protagen AG
pDONR223-ADA2A	Gateway®-adapted donor vector containing human *ADA2A*	Protagen AG
pDONR223-AQP4	Gateway®-adapted donor vector containing human *AQP4*	Protagen AG
pDONR223-CHRM3	Gateway®-adapted donor vector containing human *CHRM3*	Protagen AG
pDONR223-CXCR4	Gateway®-adapted donor vector containing human *CXCR4*	Protagen AG
pDONR223-MAG	Gateway®-adapted donor vector containing human *MAG*	Protagen AG
pDONR223-MOG	Gateway®-adapted donor vector containing human *MOG*	Protagen AG
pDONR223-SLC30A8	Gateway®-adapted donor vector containing human *SLC30A8*	Protagen AG
pEX-A-VpU_Rhodi	pEX-A derivative carrying a *Rhodobacter capsulatus* codon optimized variant of the *VpU* gene	Eurofins mwg operon
pUC57-At1AR_R.c.	pUC57 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of the *AGT1* gene	Euorfins mwg operon
pUC57-CXCR4_R.c.	pUC57 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of the *CXCR4* gene	Euorfins mwg operon
pUC57-A2AR_R.c.	pUC57 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of the *A2AR* gene	Euorfins mwg operon
pUC57-CHRM3_R.c.	pUC57 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of the *CHRM3* gene	Euorfins mwg operon
pRhotHi-bop	pRhotHi-2 derivative containing *bop* gene (*Nde*I /*Xho*I)	[240]
pRhotHi-YFP	pRhotHi-derivative containing the *EYFP* gene	[241]
$pRhon_1Hi$-YFP	$pRhon_1Hi$- derivative containing the *EYFP* gene	[242]
$pRhon_2Hi$-YFP	$pRhon_2Hi$- derivative containing the *EYFP* gene	[240]
$pRhon_3Hi$-YFP	$pRhon_3Hi$- derivative containing the *EYFP* gene	[240]
$pRhon_4Hi$-YFP	$pRhon_4Hi$- derivative containing the *EYFP* gene	[240]
pBlueScript-ADA2A	pBlueScript derivative containing *ADA2A* 1245 bp PCR product (*Sma*I)	This work
pBlueScript-AGT1	pBlueScript derivative containing *AGT1* 1088 bp PCR product (*Sma*I)	This work
pBlueScript-AQP4	pBlueScript derivative containing *AQP4* 978 bp PCR product (*Sma*I)	This work
pBlueScript-CHRM3	pBlueScript derivative containing *CHRM3* 1779 bp PCR product (*Sma*I)	This work
pBlueScript-CXCR4	pBlueScript derivative containing *CXCR4* 1065 bp PCR product (*Sma*I)	This work
pBlueScript-MAG	pBlueScript derivative containing *MAG* 1889 bp PCR product (*Sma*I)	This work
pBlueScript-MOG	pBlueScript derivative containing *MOG* 896 bp PCR product (*Sma*I)	This work
pBlueScript-SLC30A8	pBlueScript derivative containing *SLC30A8* 971 bp PCR product (*Sma*I)	This work
pRhotHi-ADA2A	pRhotHi-2 derivative containing *ADA2A* from pBlueScript-ADA2A (*Nde*I /*Xho*I)	This work
pRhotHi -AGT1	pRhotHi-2 derivative containing *AGT1* from pBlueScript-AGT1 (*Nde*I /*Xho*I)	This work
pRhotHi -AQP4	pRhotHi-2 derivative containing *AQP4* from pBlueScript-AQP4 (*Nde*I /*Xho*I)	This work
pRhotHi -CHRM3	pRhotHi-2 derivative containing *CHRM3* from pBlueScript-CHRM3 (*Nde*I /*Sal*I)	This work
pRhotHi -CXCR4	pRhotHi-2 derivative containing *CXCR4* from pBlueScript-CXCR4 (*Nde*I /*Xho*I)	This work
pRhotHi -MAG	pRhotHi-2 derivative containing *MAG* from pBlueScript-MAG (*Nde*I /*Xho*I)	This work
pRhotHi -MOG	pRhotHi-2 derivative containing *MOG* from pBlueScript-MOG (*Nde*I /*Xho*I)	This work
pRhotHi -SLC30A8	pRhotHi-2 derivative containing *SLC30A8* from pBlueScript-SLC30A8 (*Nde*I /*Xho*I)	This work
pRhotHi-VpU	pRhotHi-2 derivative containing *VpU* from pEX-A-VPU_Rhodi (*Nde*I /*Xho*I)	This work

pRhon$_5$Hi-ADA2A	pRhon$_5$Hi-2 derivative containing *ADA2A* from pRhotHi-ADA2A (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi -AGT1	pRhon$_5$Hi-2 derivative containing *AGT1* from pRhotHi-AGT1 (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi -AQP4	pRhon$_5$Hi-2 derivative containing *AQP4* from pRhotHi-AQP4 (*Nde*I /*Xho*I)	[243]
pRhon$_5$Hi -CHRM3	pRhon$_5$Hi-2 derivative containing *CHRM3* from pRhotHi-CHRM3 (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi -CXCR4	pRhon$_5$Hi-2 derivative containing *CXCR4* from pRhotHi-CXCR4 (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi -MAG	pRhon$_5$Hi-2 derivative containing *MAG* from pRhotHi-MAG (*Nde*I /*Xho*I)	[243]
pRhon$_5$Hi -MOG	pRhon$_5$Hi-2 derivative containing *MOG* from pRhotHi-MOG (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi -SLC30A8	pRhon$_5$Hi-2 derivative containing *SLC30A8* from pRhotHi-SLC30A8 (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi-VpU	pRhon$_5$Hi-2 derivative containing *VpU* from pRhotHi-VpU (*Nde*I /*Xho*I)	This work
pRhon$_5$Hi-YFP	pRhon$_5$Hi- derivative containing the *EYFP* gene	This work
pRhon$_5$Hi-YFP-His	pRhon$_5$Hi- derivative encoding the EYFP fused to a C-terminal His$_6$-tag	This work
pRhotHi-YFP-His	pRhotHi- derivative encoding the EYFP fused to a C-terminal His$_6$-tag	[213]
pRhon$_5$-At1AR-Co	pRhon$_5$Hi-2 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of *AGT1* (*Nde*I /*Xho*I)	This work
pRhon$_5$-CXCR4-Co	pRhon$_5$Hi-2 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of *CXCR4* (*Nde*I /*Xho*I)	This work
pRhon$_5$-A2AR-Co	pRhon$_5$Hi-2 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of *ADA2A* (*Nde*I /*Xho*I)	This work
pRhon$_5$-CHRM3-Co	pRhon$_5$Hi-2 derivative carrying a *Rhodobacter capsulatus* codon optimized variant of *CHRM3* (*Nde*I /*Xho*I)	This work

4 Primers

All primers were purchased in a lyophilized state from MWG Biotech AG and dissolved in a volume of nuclease free water to achieve a concentration of 10 pmol/µl. Restriction recognition sites added to the gene of interest are underlined.

Table II-4: Oligonucleotides used in this work

Name	Sequence (5´→ 3´)	Length (bp)	Application
AO409_ADA2A_FW	CATATGCCCATCATGGGCTCCTCGGT GTACATC	33	Amplification of the *ADA2A* gene by PCR; forward primer
AO409_ADA2A_RV	CTCGAGGGACACTCCTGCTCCATCCT GG	28	Amplification of the *ADA2A* gene by PCR; reverse primer
AO409_AGT_FW	GCCATATGATTCTCAACTCTTCTACTG AAGATGG	34	Amplification of the *AGT1* gene by PCR; forward primer
AO409_AGT_RV	CTCGAGCTCAACCTCAAAACATGGTG C	27	Amplification of the *AGT1* gene by PCR; reverse primer

AO409_AQP4_FW	CATATGAGTGACAGACCCACAGCAAGGC	28	Amplification of the *AQP4* gene by PCR; forward primer
AO409_AQP4_RV	CTCGAGTACTGAAGACAATACCTCTCCAGATTGG	34	Amplification of the *AQP4* gene by PCR; reverse primer
AO409_CHRM3_FW	GCCATATGACCTTGCACAATAACAGTACAACCTCG	35	Amplification of the *CHRM3* gene by PCR; forward primer
AO409_CHRM3_RV	GTCGACCAAGGCCTGCTCGGGTG	23	Amplification of the *CHRM3* gene by PCR; reverse primer
AO409_CXCR4_FW	CATATGGAGGGGATCAGTATATACACTTCAG	31	Amplification of the *CXCR4* gene by PCR; forward primer
AO409_CXCR4_RV	CTCGAGGCTGGAGTGAAAACTTGAAGACTC	30	Amplification of the *CXCR4* gene by PCR; reverse primer
AO409_MOG_FW	GCCATATGGCAAGCTTATCGAGACCCTCTCTG	32	Amplification of the *MOG* gene by PCR; forward primer
AO409_MOG_RV	CTCGAGCCTCCCAGGAGGAGTCTTCCCT	28	Amplification of the *MOG* gene by PCR; reverse primer
AO409_MAG_FW	GCCATATGATATTCCTCACGGCACTGCC	28	Amplification of the *MAG* gene by PCR; forward primer
AO409_MAG_RV	CTCGAGCTTGACCCGGATTTCAGCAT	26	Amplification of the *MAG* gene by PCR; reverse primer
AO409_SLC30A8_FW	GCCATATGTACCACTGCCACAGTGGCTCC	29	Amplification of the *SLC30A8* gene by PCR; forward primer
AO409_SLC30A8_RV	CTCGAGGTCACAGGGGTCTTCACAGAAAAG	30	Amplification of the *SLC30A8* by PCR; reverse primer
Pnif-fw	AATCGCTAGCTCCCGACAGAGGG	23	Amplification of the *nifHDK*-promoter region for constructing the pRhon$_5$Hi-2 vector; forward primer
Pnif-rv	CGATTCTAGACGGCCAGGTGCA	22	Amplification of the *nifHDK*-promoter region for constructing the pRhon$_5$Hi-2 vector; reverse primer

5 Culture media and supplements

Culture media used in this work were sterilized by autoclaving (20 min at 121 °C and 2 bar). Heat-sensitive components such as antibiotics were passed through a syringe filter unit (0.2 µm) and added to lukewarm medium. Medium components for PY and RCV minimal medium were autoclaved separately and combined afterwards.

Table II-5: Components used for cultivation media

Cultivation medium	Component	Amount
	E. coli	
LB-medium	Tryptone /peptone	10 g
	NaCl	5 g
	Yeast extract	5 g
	Deionized water	ad 1000 ml
LB Agar	Agar-agar Kobe-I	15 g
	LB medium	ad 1000 ml
	Rhodobacter capsulatus	
PY Agar	Bacto Peptone	10 g
	Yeast extract	0.5 g
	Agar	20 g
	Deionized water	ad 1000 ml
Sterilized separately and added afterwards	1 M $MgCl_2$	2 ml
	1 M $CaCl_2$	2 ml
	0.5 % $FeSO_4$	2.4 ml
PY-Fe medium	Bacto Peptone	10 g
	Yeast extract	0.5 g
	Deionized water	ad 1000 ml
Sterilized separately and added afterwards	1 M $MgCl_2$	2 ml
	1 M $CaCl_2$	2 ml
RCV minimal medium	10 % DL-malic acid	40 ml
	1 % EDTA	2 ml
	20 % $MgSO_4$	1 ml
	Trace element solution	1 ml
	7.5 % $CaCl_2$	1 ml
	0.5 % $FeSO_4$	2.4 ml
	0.1 % Thiamine	1 ml
	Phosphate buffer	9.6 ml
	Deionized water	ad 1000 ml
Sterilized separately and added afterwards	10 % $(NH_4)_2SO_4$ (RCV + N)	
	or	10 ml
	10 % serine solution (RCV + S)	
RCV + BR	15 mM *all-trans* retinal (Ethanol)	1 ml

	RCV + S (pH 6)			ad	1000 ml
	RCV medium supplements				
0.5 % $FeSO_4$ solution	$FeSO_4$	1	g		
	32 % HCl solution	1	ml		
	Deionized water			ad	200 ml
Phosphate buffer	KH_2PO_4	81.30	g		
pH 6.8	K_2HPO_4	78.70	g		
				ad	500 ml
Trace element solution	$MnSO_4$ * 1 H_2O	0.4	g		
	H_3BO_3	0.7	g		
	Cu $(NO_3)_2$ * 3 H_2O	0.01	g		
	$ZnSO_4$ * 7 H_2O	0.06	g		
	Na_2MoO_4 * 2 H_2O	0.02	g		
	Deionized water			ad	250 ml

Table II-6: Antibiotics used in this work

Antibiotic	Stock solution [mg/ml]	Final concentration [µg/ml] *Escherichia coli*	Final concentration [µg/ml] *Rhodobacter capsulatus*
Ampicillin	100	100	-
Gentamycin	10	10	4
Kanamycin	100	50	25
Streptomycin	200	-	200
Tetracycline	10	10	0.25

Table II-7: Antibodies used in this work

Antigen	Antibody (AB)	Conjugate	Requires Secondary AB	Dilution
His-tag	Polyclonal Anti His-HRP (Roth)	HRP	No	1 : 10 000
GFP	Living Colors GFP Monoclonal (Clontech)	-	Yes : Anti-Rabbit	1 : 20 000
NifH	Polyclonal [200]	-	Yes : Anti-Rabbit	1 : 50 000
Rabbit IgG	Secondary Antibody (Bio-Rad)	HRP	No	1 : 10 000

6 Buffer and solutions

Buffers were adjusted to a particular pH using solutions of hydrochloric acid (32 %) or sodium hydroxide (10 M), respectively.

Table II-8: Buffer and solutions used in this work

Buffer / solution	Component	Content	
	Competent cells		
$MgCl_2$ solution	$MgCl_2$	100	mM
$CaCl_2$ solution	$CaCl_2$	100	mM
	Glycerol	15	% (w/v)
	Polymerase chain reaction (PCR)		
Pfu buffer (10 x)	Tris (pH 8,8)	200	mM
	KCl	100	mM
	$(NH_4)_2SO_4$	60	mM
	$MgSO_4$	20	mM
	Triton x-100	1	% (v/v)
	BSA	1	mg/ml
	Agarose gel electrophoresis		
DNA sample buffer (5x)	EDTA	100	mM
	Glycerol	43	% (v/v)
	Bromphenol blue	0,05	% (w/v)
TBE buffer (5x)	Tris	89	mM
pH 8.3	Boric acid	89	mM
	EDTA	2,5	mM
	Plasmid preparation		
Solution I	Glucose	50	mM
pH 8	Tris	25	mM
	EDTA	10	mM
Solution II	NaOH	200	mM
	SDS	1	%
Solution III	Potassium acetate	5	M
	Formic acid	14	% (v/v)
RNase solution	RNase	20	µg/ml
pH 8	Tris	10	mM
	EDTA	1	mM

Cell disruption			
SP - buffer pH 7.2	K_2HPO_4	40	mM
	KH_2PO_4	22	mM
	NaCl	150	mM
	Complete Protease Inhibitor Cocktail (EDTA-free)	1	tablet
SDS-PAGE			
SDS-PAGE sample buffer	Tris	50	mM
	Glycerol	10	% (v/v)
	SDS	4	% (w/v)
	2-mercaptoethanol	0.03	% (w/v)
SDS-PAGE running buffer (10x) pH 8.8	Tris	25	mM
	Glycine	192	mM
	SDS	0.1	% (w/v)
SDS-PAGE separating gel buffer (4 x) pH 8.8	Tris	1.5	M
SDS-PAGE stacking gel buffer (4 x) pH 6.8	Tris	0.5	M
SDS-PAGE separating gel solution (12 %) ; 2 mini-gels	Acrylamide/bisacrylamide	4	ml
	Separating gel buffer (4 x)	2.5	ml
	Deionized water	3.4	ml
	SDS (10 %)	100	µl
	APS (10 %)	100	µl
	TEMED	10	µl
SDS-PAGE stacking gel solution (2 mini-gels)	Acrylamide/bisacrylamide	0.83	ml
	Stacking gel buffer (4 x)	1.3	ml
	Deionized water	2.8	ml
	SDS (10 %)	50	µl
	APS (10 %)	50	µl
	TEMED	10	µl
Western Blot			
PBS buffer (10 x)	NaCl	80	g
	KH_2PO_4	2	g
	NaH_2PO_4	11.1	g
	KCl	2	g
	Deionized water		ad 1000 ml
PBS-T buffer	10 x PBS buffer	100	ml
	Deionized water	900	ml
	Tween 20	0.05	% (v/v)
Blocking solution	Skim milk powder	1.5	g
	PBS-T		ad 50 ml

Transfer buffer (10 x)	Tris	30	g	
	Glycine	112	g	
	Deionized water		ad	1000 ml
Transfer buffer (1 x)	Transfer buffer (10 x)	100	ml	
	Denatured ethanol	100	ml	
	Deionized water		ad	1000 ml
ECL solution A	Luminol	50	mg	
	Tris (100 mM)	500	ml	
ECL solution B	p-coumaric acid	10	mg	
	DMSO	10	ml	
ECL solution C	Hydrogen peroxide	30	%	(w/v)
ECL solution complete	ECL solution A	1	ml	
	ECL solution B	0.1	ml	
	ECL solution C	0.3	µl	
Protein staining				
Coomassie staining solution	Coomassie Brilliant Blue	0.1	%	(w/v)
	Ethanol	42	%	(v/v)
	Acetic acid	16	%	(v/v)
	Deionized water	42	%	(v/v)
Coomassie destaining solution	Ethanol	20	%	(v/v)
	Acetic acid	7	%	(v/v)
	Deionized water	73	%	(v/v)
Amidoblack staining solution	Amidoblack	0.1	%	(w/v)
	Ethanol	45	%	(v/v)
	Acetic acid	10	%	(v/v)
Protein quantification				
Bradford solution	Coomassie Brilliant Blue	20	mg	
	Ethanol (96 %)	25	ml	
	Orthophosphoric acid	50	ml	
	Deionized water	500	ml	
Solubilization				
Solubilization buffer	Tris	20	mM	
pH 8	NaCl	100	mM	
	Glycerol	10	%	(v/v)
	2-mercaptoethanol	4	mM	
	Detergent		**Table II-10**	
	Complete Protease Inhibitor Cocktail (EDTA-free)	1	tablet	

Protein purification				
Wash buffer	Tris	20	mM	
	NaCl	300	mM	
	Glycerol	10	%	(v/v)
	2-mercaptoethanol	4	mM	
	Detergent	**Table II-10**		
	Imidazole	10 – 300	mM	
	Complete Protease Inhibitor Cocktail (EDTA-free)	1	tablet	

7 Equipment and consumable materials

Table II-9: Consumable materials used in this work

Material	Producer
Microbiology Anaerocult A	Merck
Cellulose acetate membrane filter	Schleicher & Schüll
Cellulose acetate syringe filter	Whatman
Ni-NTA Superflow	Qiagen
PVDF membrane	Biorad
NuPAGE®Bis-Tris precast gels	Life technologies

All devices and materials not explicitly listed were conforming to laboratory standards.

8 Software

Clone-Manager 9.0	Scientific & Educational software
AIDA	Raytest
Graphical codon usage analyzer	http://gcua.schoedl.de/
Basic local alignment search tool	http://blast.ncbi.nlm.nih.gov/Blast.cgi
TMHMM Server, v.2.0	http://www.cbs.dtu.dk/services/TMHMM/
Adobe Illustrator CS4	Adobe Systems Incorporated
UCSF Chimera	http://www.cgl.ucsf.edu/chimera/

Techniques described in this section were based on protocols developed in the scope of this work or described by preceding works of Malach [270], Heck [328], Katzke [213] and Pünder [243].

9 Techniques in Microbiology

9.1 Cultivation of bacteria

9.1.1 Cultivation of *E. coli* strains

The *E. coli* strains BL21 (DE3) and C43 (DE3) were employed for T7 RNAP driven expression of genes, while the *E. coli* strain S17-1 was used for bi-parental mating. The strain DH5α was used exclusively for molecular cloning experiments of plasmid DNA. *E. coli* strains were cultivated routinely in LB medium supplemented with a particular antibiotic (**Table II-6**). Depending on the application, cells were grown either on agar plates or in liquid culture for at least 16 h at 37 °C. Liquid cultures were inoculated with a single colony from a freshly streaked agar plate and grown in Erlenmeyer flasks under constant agitation (130 rpm).

9.1.1.1 Heterologous expression of genes using a T7 RNA polymerase based system

E. coli strains BL21 (DE3) and C43 (DE3) were employed for T7 RNAP driven expression of genes [169]. Competent cells were transformed (**II.10.2**) with 50 ng of plasmid DNA and incubated on antibiotic containing agar plates overnight at 37 °C. A single colony from these agar plates was used for inoculating 10 ml LB medium supplemented with antibiotic (preculture) and incubated for at least 16 h at 37°C under agitation (130 rpm). Subsequently, a 500 ml flask filled with 50 ml LB medium and antibiotic was inoculated with preculture to an optical density ($OD_{580\ nm}$) equaling 0.05. When the growing culture reached an $OD_{580\ nm}$ of 0.5 – 0.7, gene expression was induced by adding 0.4 mM IPTG to the medium. Samples were taken at the logarithmic ($OD_{580\ nm}$ ~ 1) and the stationary growth phase (24 h after induction). Cell suspensions were centrifuged for 1 min at 8,000 x g in a table top centrifuge and the obtained cell pellets stored at -20°C until further use.

9.1.2 Cultivation of *Rhodobacter capsulatus* strains [238]

R. capsulatus strain B10S was used for heterologous expression of genes cloned into the vectors pRhokHi-2 and pRhonHi-2, whereas the mutant strains TD22 was employed for P_{nifH} driven expression and the B10S-T7 strain for T7 RNAP driven expression, respectively. *R. capsulatus* strains were grown photoheterotrophically under high light intensity illumination at 30 °C in RCV minimal medium supplemented with different nitrogen sources (1 mM serine, 15 mM NH_4SO_4 or dinitrogen gas) or on PY

agar plates supplemented with antibiotics. Liquid cultures were grown in different gastight vessels. Small scale cultures were cultivated in Hungates (10 ml) and Erlenmeyer flasks (100 ml), whereas large scale cultures were grown in flat panels (600 ml) or Schott-flasks (500 ml). For photoheterotrophic cultivation, these vessels were flushed with argon or dinitrogen to create an oxygen-free atmosphere. Subsequently, these cultures were illuminated by six bulb-lights (60 W light bulb, Osram, Germany) or by 120 high power infrared LED panels (λ_{max} = 856 nm, SFH 4257, Osram, Germany) for 3 - 5 days. To grow *R. capsulatus* cells on PY agar plates, freshly streaked agar plates, together with a gas-pak sachet, were put into air-tight jars. The gas-pak sachet is a disposable carbon dioxide/hydrogen gas-generating system (Anaerocult) that allows for an oxygen-free atmosphere within the container. After sealing the jar the container was illuminated for 2 - 3 days by bulb-light illumination.

9.1.2.1 Heterologous expression of genes using a T7 RNA polymerase based system (pRhotHi-2)

Heterologous expressions of genes under the control of a T7 RNAP were performed in the *R. capsulatus* B10S-T7 strain [213]. This mutant strain carries a chromosomally integrated copy of the T7 RNAP gene under the control of a host specific fructose promoter. The gene of interest was introduced to *R. capsulatus* by conjugation (**II.10.3**) and transformed cells from an agar plate used to start a 10 ml preculture. The medium of the preculture was composed of RCV minimal medium supplemented with NH_4^+ as a nitrogen source and antibiotic for plasmid maintenance, while oxygen free atmosphere was achieved by flushing the vessels with argon. Precultures were grown photoheterotrophically for 2 – 3 days (**II.9.1.2.**). The induction of protein production was realized by adding 8 mM fructose to the medium of the main culture. Subsequently, the main culture (inoculation $OD_{660\ nm}$ ~ 0.05) was cultivated phototrophically and samples were taken at the logarithmic ($OD_{660\ nm}$ ~ 1) and the stationary growth phase (OD ~ 2 - 3) respectively. Cells were centrifuged for 10 min at 16,000 x g, the supernatant discarded and the pellet frozen at -20°C.

9.1.2.2 Heterologous expression of genes under the control of the *nifHDK* promoter (pRhonHi-2 vectors)

With the exception of two deviations, the experimental steps were the exact same as described in the section before. Expression vectors of the pRhonHi-2 series were employed in the bacterial strains B10S and the *nifHDK* deletion mutant TD22. Target gene expression was induced by cultivating expression cultures in RCV minimal medium supplemented with either serine (1 mM) or dinitrogen as the sole source of nitrogen (nitrogen-limiting conditions), while target gene repression was realized by cultivating

expression cultures in minimal medium supplemented with NH_4^+ (nitrogen-sufficient conditions). For auto-induction medium, RCV minimal medium was supplemented with two nitrogen sources at the same time, 1 mM serine and 2.5 mM – 10 mM NH_4^+.

9.2 Cryopreservation of bacteria

For long term storage of *E. coli* cells, 800 µl of logarithmically grown cells were mixed with 200 µl of glycerol and stored at -80 °C. For cryogenic storage of *R. capsulatus*, cell material was scraped off of a freshly streaked agar plate and resuspended in 1.5 ml of RCV minimal medium. Subsequently, this suspension was centrifuged (10 min, 16,000 x g) and the obtained cell pellet resuspended in 1 ml PY-Fe medium. This mixture was then mixed with equal amounts of glycerol and stored at -20 °C for long term storage.

9.3 Optical density measurement of bacteria

The optical density of growing cultures was spectroscopically measured to quantify different culture parameters such as biomass or growth behavior. Typically, the solvent of the sample or water was used as the blank reference for measurement. The wavelength used for measuring the absorption differed with regard to the deployed strain. The optical density of *E. coli* cells was measured routinely at wavelength 580 nm ($OD_{580\ nm}$), whereas *R. capsulatus* cells were measured at a wavelength of 660 nm ($OD_{660\ nm}$).

10 Transfer of DNA into bacterial cells [236]

10.1 Preparation of competent *E. coli* cells

This protocol was applied to all *E. coli* strains used in this work. 100 ml LB medium was inoculated with 1 ml of a freshly grown preculture (**II.9.1.1**) and incubated at 37 °C under agitation (130 rpm). At an $OD_{580\ nm}$ ~ 0.4 – 0.6 cells were centrifuged for 10 min (4,000 x g, 4°C) and the resulting supernatant discarded. All following steps were carried out on ice. The obtained pellet was resuspended in 25 ml of ice-cold $MgCl_2$ buffer and incubated for 30 min – 60 min. Subsequently, the cell suspension was subjected to centrifugation for 10 min (4,000 x g, 4°C) and the resulting cell pellet carefully resuspended in 10 ml of prechilled $CaCl_2$ buffer. Afterwards, competent cells were separated into sterile reaction tubes (200 µl aliquots) and stored at -80 °C until further use. Verification of competent cells sensitivity

towards different antibiotics was accomplished by means of an antibiogram. To this end, 5 ml of LB medium, supplemented with an antibiotic of choosing, was inoculated using 20 µl of competent cells and incubated overnight at 37 °C under agitation (130 rpm). Turbidity of grown cultures allowed for predictions of competent cells sensitivity towards a particular antibiotic.

10.2 Transformation of plasmid DNA into *E. coli*

Transfer of plasmid DNA into competent *E. coli* cells was carried out using the heat shock method. 2 µl of purified plasmid DNA (**II.12.1**) or 10 µl of a ligation reaction mixture (**II.11.6**) was added to 200 µl competent cells (**II.10.1**). This mixture was chilled on ice for 30 min. Subsequently, the reaction tube was incubated for 90 sec at 42 °C and once again put on ice for 2 min After addition of 700 µl LB medium, the cell suspension was incubated for 2 h in a thermomixer (37 °C, 800 rpm). To select clones carrying the desired plasmid DNA, the transformation mixture was spread on agar plates supplemented with an antibiotic and incubated for at least 16 h at 37 °C. The plasmid contains a gene that confers resistance to a particular antibiotic, therefore only cells that have successfully received the plasmid DNA can survive on antibiotic containing agar plates.

10.3 Conjugational transfer of plasmid DNA into *R. capsulatus* [330]

Plasmid DNA was introduced into *R. capsulatus* strains by conjugational transfer. To this end, the plasmid of interest was first transformed (**II.10.2**) into *E. coli* S17-I cells and 20 – 30 colonies from the agar plate resuspended in 1 ml of PY-Fe medium. In the meantime, cell material from a freshly streaked *R. capsulatus* agar plate was resuspended in 5 ml RCV minimal medium. 1 ml of this cell suspension was gently mixed with 500 µl of the *E. coli* donor cells and the resulting cell suspension centrifuged for 10 min at 16,000 x g. Obtained cell pellet was resuspended in 100 µl of the supernatant and transferred onto a membrane filter, which was placed on a PY agar plate without an antibiotic. After incubation at 30 °C in the dark, the membrane filter was placed in a reaction tube with 1 ml RCV minimal medium. Cell material was peeled off the filter by vigorous shaking (vortexing) and the cell suspension once again subjected to centrifugation for 10 min at 16,000 x g. Cell pellet was resuspended in 100 µl of the supernatant and streaked on antibiotic containing PY agar plates. The agar plates were incubated phototrophically (**II.9.1.2**) for two days and seeded on agar plates once again. Cell material from these agar plates where then used to start precultures.

11 Techniques in Molecular biology

11.1 Molecular cloning

Recombinant DNA was generated by means of molecular cloning as described by Sambrook *et al.* [244]. The first step of cloning a gene was either to amplify the gene by PCR or to clone a gene directly from one plasmid to another (subcloning). The gene of interest, together with the target vector, was cleaved by restriction enzymes and subsequently subjected to agarose gel electrophoresis. Resulting DNA molecules were extracted from gels by using gel elution kits, while the ends of the corresponding DNA fragments permanently attached to each other by phosphodiester bonding, facilitated by a ligation reaction. Successful recombination of DNA molecules was verified by blue white screening or by restriction pattern analysis of isolated plasmid DNA. Final verification of the inserted gene was realized by DNA sequencing.

11.2 Polymerase chain reaction (PCR) [331]

Polymerase chain reaction (PCR) was used for amplifying specific gene regions from genomic DNA or plasmid DNA, to provide DNA fragments for molecular cloning.

The following reaction components were mixed in a PCR tube:

Component	Quantity
Pfu buffer	5 µl
Pfu polymerase	1 µl
Primer I	2 µl
Primer II	2 µl
DMSO	5 µl
dNTPs (2.5 mM)	5 µl
Template DNA	1 ng - 100 ng
Nuclease-free water	x µl

This reaction mixture was adjusted with nuclease free water to a total volume of 50 µl and placed into a preheated (98 °C) thermocycler. Depending on the starting material, and the gene to be amplified, thermocycling conditions varied. Listed below are typical conditions for a PCR reaction.

Step	Temperature	Time	
Initial denaturation	98 °C	1 min (plasmid DNA)	
		5 min (genomic DNA)	
Denaturation	98 °C	1 min	30 cycles
Annealing	55 °C – 70 °C*	1 min	
Elongation	72 °C	1 min / kb	
Final elongation	72 °C	10 min	
Hold	4 °C	∞ min	

* annealing temperature = Tm Primer – 4°C

The completed PCR reaction mixture was combined with adequate amounts of DNA sample buffer and subjected to agarose gel electrophoresis (**II.11.4**). Subsequently, DNA was extracted from the gel and cloned into a cloning vector (pBlueScript KS) by blunt end ligation.

11.3 Hydrolytic cleavage of DNA by restriction enzymes [332]

Plasmid DNA was treated with restriction enzymes to obtain DNA fragments with compatible ends.
The following components were combined in a reaction tube:

Component	Quantity
Plasmid DNA	10 µl
Restriction enzyme I	1 µl
Restriction enzyme II	1 µl
Buffer	2 µl
Nuclease free water	6 µl

These reaction compounds were mixed by pipetting and incubated for at least 2 h at 37°C or overnight, respectively. The volume of the deployed plasmid DNA varied with respect to the material used. Volume of DNA from small preparations equaled 10 µl, whereas volume of DNA from large scale preparations amounted to 1 µg DNA. The resulting difference in total volume was adjusted with water accordingly. Finally, this reaction was mixed with 5 µl DNA sample buffer and subjected to agarose gel electrophoresis.

11.4 Agarose gel electrophoresis [244]

DNA molecules were separated in a matrix of agarose by applying an electrical field. Typically, agarose gel electrophoresis was used for two different applications: for restriction pattern analysis of digested DNA (**II.11.3**) and for extraction of DNA molecules (**II.11.5**). Agarose gels were cast with a percentage of 0.8 – 1. To this end, agarose was dissolved in TBE buffer (0.5 x) and heated up until a clear solution formed. The cooled solution (50 °C) was then mixed with 10 µl – 25 µl ethidium-bromide and poured into a cast to set. Samples, together with a molecular-weight marker, were loaded into designated wells in the gel and the electrophoresis performed according to manufacturer's instructions (Bio-Rad), using a voltage of 135V for 30 minutes. The ethidium bromide in the gel intercalates into DNA molecules and emits light of a specific wavelength, which can be visualized under UV light. The visualized bands can be used to estimate the DNA size and to extract a particular DNA band from the gel by using gel extraction kits.

11.5 Gel extraction from agarose gels [333]

PCR products and digested DNA samples were first subjected to agarose gel electrophoresis (**II.11.5**). Visualized DNA bands of choosing were cut out as gel slices and the DNA extracted according to manufacturer's instructions (innuPREP Gel Extraction Kit-Analytik Jena). Typically, PCR products were eluted with 50 µl preheated water (50 °C), digested vector DNA with 20 µl and digested insert DNA with 12 µl.

11.6 Ligation [334]

Ligation reactions were performed in order to join two DNA fragments with compatible ends. The joining of DNA fragments was catalyzed by a T4 DNA ligase, which forms a phosphodiester bond between the 3´-hydroxyl of one DNA fragment and 5´-phosphoryl of another.

Following components were mixed in a reaction tube:

Component	Quantity
Insert DNA	12 µl
Vector DNA	6 µl
Buffer	2 µl
T4 DNA ligase	1 µl

Ligation mixtures were incubated for 1 h at room temperature and subsequently stored overnight at 4°C. Depending on the copy number of the vector used for cloning, 1 µl – 10 µl from the ligation mixture was used for transformation into *E. coli* DH5α cells (**II.10.2**). Liquid cultures were inoculated with a single colony, the plasmid DNA isolated and successful cloning of the gene verified by restriction pattern analysis or blue white screening and finally by DNA sequencing.

11.7 Blue white screening

Blue white screening was used solely for blunt end ligation of PCR products into the cloning vector pBlueScript KS. The PCR product was inserted into the gene encoding the α-peptide of the β-galactosidase at the recognition site of the restriction enzyme *Sma*I. The cloning vector contains one half of the gene encoding the β-galactosidase and the transformed cells the other half. Each half of the enzyme on its own is inactive, yet both parts can form a fully active enzyme when they are in close proximity. Successfully inserted genes disrupt one half of the β-galactosidase, so that a functional form of the enzyme can't be formed. With a simple colorimetric assay the formation of an active β-galactosidase can be detected and thereby used for screening of cells containing the cloning vector carrying an inserted gene. The agar plates used for this screening are supplemented with a colourless substance (X-gal), which upon cleaving by an active β-galactosidase forms a bright blue pigment, lending cells producing an active enzyme a blue coloration [244]. This way, cells containing successfully cloned PCR products can be differentiated by their pigmentation. Three white clones were picked routinely, the plasmid DNA isolated (**II.12.1**) and verified by restriction pattern analysis (**II.11.3**). Finally, the sequence of the gene of interest was verified by DNA sequencing.

11.8 DNA sequencing

For DNA sequencing, services offered by companies MWG operon and Sequiserve GmbH were employed.

11.9 Isolation of nucleic acids [335]

11.9.1 Plasmid DNA preparation

Plasmid preparation was performed in small scale (5 ml – 25 ml) with the peqGOLD XChange Plasmid midi kit according to manufacturer's instructions. Typically, the purified plasmid DNA was eluted in 50 µl preheated (65 °C) nuclease-free water and stored at -20 °C. A classical alkaline/SDS lysis procedure in combination with an ethanol precipitation was performed to isolate plasmid DNA from large scale cultures. To this end, 50 ml TB medium was inoculated with a toothpick of a single colony from a freshly streaked agar plate. This culture was grown for 16 h at 37°C under agitation (130 rpm) and subjected to centrifugation (10 min, 6,000 x g, 4 °C). The supernatant was discarded and the pellet resuspended in 7 ml solution I together with 1 ml of lysozyme (1 mg/ml) and 500 µl RNase solution. The lysozyme treated cells were incubated for 10 min at RT and then mixed gently with 20 ml of solution II. Following incubation for 10 min on ice, the suspension was treated with 15 ml of solution III and once again chilled for 10 min on ice. The mixture was subjected to centrifugation for 10 min at 16,000 x g (4 °C) in order to pellet unwanted compounds such as cell debris, proteins and chromosomal DNA. The supernatant was filtrated (folded filter) into two reaction tubes (50 ml) and the plasmid DNA purified by ethanol precipitation. To this end, the filtrate was mixed with 15 ml isopropanol and centrifuged for 15 min at 16,000 x g (4 °C). The resulting supernatant was discarded and the pellet washed thrice with 70 % Ethanol (5 min, 16,000 x g and 4 °C). The obtained pellet was either loft dried or incubated for 30 min at 37 °C. Subsequently, the purified DNA was resuspended in 250 µl of nuclease-free water and incubated for 30 min at 55 °C under gentle agitation. Purity and veracity of the isolated plasmid DNA was verified by spectroscopic analysis and agarose gel electrophoresis respectively.

11.9.2 Genomic DNA extraction

Genomic DNA was extracted according to manufacturer's instructions (DNeasy Blood & Tissue Handbook, Qiagen). The extracted genomic DNA was eluted in 500 µl nuclease-free water.

12 Techniques in Protein Biochemistry

12.1 Membrane fractionation

12.1.1 Small scale

Cells corresponding to an optical density of 1 (*E. coli*) or 3 (*R. capsulatus*) were harvested by centrifugation (10 min, 16,000 x g,) and stored for at least one day at -20 °C. The cell pellet was resuspended in 800 µl of SP- buffer supplemented with lysozyme (1 mg/ml) and mixed with 0.4 g glass beads. The reaction tubes were then placed in a bead mill homogenizer and cells disrupted mechanically for 15 min at max frequency. Cell debris and inclusion bodies were removed by low speed centrifugation (15 min, 10,000 x g and 4 °C). Finally, 600 µl of the supernatant was subjected to ultracentrifugation (2 h, 220,000 x g and 4 °C) to separate membrane fraction from the soluble fraction. The supernatant was discarded and the pellet containing the membrane fraction collected for further analysis.

12.1.2 Large scale

This protocol was applied to *R. capsulatus* liquid cultures exceeding volumes of 0.2 l. Cells were harvested by two low speed centrifugation steps. To minimize culture volume, the culture was first centrifuged at 8,000 x g for 1 h – 2 h (logarithmic phase = 1 h, stationary phase = 2 h) and subsequently subjected to centrifugation at 16,000 x g for 1 h – 2 h, without brakes. The supernatant was discarded and the resulting cell pellet stored at -20 °C for at least one day. The cell pellet was resuspended in SP buffer supplemented with lysozyme (1 mg / ml) and cell disruption was performed by passing cells 3 – 5 times through a french press. After cell disruption all steps were carried out on ice with prechilled buffers and components. Removal of cell debris and inclusion bodies was achieved by low speed centrifugation at 16,000 x g for 1 h (4 °C, without brakes). The supernatant was collected and subjected to ultra-centrifugation at 220,000 x g for 3 h (4 °C). The resulting membrane pellet was used for further downstream processes.

12.2 Solubilization

Most techniques used for characterizing proteins, such as protein purification or crystallization, are designed primarily for aqueous solutions. Solubilization was used for extracting membrane proteins from the membrane in water-soluble detergent micelle complexes. Membrane proteins were solubilized and purified based on a modified guideline provided by Newby *et al.* [245].

Detergent Screening

The protein of interest was produced in *R. capsulatus* cells (**II.9.1.2**) in a volume of 200 ml – 400 ml RCV minimal medium supplemented with an antibiotic. Cultures were grown photoheterotrophically for 2 - 3 days and illuminated by either bulb light or infrared light, respectively. The membrane fraction was isolated (**II.12.1**), resuspended in solubilization buffer (g/ml) and mixed with equal volumes of detergent solution (**Table II-10**). This mixture was incubated at 4 °C overnight with gentle agitation and subsequently subjected to centrifugation at 220,000 x g for 2 h (4 °C). The supernatant contained solubilized proteins, while proteins not extracted by the detergent remained in the pellet fraction. Detergent solubilization efficiency was determined by comparative analysis of the two fractions with regard to their protein of interest content.

Table II-10: Detergents and concentrations used in this work

Detergent	Stock solution	Final concentration	Concentration in purification buffer
N-dodecyl-b-D-maltopyranoside (**DDM**)	40 mM	20 mM	0.5 mM
octyl-b- D-glucopyranoside (**OG**)	540 mM	270 mM	40 mM
3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (**CHAPS**)	300 mM	150 mM	16 mM
n-Dodecylphosphocholine (**FC12**)	40 mM	20 mM	4 mM
n-Hexadeclphosphocholine (**FC16**)	4 % (w/v)	2 % (w/v)	-

12.3 Protein purification

Proteins were purified by using immobilized metal ion chromatography (IMAC). A genetically encoded His_6-Tag was placed at the C-terminus of the target protein and produced recombinantly in *R. capsulatus* (**II.9.1.2**). Purification of proteins was performed at 4 °C with prechilled buffers, while columns used for purification were purchased from Qiagen GmbH.

12.3.1 Purification of YFP

R. capsulatus cells were grown photoheterotrophically for 2 - 3 days in specially made photo reactors (flat panels). For purification of YFP-His_6, a culture volume of 600 ml was harvested and stored at -20°C for one day. Subsequently, cells were disrupted and fractionated (**II.13.1.2**). Membrane fraction was discarded and the cleared lysate utilized for affinity purification by IMAC. Cleared lysate was loaded onto a Ni-NTA Superflow Column (Qiagen), packed with 3 ml Ni-NTA resin. Prior to use, column was equilibrated with wash buffer 10. After the cleared lysate was drawn through the column, the column was washed twice with 15 ml wash buffer 10, once with wash buffer 25 and once with wash buffer 40, whereas elution was achieved by addition of 10 ml buffer E. For long term storage and functional characterization, eluted protein was concentrated to a volume of 0.5 ml – 1 ml by membrane ultrafiltration using concentrators (Vivaspin). To this end, buffer of the eluted protein was exchanged with protein storage buffer according to manufacturer's instructions for at least 3 times. Purified proteins were stored at 4 °C.

12.3.2 Purification of Bacteriorhodopsin

Bacteriorhodopsin was produced heterologously in *R. capsulatus* by employing the pRhonHi-2 vector and purified by using immobilized metal ion chromatography (IMAC). Purification of protein was performed at 4 °C with prechilled buffers, while columns used for purification were purchased from Qiagen GmbH. *R. capsulatus* TD22 cells were grown photoheterotrophically for 2 - 3 days in specially made photo reactors (flat panel bioreactor) under infrared light illumination. Cells were cultivated in RCV minimal medium (pH = 6) supplemented with 15 mM all-*trans* retinal (ethanol) and 1 mM serine as the sole nitrogen source. For purification, a culture volume of 1l was harvested and stored at -20 °C for at least one day. Subsequently, cells were disrupted and fractionated. The membrane fraction was collected and the proteins solubilized by 20 mM DDM overnight under gentle agitation (**II.13.2**). The resulting solubilizate was subjected to affinity purification by IMAC. All buffers used for the purification of the membrane protein contained 0.5 mM DDM, to maintain detergent-protein micelles at all times. The solubilizate was mixed with equal volumes of wash buffer 10 and incubated with 3 ml Ni-NTA resin (Qiagen) for 1 h at 4 °C under gentle agitation. This mixture was loaded onto a Ni-NTA Superflow Column (Qiagen) and drawn through the column by gravity flow. The column was washed four times with 15 ml wash buffer 10, once with wash buffer 25 and once with wash buffer 40, and finally eluted with 10 ml buffer E. For long term storage and functional characterization, eluted protein was concentrated to a volume of 0.5 ml – 1 ml by membrane ultrafiltration using concentrators (Vivaspin). To

this end, buffer of the eluted protein was exchanged with protein storage buffer according to manufacturer's instructions for at least three times. Purified proteins were stored at 4 °C.

12.4 Protein quantification [335]

Protein concentration of solutions was quantified routinely by the Bradford protein assay. Serial dilutions of the protein sample were prepared with distilled water (1:10, 1:100, and 1:1000) and 100 µl of these dilutions mixed with 900 µl Coomassie Blue solution. Following incubation for 5 min at RT, the mixture was spectroscopically measured at 595 nm. The solvent of the sample was used as a reference, whereas a calibration curve determined with bovine serum albumin solution was used for determining the concentration of the sample.

12.5 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) [336]

SDS-PAGE was performed to separate proteins according to their electrophoretic mobility in an electrical field. In general, SDS-PAGE was performed with either precast gels, using the Novex® NuPAGE® SDS-PAGE Gel system (4 % - 12 % Bis-Tris gels), or self-made gels, employing the Mini-PROTEAN 3 Cell system (BioRad, München, Germany). Acrylamide gels (5 % stacking gel and 12 % separating gel) were poured between two glass plates and fully polymerized gels placed into gel-running tanks filled with buffer. Samples were mixed with SDS sample buffer and incubated for 10 min at 99°C or for 30 min at 37 °C in case of aggregation-prone proteins. Subsequently, proteins were loaded into designated wells of the gels and electrophoresis performed at 100 V for approximately 2 h. SDS gels were either stained with Coomassie Brilliant Blue R-250 solution or further processed for western blots.

12.6 Western Blot [337]

The western blot was used for the transfer of proteins from SDS-gels onto PVDF membranes. Electroblotting of proteins was routinely performed by employing the Mini Trans-Blot® Cell from Bio-Rad. Protein samples were subjected to SDS-PAGE (**II.12.5**) and the resulting SDS-gels washed twice with Blotting-buffer for 10 min at RT under gentle agitation. Meanwhile, a piece of PVDF membrane was cut to size of the SDS-gel and incubated for one min in denatured ethanol. Subsequently, the PVDF membrane was washed for 5 min in distilled water and for 10 min in Blotting-buffer. After this, the gel holder cassette (black surface at the bottom) was assembled in the following order: foam pad, two Whatman® filter paper, SDS-gel, PVDF membrane, two Whatman® filter paper, foam pad. This

cassette was added to the electrode assembly and finally placed into a buffer tank. A cooling unit was inserted into the buffer chamber and the tank filled with Blotting-buffer. Electroblotting was performed for 15 min at 150 A and 30 min at 300 A. Finally, the membrane was incubated over night with blocking buffer under gentle agitation (4 °C) and further processed for immunological detection.

12.7 Immunological detection of proteins on PVDF membranes

Typically, proteins analyzed for this work were fused C-terminally with a His_6-tag. Therefore, a polyclonal antibody conjugated to a horseradish peroxidase (Anti His-HRP – Roth, 1 mg/ml) was employed for immunological detection of His-tagged proteins. PVDF Membranes treated with blocking solution were washed three times with PBS-T (5 min incubation at RT) and incubated for 1 h with an antibody solution (**Table II-7**) under gentle agitation. The antibody solution was discarded and the membrane washed again three times with PBS-T. Finally, the membrane was prepared for detection by chemiluminescence. To this end, the membrane was incubated with the ECL- solution complete that contains the substrate for the horseradish peroxidase (HRP). The biochemical reaction catalyzed by this enzyme forms a chemiluminescent intermediate, which can be detected by a camera based detection unit (STELLA – adjustement "sensitive chemoluminescence"). After chemiluminescent detection of target proteins, the overall transfer of proteins onto the PVDF membrane was verified by amido black staining.

A deviation was done for the immunological detection of YFP and NifH. For these proteins primary antibodies were deployed (**Table II-7**) that were lacking a HRP-conjugate. Consequently, a secondary antibody raised against a primary antibody was used (Anti-Rabbit Bio-Rad) for detection. The procedure was the same as described above, with the exception that after Electroblotting (**II.12.6**) PVDF membrane was not blocked overnight, but for 1 h at RT and subsequently the membrane incubated in primary antibody solution (resuspended in blocking-solution) overnight.

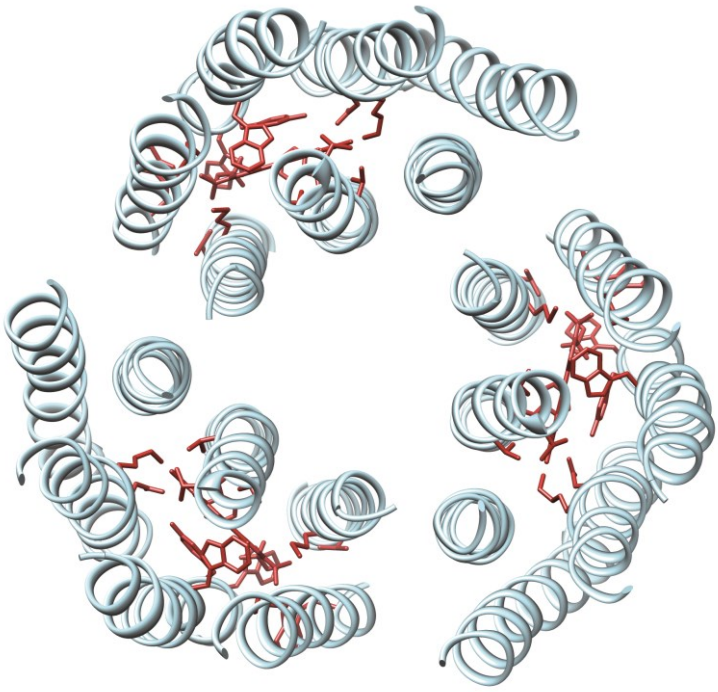
12.8 Amido black staining of PVDF membranes

Electroblotted membranes were stained with amido black solution to analyze the overall transfer of proteins onto the membrane. To this end, membranes were incubated for 5 min with amido black solution. Subsequently, the solution was discarded and the membrane rinsed a couple of times in distilled water. Finally, membranes were loft-dryed until blue-black bands representing the proteins formed.

12.9 Promoter strength characterization of pRho constructs in *R. capsulatus*

For promoter strength characterization studies, pRho-constructs containing an *EYFP* gene were introduced into *R. capsulatus* B10S cells by conjugation (**II.9.1.2**). Cells were cultivated photoheterotrophically under bulb light illumination for three days in RCV minimal medium supplemented with kanamycin and different nitrogen sources (15 mM NH_4SO_4, 1 mM serine or dinitrogen gas). Cultures were harnessed (OD_{660nm} = 2) by centrifugation and stored at -20°C for at least one day. Pellets were resuspended in 1000 µl of SP- buffer supplemented with lysozyme (1 mg/ml) and mixed with 0.4 g glass beads. The reaction tubes were then placed in a bead mill homogenizer and cells disrupted mechanically for 15 min at max frequency. Cell debris and inclusion bodies were removed by low speed centrifugation (15 min, 10,000 x g and 4°C). YFP fluorescence was measured spectroscopically (excitation 488 nm, detection 515 nm) and protein accumulation was verified by western blotting with GFP specific antibodies (**II.12.7**).

CHAPTER III: RESULTS



Bacteriorhodopson (PDB file 1BRD)

1 Construction and characterization of the pRhonHi-2 expression vector

While for some membrane proteins standardized expression conditions are a viable strategy for efficient production, toxic or intricate membrane proteins can be very demanding with respect to the deployed expression plasmid. To address the special needs for membrane protein expression in *R. capsulatus*, the first step in this work was concerned with the construction of a new expression vector that is particularly suited to produce membrane proteins. Before constructing a new expression plasmid, one needs to consider basic elements needed for expressing target genes. The pRho expression vector series was already shown to be optimal for the constitutive and inducible expression of soluble proteins in *R. capsulatus* [213]. For this reason, the new expression vector was based on this vector series, but changed with regard to the utilized promoter. Although the pRho vector series contains the expression vector pRhotHi-2, which is characterized by a strong T7 promoter, it may not be optimally suited for the delicate process of membrane protein production. In bacteria, the transcription of a gene is inseparably connected to the translation of the mRNA, since both processes occur at the same time. During transcription of the gene, the elongating mRNA chain is bound by the ribosome and translation at the ribosome is initiated [246]. Considering that many membrane proteins are co-translationally translocated into the membrane (**I.2.1.1**), problems can arise from an expression system that relies on a viral RNA polymerase that doesn't act in concert with a host specific ribosome, therefore the new expression vector was based on the host-specific *nifHDK* promoter.

The *nifHDK* promoter initiates the transcription of the structural genes encoding the Mo-nitrogenase, which is the key enzyme of nitrogen fixation **(I.3.1)**. It offers many properties that make it especially suited for expressing membrane proteins. Firstly, the gene product regulated by this promoter can make up to 30 % of total cellular proteins in *R. capsulatus* [247], which demonstrates its strength. Apart from being a very strong promoter, the promoter is tightly regulated by the nitrogen source in the cultivation medium. Among other environmental factors, *nif*-genes are strictly controlled by ammonium. The ammonium control occurs at three different levels, two of which affect the transcription of the genes. As illustrated in **Figure III-I**, expression studies conducted in *R. capsulatus* B10S demonstrate a strict repression of the chromosomal P_{nifH} activity by NH_4^+ in the cultivation medium. Furthermore, P_{nifH} regulated expression of the NifH protein can easily be induced by growing the bacterium under nitrogen-limiting conditions with alternative nitrogen sources such as serine or dinitrogen.

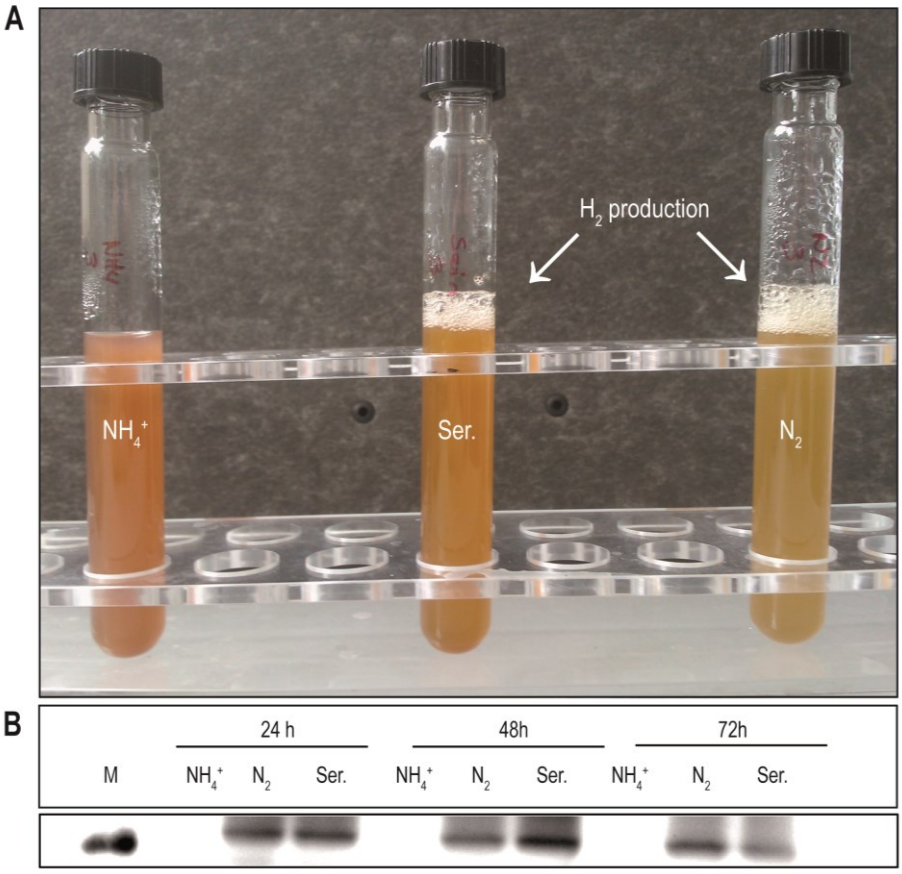


Figure III-1: Ammonium control of *nifH* expression in *R. capsulatus*
(A) Photographic picture of *R. capsulatus* B10S cultures grown anaerobically in RCV minimal medium for three days under bulb light illumination. Under nitrogen-limiting conditions (ser. = serine, N_2 = dinitrogen) *R. capsulatus* cells induce the expression of *nif*-genes and consequently produce Mo-nitrogenase. The reaction catalyzed by this enzyme leads to the formation of H_2, which is indicated by bubbles (arrows) in the gas-tight culture vessels. Under nitrogen-sufficient conditions (NH_4^+) expression of *nif*-genes is repressed, which leads to the inhibition of Mo-nitrogenase production. (B) Immunological detection of NifH protein in *R. capsulatus* whole cell extracts ($OD_{660\ nm}$ = 0.15) demonstrates inhibition of NifH production in the presence of NH_4^+, while utilizing N_2 or serine as the sole nitrogen source leads to the accumulation of NifH.

This feature of the promoter illustrates an easy and cost-effective manner to regulate promoter activity by simple alteration of the nitrogen source. Lastly, the nitrogen fixation in *R. capsulatus* is tightly linked to the anoxygenic photosynthesis of the organism [194]. Considering that the reason for evaluating *R. capsulatus* is its remarkable membrane physiology, choosing a promoter that is activated under phototrophic conditions facilitates the exploitation of the organisms physiology for the production of membrane proteins.

1.1 Construction of the pRhonHi-2 expression vector

Prior to this thesis, the construction of a pRho vector based on the *nifHDK* promoter has been explored [240, 242, 248] and resulted in the construction of the four expression vectors pRhon$_1$Hi-2 – pRhon$_4$Hi-2. The assumed "core region" of the *nifHDK* promoter is comprised of the binding region of the NifA 1/ NifA 2 transcription activator (UAS), the integration host factor (IHF), the transcription starting point and the ribosome binding site. For the vector construction, the T7 promoter region of the pRhotHi-2 vector was exchanged with this "core region" by means of molecular cloning (pRhon$_1$Hi-2) and assessed for its ability to express a reporter protein. Surprisingly, target gene expression mediated by this expression vector was greatly inferior to that described in literature [242].

The IHF protein is known to introduce sharp bends into the DNA that facilitates the physical interaction of components needed for transcription of genes. The presence of this regulative element led to the assumption that there may be additional elements needed for the full activation of the promoter, which are placed upstream of the promoter. Additional constructs containing expanded upstream regions of the *nifHDK* promoter were cloned and termed pRhon$_2$Hi-2, pRhon$_3$Hi-2 and pRhon$_4$Hi-2, respectively. However, none of these constructs demonstrated distinct changes in target gene expression [240].

The importance of the *nifHDK* operon for the nitrogen fixation has led to the construction of promoter fusion constructs, which have been used to analyze the regulation of the *nifHDK* promoter in response to different environmental factors. Therefore, reference vectors that harbor an active *nifHDK* promoter already exist and can be consulted for clarification of missing regulative elements in the pRhonHi-2 series. As illustrated in **Figure III-2**, a comparison of the pRhonHi-2 vectors with one construct that harbors a *nifH-lacZ* fusion cassette (pPHU266 [249]) demonstrates that the genomic regions implemented in the pRhonHi-2 vectors differ only with respect to a small downstream region from the promoter region present in the pPHU266 vector.

In order to ensure that problems encountered with the pRhonHi-2 vectors were in fact attributed to missing regulative elements present downstream of the promoter, a new pRho construct (pRhon$_5$Hi-2) was constructed. The genomic region of *R. capsulatus* containing the "core region" and downstream sections of the *nifHDK* promoter was amplified by PCR and exchanged by means of molecular cloning (*Nhe*I/*Xba*I) with the T7 promoter of the pRhotHi-2 vector.

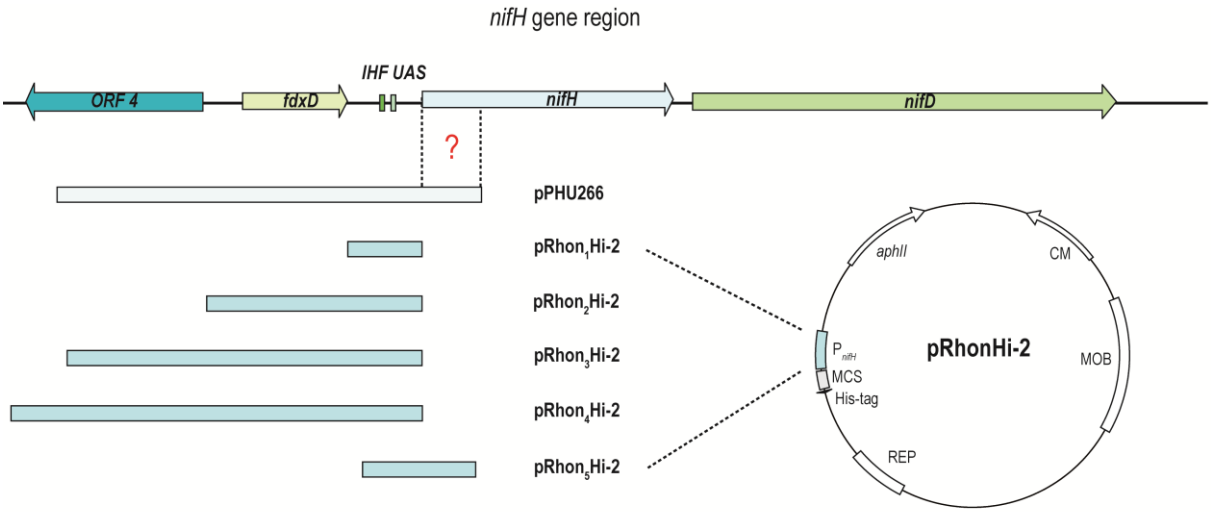


Figure III-2: Schematic depiction illustrating differences of pRhonHi-2 vectors with respect to implemented *R. capsulatus* genomic regions
At the top, the *nifH* gene region of *R. capsulatus* is shown. This excerpt of the chromosomal region includes the *nifH* gene, the upstream region that contains the *nifHDK* promoter, a gene encoding a ferredoxin (*fdxD*) and an open reading frame (ORF4), which encodes a putative catabolite repressor protein. To construct a *nifHDK* promoter based expression plasmid, the assumed promoter region was implemented in the pRhotHi-2 vector, resulting in the pRhon$_1$Hi-2 vector. Since mediated target gene expression did not meet with expected success, the problems were attributed to missing regulative elements present in the upstream region of the gene. However, expanding the upstream regions (pRhon$_1$Hi-2 – pRhon$_4$Hi-2) did not demonstrate a noticeable difference in mediated target gene expression. A comparison with the successfully employed *nifH-lacZ* fusion plasmid pPHU266 illustrates that the pRHon$_4$Hi-2 vector differs only with regard to the downstream region of the *nifH* gene, which suggests regulative elements important for full promoter activity in this region. Hence the pRhonHi-2 variant 5 was constructed. The broad-host range expression vector harbors two antibiotic resistance genes (chloramphenicol and kanamycin), an origin of replication (REP) and an origin of transfer (MOB). Target genes can be integrated into the multiple cloning site (mcs) and fused to a hexahistidine-tag (His$_6$-tag) encoding sequence, allowing affinity purification and immunological detection of the recombinant protein. *fdxD* : ferredoxin, ORF = open reading frame, IHF = integration host factor, UAS = upstream activator sequence.

The pRhonHi-2 variant 5 leads to a transcriptional fusion of target gene and *nifH*-gene region, while a stop codon prevents a translational fusion of target gene and NifH-protein. Subsequently, the new expression vector was evaluated with regard to other pRhonHi-2 constructs and its suitability as an expression plasmid.

1.2 Target gene expression mediated by the pRhonHi-2 vector

A reporter gene encoding the yellow fluorescent protein (*EYFP*, Clontech) was employed as a means to measure the level and regulation of target gene expression. The pRhonHi-2 vectors 1 - 5 containing the reporter gene were introduced into the *R. capsulatus* wild type strain B10S by conjugational transfer and the resulting expression strains grown photoheterotrophically. To this end, precultures were grown under high light intensity conditions (bulb light illumination) in small scale culture tubes (Hungates) under anaerobic conditions. Cultures were cultivated in RCV minimal medium supplemented with

malate as the carbon source and ammonium (NH_4^+) as the sole nitrogen source, while the addition of kanamycin kept selective pressure on cells. In order to analyze the manner in which target gene expression can be induced, the test cultures were grown with different sources of nitrogen: NH_4^+, serine (S), and dinitrogen (N_2). Target gene expression was indirectly quantified by fluorescence measurement and by means of western blotting (**II.12.9**).

Are genetic elements downstream of the *nifHDK* promoter needed for full promoter activation?

In order to determine if the implemented genomic region was the problem encountered in the pRhonHi-2 vector series, target gene expression mediated by the pRhon$_5$Hi-2 vector was compared to the pRhonHi-2 vector variants 1 - 4. The result of this comparison is depicted in **Figure III-3**.

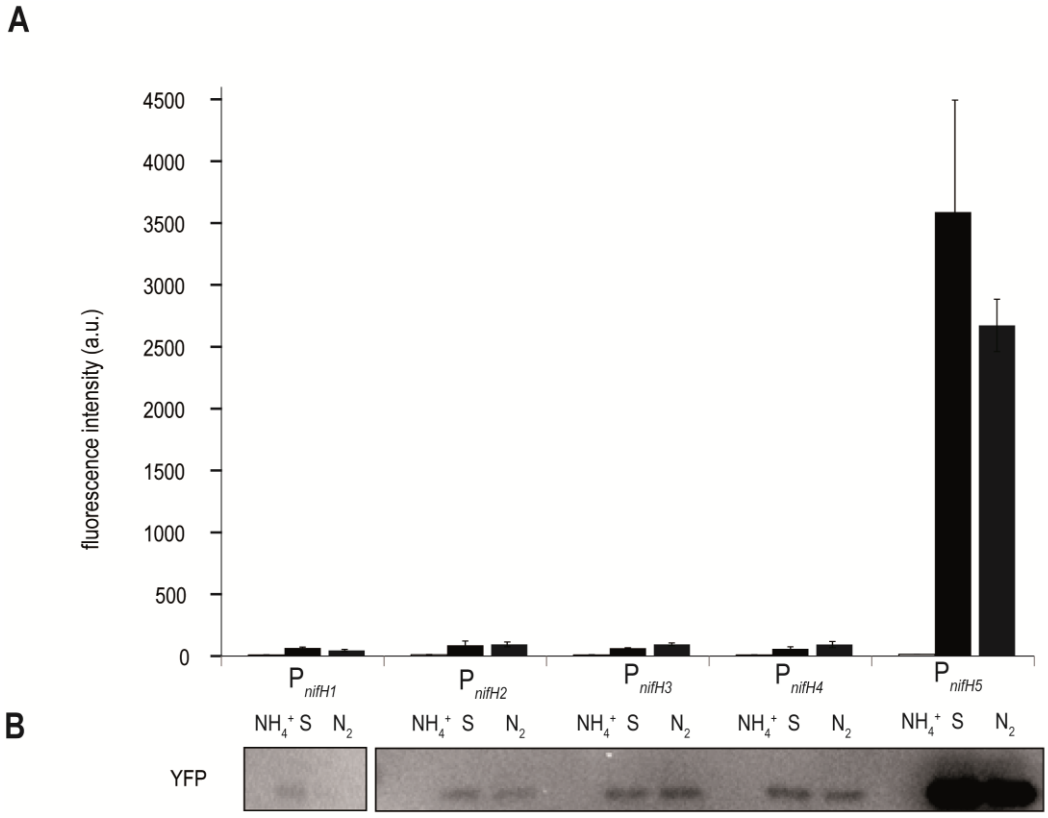


Figure III-3: Comparison of YFP expression mediated by different pRhonHi-2 vectors
To investigate whether the downstream region of the *nifH* gene is relevant for target gene expression, a new variant was constructed (P_{nifH5}). The pRhonHi-2 vectors containing the EYFP gene were expressed in *R. capsulatus* cells under photoheterotrophic conditions. Cultures were grown either with ammonium (NH_4^+), serine (S) or dinitrogen (N_2) as the sole source of nitrogen in the medium, to determine if target genes can be strictly repressed and strongly induced by simple alteration of the nitrogen source. YFP expression was determined by means of spectroscopy (A) and Western blotting with GFP specific antibodies (B).Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations. a.u.: arbitrary units

The expression study illustrates that cultures employing the pRhon$_5$Hi-2 vector are characterized by a distinctly higher YFP accumulation and a higher relative fluorescence than cultures employing the other pRhonHi-2 vectors. Under nitrogen-limiting conditions (serine as the sole nitrogen source) the pRhon$_5$Hi-2 vector reaches at least 40 times stronger expression of YFP than the other pRhonHi-2 vectors. Considering that the new construct differs primarily with regard to the downstream region of the *nifHDK* promoter, this expression study underlines the importance of this genomic region for promoter activity.

How strong is the mediated gene expression compared to other pRho vectors?

To evaluate the promoter strength of the pRhon$_5$Hi-2 construct, the reporter gene was comparatively expressed with established pRho vectors (**I-3.3**) pRhokHi-2 and pRhotHi-2 (**Figure III-4**).

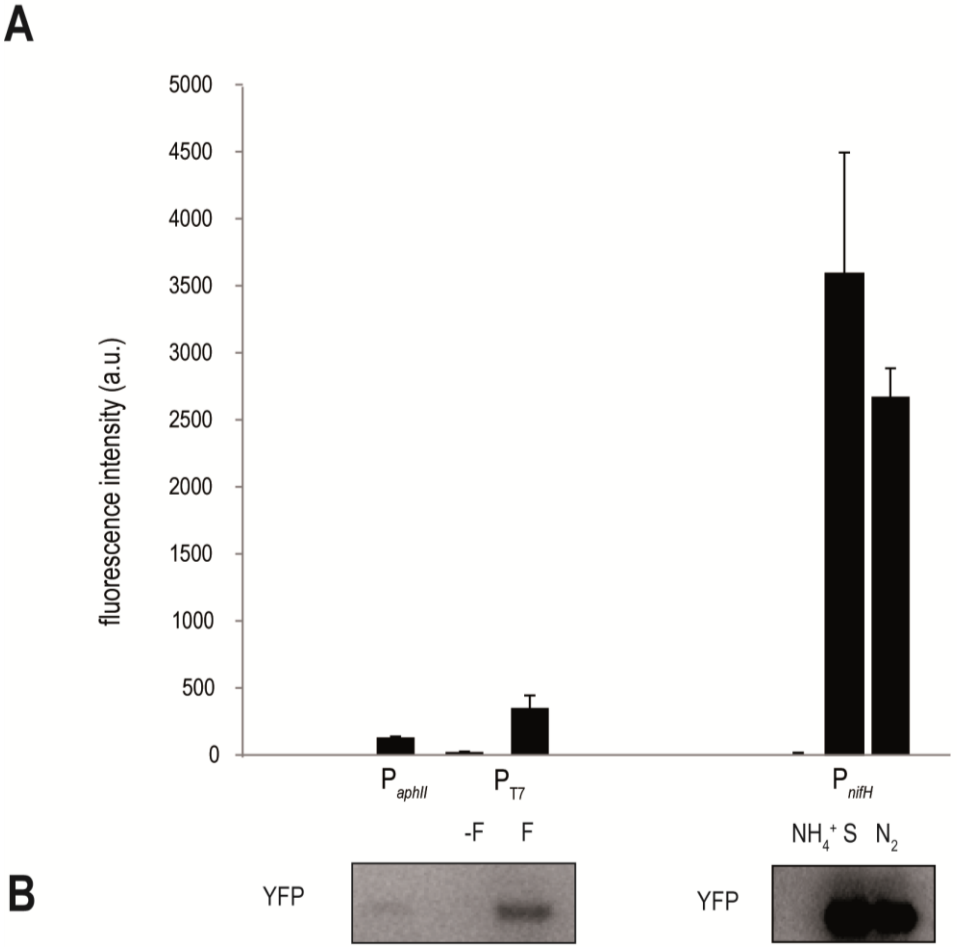


Figure III-4: Target gene expression mediated by different pRho vectors
To evaluate the promoter strength of the pRhon$_5$Hi-2 vector (P_{nifH}), target gene expression mediated by the expression plasmids pRhokHi-2 (P_{aphII}) and pRhotHi-2 (P_{T7}) was comparatively analyzed (see **II-9.1.2.1** for experimental procedure). The pRhokHi-2 vector mediates a constitutive expression of target genes, whereas the pRhotHi-2 vector facilitates an inducible expression of target genes by simple addition of fructose (F) into the culture medium. Target gene expression was quantified by fluorescence analysis (A) and by means of Western blotting (B). Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations. a.u.: arbitrary units. NH_4^+ = ammonium, S = serine, N_2 = dinitrogen

A comparison with other pRho vectors revealed that the pRhokHi-2 vector mediates the weakest fluorescence (114 a.u.), followed by the pRhotHi-2 vector (348 a.u.) and succeeded by the new pRhon$_5$Hi-2 vector (N_2 = 2672 a.u.; Ser. = 3587 a.u.). The results obtained by the fluorescence measurements (**Figure III-4A**) are in general agreement with the results observed for the Western blot analysis (**Figure III-4B**), which demonstrates that out of all the analyzed expression plasmids the pRhon$_5$Hi-2 vector mediates the strongest accumulation and activity of YFP. Furthermore, expression analysis confirmed the target gene expression to be strictly repressed by NH_4^+ in the culture medium (15 a.u.), while nitrogen-limiting conditions induce target gene expression, as can be seen when expression strains are cultivated in minimal medium supplemented with serine (induction factor 234) or N_2 (induction factor 174) as the sole nitrogen source. A different impact on protein production could be achieved with respect to the utilized nitrogen source. Utilizing dinitrogen (N_2) instead of serine (S) resulted in a reduced expression of the reporter gene. Consequently, serine was chosen as the sole nitrogen source for inducing target gene expression with the pRhonHi-2 vector in *R. capsulatus*. From here on, the pRhon$_5$Hi-2 expression vector will be simply referred to as pRhonHi-2.

1.3 Influence of the employed culture vessel on pRhonHi-2 driven protein production

In order to establish a generic protocol for the efficient purification of proteins employing the pRhonHi-2 expression vector, a simple upscaling of *R. capsulatus* cultures was performed. The expression studies up until this point were all performed in Hungates (10 ml – 15 ml), so for upscaling, a large scale culture in 500 ml Schott flasks was inoculated with *R. capsulatus* B10S cells harboring the pRhonHi-YFP vector. As a reference expression system, *R. capsulatus* B10S-T7 cultures containing a pRhotHi-YFP vector were prepared likewise and both cultures incubated under standardized photoheterotrophic cultivation conditions. Protein production was induced by nitrogen-limiting conditions (serine as the sole nitrogen-source) in cultures employing the pRhonHi-2 vector and 8 mM fructose in *R. capsulatus* B10S-T7 cells producing YFP-His$_6$ utilizing the pRhotHi-2 vector. Cells were harvested by centrifugation and mechanically disrupted (**II.12.3.1**). The obtained cleared lysate was then used for isolating YFP-His$_6$ molecules by means of immobilized metal ion affinity chromatography (IMAC) as describe in **II-12.3.1**. Surprisingly, the purification study reflected unexpected results regarding the achieved protein yield. While for the pRhotHi-2 vector a protein yield of 6.0 mg/l culture could be noted, the pRhonHi-2 vector led to an YFP-His$_6$ production accounting for 9.5 mg/l culture, which accounts for a factor difference of ~ 1.6. Although this yield is slightly better than the T7 RNAP based system, it is not

in accordance with the expression study conducted before (**III.3.1.3**), where the P_{nif} - based system illustrated a difference in mediated expression by a factor of approximately 10.

It is known that results obtained from small scale cultures are rarely suitable to predict results obtained with large scale cultures, since culture conditions can be distinctly different. Especially with phototrophic organisms, a typical problem of upscaling is self-shading, which can severely affect phototrophic growth [213]. This phenomenon is particularly pronounced when high cell densities are reached and the light absorption of cells influence the light intensity within the culture. Naturally, a reduced light intensity would affect the phototrophic growth of the organism and consequently the biosynthetic capacity. However, the reference expression system (B10S-T7) was subjected to the same cultivation conditions, which is why the observed discrepancy between the two expression plasmids are more likely attributed to altered cultivation conditions that specifically target the *nifHDK*-promoter driven expression, but not the T7-promoter mediated YFP-His_6 production. Considering that the main difference between the expression studies and the purification study was the employed culture vessel, it stands to reason if the observed effect is connected to the employed culture vessel.

Does the culture vessel influence pRhonHi-2 mediated expression?

In order to analyze the impact of the culture vessel on protein production, the purification study was repeated with cell material obtained from cultures cultivated in Hungates. The obtained protein yields are depicted comparatively with results achieved when Schott flasks were employed in **Table III-1**.

Table III-I: Protein yields obtained by employing different culture vessels

	Schott flasks	Hungates
pRhotHi-2	6.0 mg/ml	8.0 mg/ml
pRhonHi-2	9.5 mg/ml	38.0 mg/ml

As expected, the result of the purification study performed with material from small scale cultures was in general agreement with results observed in expression studies conducted before. Employing the pRhotHi-2 vector led to a protein yield equaling 8 mg/l, which is similar to the obtained yield in Schott flasks, where yields of 6 mg/l could be achieved. In contrast, utilizing the pRhonHi-2 vector in Hungates

resulted in yields corresponding to 38 mg/l of YFP-His_6, emphasizing a huge discrepancy with the results obtained in Schott flasks (9.5 mg/l). Since Hungates and Schott flasks differ greatly with respect to glass thickness and surface-to-volume ratio (**Figure III-7**), it is very likely that cells are subjected to different light conditions in both culture vessels. These results indicate that the pRhonHi-2 driven expression of target genes seems to be affected by the prevailing light conditions in the employed culture vessel.

Considering SDS-PAGE gel illustrations of purification studies (**Figure III-5B**), a noticeable band (red arrows) was noted to accompany the YFP band in *R. capsulatus* cells expressing genes with pRhonHi-2 vector.

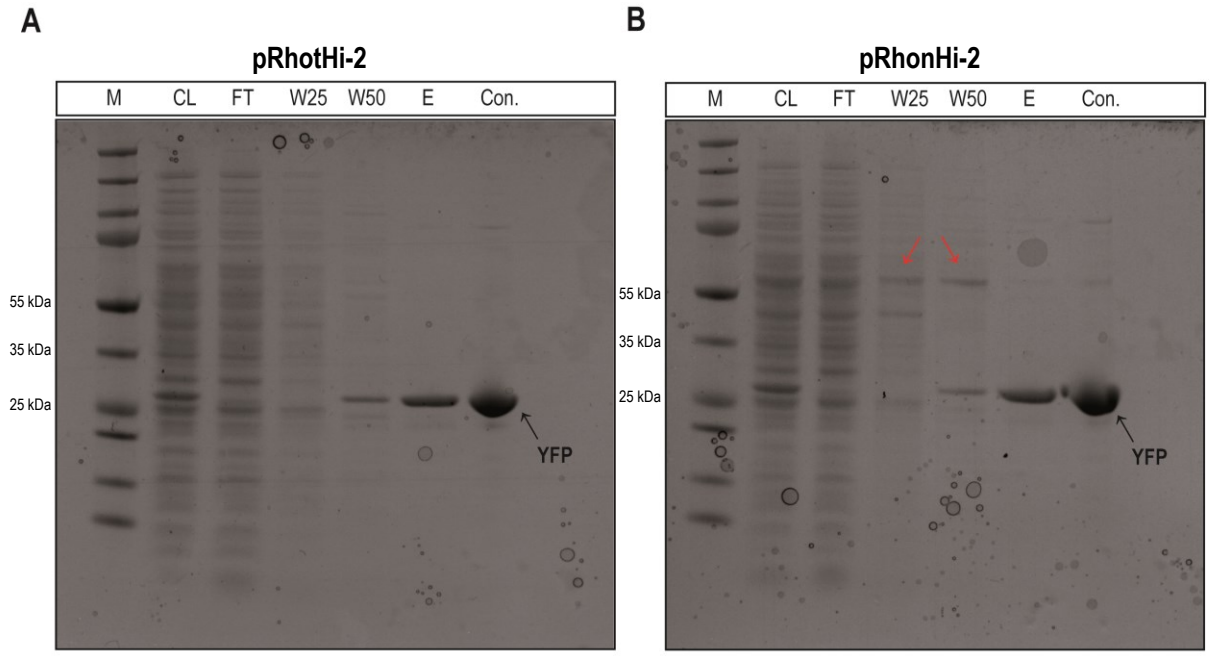


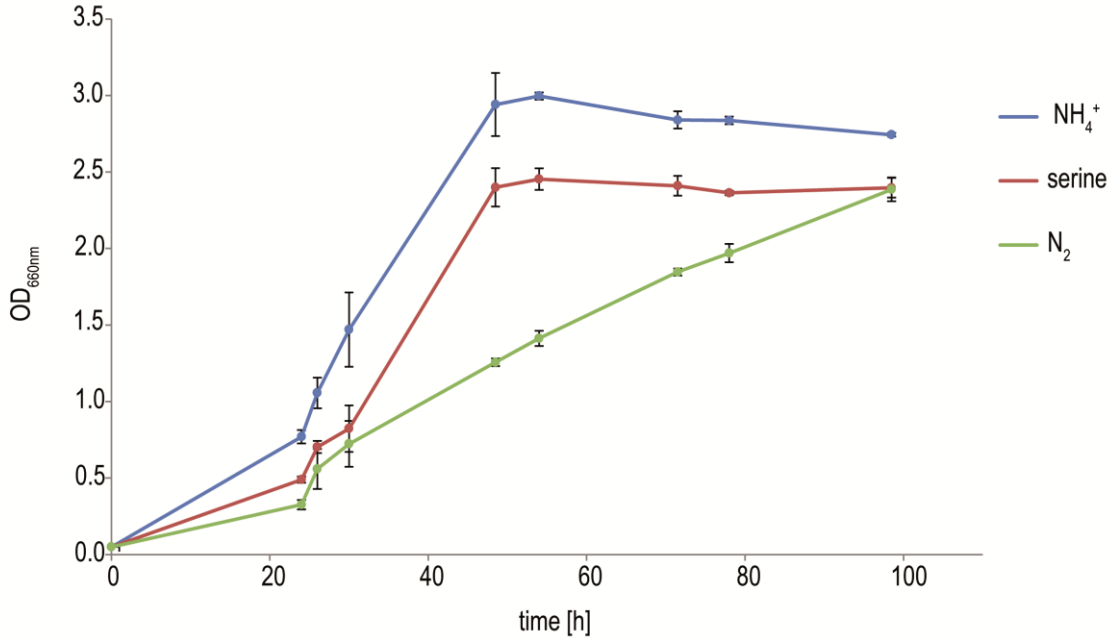
Figure III-5: Comparative study of YFP-His_6 purification in *R. capsulatus* employing different pRho vectors

Illustrated are Coomassie stained SDS gels depicting different steps of a YFP-His_6 affinity purification conducted with cell material obtained from *R. capsulatus* cells employing either the pRhotHi-2 vector (A) or the pRhonHi-2 vector (B). Cells were grown photoheterotrophically for two days in 500 ml Schott flasks under bulb light illumination. While for pRhotHi-2 mediated YFP-His_6 production B10S-T7 cells were employed, for pRhonHi-2 driven YFP-His_6 production B10S cells were utilized. For purification, cultures were harvested by centrifugation, mechanically disrupted and the obtained cleared lysate used for affinity purification. Buffer of eluted proteins was exchanged and proteins concentrated by the employment of concentrators. The pRhotHi-2 mediated protein purification yielded 6 mg/l culture YFP-His_6, whereas pRhonHi-2 driven production yielded 9.5 mg/l culture. Interestingly, the purification study revealed a band (red arrow) in the Coomassie stained SDS gel that was produced in high quantities in *R. capsulatus* cells grown under nitrogen-limiting conditions. M = protein marker, CL = cleared lysate, FT = flow through, W25 = wash fraction 25 mM imidazole, wash fraction 50 mM imidazole, E = elution fraction, Con. = eluted protein concentrated by means of concentrators.

This band corresponds most likely to the NifDK-proteins from the Mo-nitrogenase complex and is a byproduct of physiological conditions that lead to induction of the chromosomal *nifHDK* promoter. It could be shown that the employed culture vessel had a negative impact on protein production, another factor which could potentially influence the protein production in the newly developed expression system is the manner in which gene expression is induced with the pRhonHi-2 vector.

1.4 Influence of the Mo-nitrogenase production on pRhonHi-2 mediated gene expression and growth behavior of *R. capsulatus*

Although the applicability of the pRhonHi-2 vector could be shown, the cultivation under nitrogen-limiting conditions could still have physiological repercussions for *R. capsulatus*, since these conditions lead to the induction of all *nif*- promoters present in *R. capsulatus* [197]. Cellular growth was investigated as a measure of cellular stress under different cultivation conditions with respect to the nitrogen source.
Recorded growth curves, depicted in **Graph III-I**, illustrate that nitrogen-limiting conditions have a negative impact on cell growth.



Graph III-1: Growth behavior of *R. capsulatus* B10S cultures utilizing different nitrogen sources
R. capsulatus B10S cells were grown photoheterotrophically in RCV minimal medium supplemented with different nitrogen sources: NH_4^+, N_2 and serine. As a diazotrophic organism, *R. capsulatus* can grow without external sources of fixed nitrogen and is able to utilize atmospheric dinitrogen as the sole nitrogen source. Utilizing N_2 or serine as the sole nitrogen source (nitrogen-limiting conditions) in the culture medium activates the expression *nif*-genes of the organism. Here shown is the impact of various nitrogen sources on growth behavior of *R. capsulatus* B10S cultures. Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations.

Compared to cells grown under nitrogen-sufficient conditions (NH_4^+), cells grown with serine and dinitrogen in particular, demonstrate a reduced growth phenotype. These effects may be attributed to the production of the Mo-nitrogenase, considering that the synthesis (~ 30 % cellular protein [247]) and activity of the *nifHDK*-encoded Mo-nitrogenase leads to an increased consumption of ATP and cell resources.

Does the production of the Mo-nitrogenase affect cell growth in *R. capsulatus*?

To analyze the effect of Mo-nitrogenase expression on recombinant protein production and growth behavior of *R. capsulatus*, a comparative analysis between the wild type strain B10S and the deletion mutant TD22 [239] was performed. This mutant strain lacks the structural genes encoding the Mo-nitrogenase (**Figure III-6**), providing an ideal platform to study side-effects of Mo-nitrogenase production.

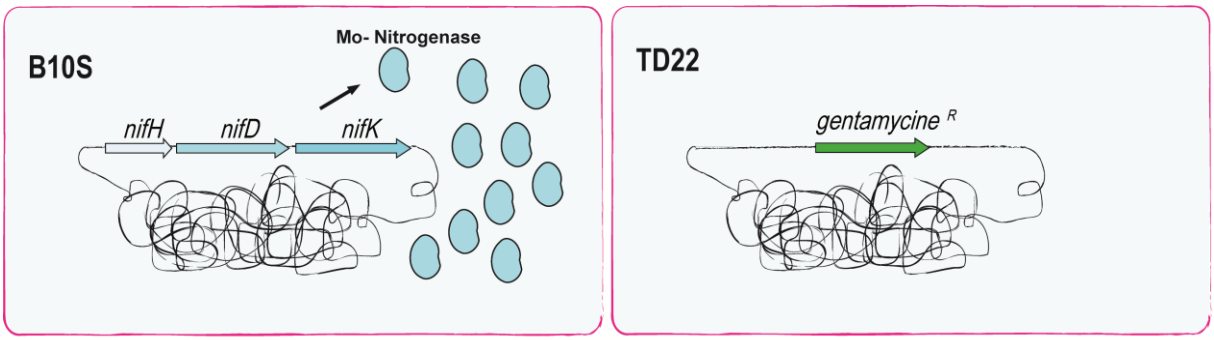
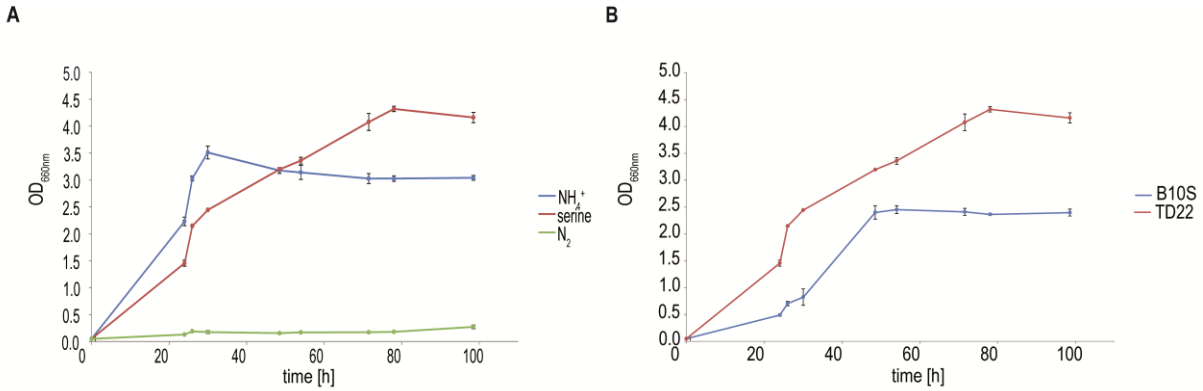


Figure III-6: Cartoon depiction illustrating differences between *R. capsulatus* strains B10S and TD22
The deletion mutant TD22 [239] was constructed by interposon mutagenesis of the B10S strain, resulting in a strain harboring a gentamycine resistance gene instead of the *nifHDK* operon. Consequently, the strain TD22 is not able to produce Mo-nitrogenase and can therefore be employed to study possible side-effects associated with production and the catalytic activity of the enzyme.

As mentioned before, nitrogen-limiting conditions led to a different growth in response to the utilized nitrogen source. To ascertain that the reduced growth phenotype is attributed to the production of the Mo-nitrogenase, growth curve experiments under nitrogen-limiting (serine or N_2) and nitrogen-sufficient (NH_4^+) conditions were performed. As illustrated in the **Graph III-2**, the growth curve experiments demonstrate very clearly that the mutant strain is characterized by a different growth behavior with respect to the utilized nitrogen source. As expected, the deletion mutant is not able to grow when N_2 is

utilized as the sole nitrogen source, since the deletion of the *nifHDK* operon prevents the production of the Mo-nitrogenase.



Graph III-2: Influence of the nitrogen source on growth behavior of *R. capsulatus* TD22 cultures
(A) *R. capsulatus* TD22 cells were grown photoheterotrophically in RCV minimal medium supplemented with different nitrogen sources: NH_4^+, N_2 and serine. With respect to the utilized nitrogen source distinct growth curves can be observed. Since the *nifHDK* deletion prevents the TD22 strain from producing the Mo-nitrogenase, the organism cannot utilize N_2 as a nitrogen source in a cultivation medium containing molybdenum. (B) Illustrated are the growth curves of *R. capsulatus* B10S and TD22 strains utilizing serine as the sole nitrogen source. A comparison between the growth behaviors of both strains illustrates differences attributed to the production of the Mo-nitrogenase. As can be deduced from the growth curve analyis, the production of the Mo-nitrogenase in the wild type strain B10S leads to a negative impact on cell growth, which can be overcome by employment of the deletion strain TD22. Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations.

Although *R. capsulatus* is able to produce an alternative Fe-nitrogenase, the production of this enzyme is strictly repressed by the presence of molybdenum in the cultivation medium. Consequently, the strain TD22 can grow with serine or NH_4^+ but not with N_2. As with the wild type strain B10S, a reduced growth is observed under nitrogen-limiting conditions, but only for 48 h, after which the culture recovers and reaches cell densities (max $OD_{660\ nm}$ = 4.3) which exceed that observed for cultures grown under nitrogen-sufficient conditions (max $OD_{660\ nm}$ = 3.5).

A direct comparison between the wild type and mutant strain (**Graph III-2B**) illustrates that the wild type strain is characterized by a much more pronounced reduced growth. This leads to the conclusion that the production of Mo-nitrogenase has a negative impact on cell growth, which can be overcome by utilizing the strain TD22.

Does the production of the Mo-nitrogenase affect pRhonHi-2 mediated YFP production in *R. capsulatus*?

Can flat panels alleviate the observed negative effect of Schott flasks on pRhonHi-2 mediated YFP production?

In order to analyze the impact of Mo-nitrogenase production on recombinant protein production, a comparative purification analysis was performed. For this, pRhonHi-YFP vectors were introduced into the B10S and the TD22 strain by conjugation (**II.10.3**). Under consideration of the culture vessel choice, the purification study was performed with cell material obtained from both small scale and large scale cultures. In order to alleviate the observed negative effects of Schott flasks, optimized culture vessels referred to as flat panels were employed for large scale cultures (**Figure III-7**).

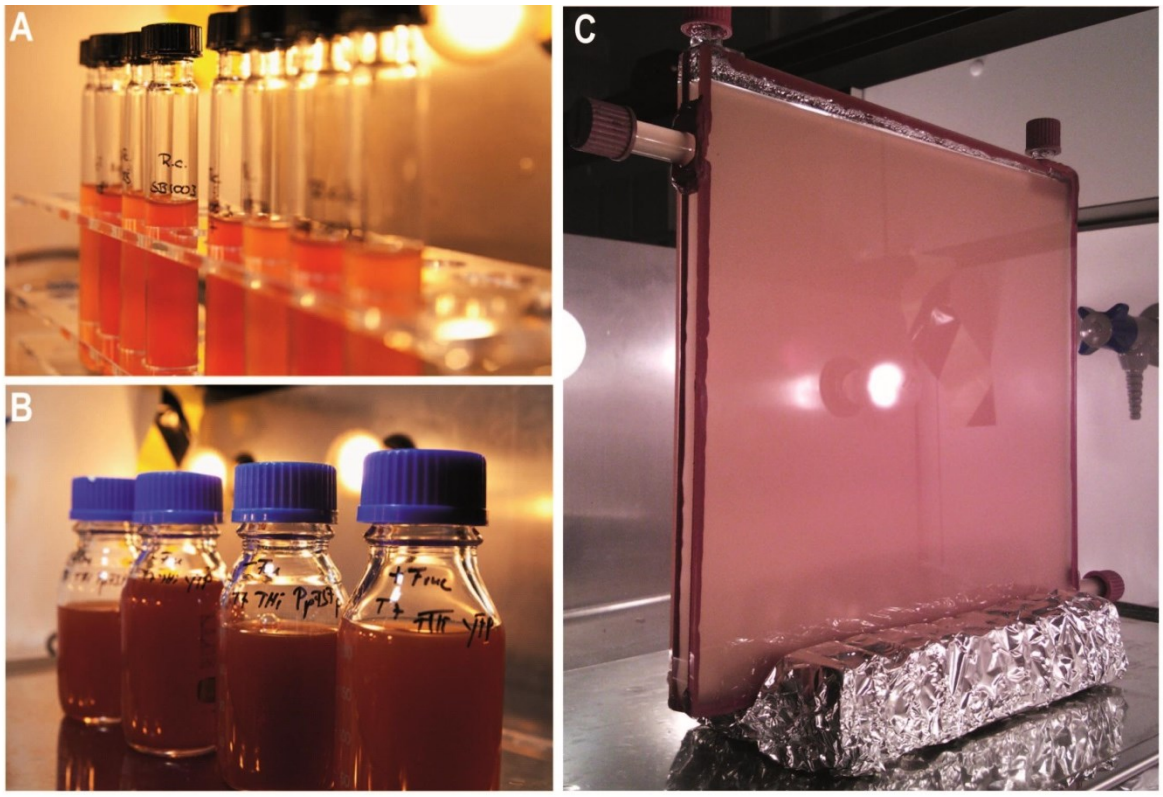


Figure III-7: Culture vessels employed for phototrophic cultivation of *R. capsulatus*
Photographic illustration of different culture vessels employed for phototrophic cultivation of *R. capsulatus* cells in this study. Small scale cultures (10 ml) were routinely cultivated in Hungates (A), whereas large scale cultures (500 ml – 600 ml) were grown in common Schott flasks (B) or specially made flat panel bioreactors (C). Pictures (A) and (B) were taken by Achim Heck.

Flat panels are characterized by thinner glass walls and a greater light exposure area than common Schott flasks due to a greater surface-to-volume ratio [213]. The result of this comparative purification study is shown in **Figure III-8**. The gel bands corresponding to the target protein (arrows) demonstrate that the deletion of the *nifHDK* operon leads to an increase in YFP-His_6 production, irrespective of the employed culture vessel. This is best reflected by the densitometry analysis showing the factor difference of YFP band intensity (arrow) between the different expression strains.

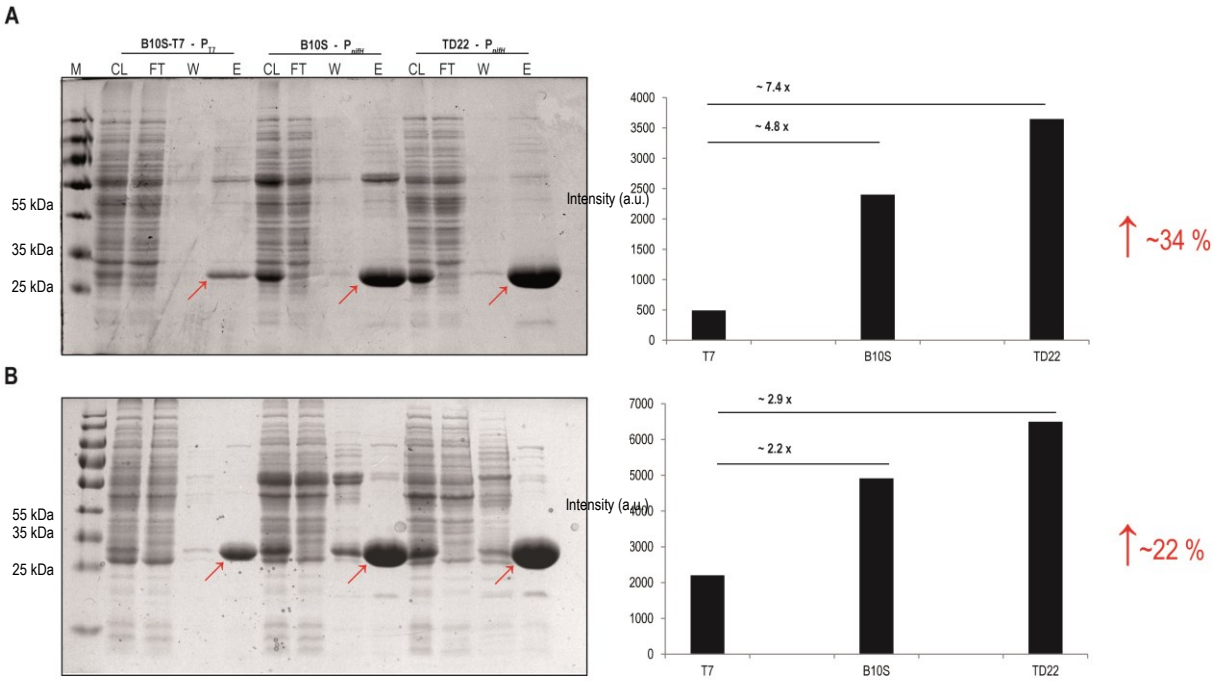


Figure III-8: Influence of employed culture vessel and Mo-nitrogenase production on pRhonHi-2 mediated YFP-His_6 production in *R. capsulatus*

In order to analyze the effect of the employed culture vessel on pRhonHi-2 mediated protein production, cultures were grown in Hungates (A) or flat panels (B). Likewise, to determine the effect of Mo-nitrogenase production on recombinant protein production, experiments were comparatively conducted in the *nifHDK* deletion mutant TD22 and B10S. Coomassie stained SDS gel illustrations (left side) demonstrate the progress of affinity purification performed with cell material obtained from *R. capsulatus* cells producing the reporter protein by pRhotHi-2 mediated expression in the B10S-T7 strain or pRhonHi-2 mediated expression in the strains B10S or TD22. On the right side, a bar chart illustrates the densitometric analysis of eluted protein bands (red arrow). The numbers in red illustrate the yield improvement of YFP production observed in the deletion mutant TD22 in comparison to the wild type strain B10S utilizing the pRhonHi-2 vector. The factor difference of quantified band intensity between different expression plasmids are noted in the bar charts. M = protein ladder, CL = cleared lysate, FT = flow through, W = wash , E = elution

While for Hungates (**Figure III-8A**) an improvement of 34 % could be noted in the deletion strain TD22, the YFP band intensity was increased by 22 % in flat panels (**Figure III-8B**). A comparison between the T7 RNA polymerase based system (pRhotHi-2) and the P_{nifH} based system (pRhonHi-2) in different cultivation vessels (Hungates vs flat panels) illustrates once again significant differences between small scale and large scale cultures. As expected, the pRhonHi-2 vector demonstrates the best results in Hungates, where a difference of ~ 7.4 in YFP band intensity could be noted between the TD22 and B10S-T7 strain, while in flat panels this difference is reduced to a factor of ~ 2.9. Although the P_{nifH} driven protein production was higher in flat panels as opposed to yields achieved in Schott flasks (improved by a factor ~ 1.5), there is still a large discrepancy between large scale cultures and small scale cultures.

Section Summary

- The downstream region of the *nifHDK* promoter seems to be of paramount importance for target gene expression
- The $pRhon_5Hi$-2 vector mediated the strongest expression among tested pRho vectors under phototrophic conditions
- Expression of target genes could be strictly repressed by NH_4^+, while nitrogen-limiting conditions (serine or N_2 as the sole nitrogen source) induce target gene expression in the pRhonHi-2 vectors
- Utilizing serine instead of N_2 led to a stronger expression of YFP and to a superior growth behavior
- Target genes could be expressed during anoxygenic photosynthesis
- pRhonHi-2 mediated expression of YFP was affected by the employed culture vessel, which might be caused by altered light conditions
- Co-production of the Mo-nitrogenase had a negative impact on pRhonHi-2 mediated protein production and cell growth. The employment of a *nifHDK* deletion mutant, incapable of producing the Mo-nitrogenase, could overcome this negative impact and led to an increased YFP production and higher growth rates

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2 Evaluation of *R. capsulatus* as an alternative platform organism to *E. coli* for membrane protein expression

In the preceding section the successful construction and characterization of the new P_{nifH} based pRhonHi-2 vector could be demonstrated. The main focus in this section was the actual evaluation of *R. capsulatus* as a platform organism for the heterologous expression of therapeutically relevant membrane proteins. To this end, the organism was evaluated with regard to its ability to accumulate membrane inserted proteins under consideration of the following factors: i) the growth phase ii) the promoter choice and iii) the co-production of Mo-nitrogenase, so that the information obtained by this analysis can be utilized for the optimal exploitation of *R. capsulatus* with regard to membrane protein production. The ability of *R. capsulatus* to produce correctly inserted membrane proteins was compared to that of the most frequently employed bacterial expression platform for membrane protein production: the *E. coli* strains BL21 (DE3) and its derivative C43 (DE3), which is optimized for the production of toxic membrane proteins (**I.2.3.1**). Two different expression plasmids were employed for this study, the T7 promoter based pRhotHi-2 vector and the newly developed pRhonHi-2 vector. While the pRhotHi-2 vector was applied to expression hosts *E. coli* BL21 (DE3), C43 (DE3) and *R. capsulatus* B10S-T7 for P_{T7} driven protein production, the vector pRhonHi-2 was utilized in the expression strains *R. capsulatus* B10S and TD22 for P_{nifH} regulated gene expression. The named expression platforms were employed for the heterologous expression of ten therapeutically relevant membrane proteins that will be given a short introduction in the following section.

2.1 Membrane proteins analyzed in this study

In order to ascertain that *R. capsulatus* is capable of expressing topologically and functionally diverse membrane proteins, ten therapeutically relevant membrane proteins were selected for this study. The membrane proteins were chosen primarily according to their physiological function, their topology and their originating organism. As discussed in the introduction, membrane proteins take part in four major biological processes: cell-cell interactions (**I.1.2.1**), solute transport (**I.1.2.2**), signal transduction (**I.1.2.3**) and energy metabolism (**I.1.2.4**). To ensure that *R. capsulatus* is capable of producing physiologically diverse membrane proteins, at least one protein involved in each of the four listed major biological processes was selected. The relevant characteristics of the analyzed membrane proteins are summarized in **Table III-2**.

Table III-2: Relevant features of membrane proteins analyzed in this study

Name	Abbreviation	Origin	MW (kDa)*	TM	Therapeutic relevance	Ref.
Myelin oligodendrocyte glycoprotein	MOG	*Homo sapiens*	33.5	3	Multiple sclerosis, acute disseminated encephalomyelitis, neuromyelitis optica, Narcolepsy	[250-253]
Myelin associated glycoprotein	MAG	*Homo sapiens*	69.0	1	HIV, kearns-sayre syndrome, peripheral neuropathy and multiple sclerosis, schizophrenia	[254-258]
Viral protein Unique	VpU	HIV-1 and SIV_{CPZ}	9.2	1	Aquired Immune Deficiency Syndrome	[259]
Aquaporine 4	AQP4	*Homo sapiens*	34.8	6	Neuromyelitis optica	[260]
Solute carrier family 30, member 8	SLC30A8	*Homo sapiens*	35.0	6	Diabetes mellitus type 2	[261]
Bacteriorhodopsin	BR	*Halobacterium salinarum*	28.2	7	GPCR prototype	[262]
Adenosine A_{2A} receptor	$A_{2A}R$	*Homo sapiens*	44.7	7	Alzheimer, Parkinson, Lesch-Nyhan syndrome, Creutfzeld-Jakob, Huntington, Insomnia, pain and drug addiction	[156, 263]
Angiotensin II receptor type 1	At1AR	*Homo sapiens*	41.0	7	Renal dysplasia and hypertension	[264, 265]
Cholinergic receptor, muscarinic 3	CHRM3	*Homo sapiens*	66.1	7	Urinary bladder disease	[266]
Chemokine receptor 4	CXCR4	*Homo sapiens*	39.7	7	HIV, breast cancer, WHIM	[267-269]

*calculated by ExPASy ProtParam tool (http://web.expasy.org/protparam/), TM = no. of transmembrane helices

As proteins playing pivotal roles in myelination of nerves, the glycoproteins Myelin oligodendrocyte glycoprotein (MOG) and Myelin-associated glycoprotein (MAG) were chosen. Among other functions, these proteins are assumed to be important as adhesion molecules providing structural integrity of myelin sheaths and thus belong to the group of proteins involved in cell-cell interactions. Structurally, both proteins are integral membrane proteins with MOG exhibiting three and MAG possessing one α-helical transmembrane domain (TM). Another protein of this group is the Viral protein unique (VpU) from the human immunodeficiency virus (HIV-1). This single-spanning integral membrane protein is important for the enhancement of virion release and can be found in the membranes of infected cells but not in the virus particles itself. The biological process Solute transport was covered by the water channel protein Aquaporine 4 (AQP4) and the zinc-efflux transporter solute carrier family 30 member 8 (SLC30), both of which are membrane proteins exhibiting six α-helical TMs. As an exemplary protein important for the biological process energy metabolism, the light driven proton pump Bacteriorhodopsin (BR) from *H. salinarum* was chosen. This integral membrane protein is characterized by seven α-helical TMs and important for converting light energy into chemical energy, by providing the light driven formation of a proton gradient across the membrane (**I-1.2.4**). Lastly, proteins important for signal transduction were represented by four GPCRs (**I-1.2.3**): angiotensin II receptor type2 (At1aR), adenosine A_{2A} receptor ($A_{2A}R$), chemokine receptor 4 (CXCR4) and the cholinergic receptor muscarinic 3 (CHRM3). As key receptors for transducing external signals across the membrane, these membrane proteins are all exhibiting seven α-helical TMs.

All membrane proteins analyzed in this work belong to the class of integral membrane proteins, which traverse the membrane by α-helical domains (**I-1.1.3**). The list of membrane proteins illustrates a diverse set of proteins that are predominantly human in origin but are complemented by a viral as well as an archaeal protein. Likewise, the list contains single-spanning as well as multi-spanning membrane proteins exhibiting up to seven TMs, so that the versatility of *R. capsulatus* to express topologically different membrane proteins can be investigated. The therapeutic relevance of the analyzed membrane proteins is ascribed to their status as diagnostic markers for certain diseases (e.g. AQP4), their direct involvement in the pathogenesis of a particular disease (e.g. At1aR) or their role as prototypes for drug targets (e.g. BR). By choosing this assortment of membrane proteins, the conducted expression study will allow the evaluation of *R. capsulatus* as an expression platform for a broad range of therapeutically relevant membrane proteins from different organisms.

2.2 Construction of pRho vectors containing genes encoding target membrane proteins

With the exception of the genes encoding BR and VpU, all target genes were provided in commercially available plasmids by the biotech company Protagen AG (Dortmund, Germany). Genes encoding the membrane proteins were amplified by PCR in order to introduce specific restriction sites to the 5´ and 3´ ends. The recognition site for *Nde*I was added to the 5´ end, whereas the recognition site for the enzyme *Xho*I was introduced to the 3´ end, except for the gene encoding CHRM3 (*Sal*I). Subsequently, the resulting PCR products were inserted into vectors pRhotHi-2 and pRhonHi-2 by means of molecular cloning (**II.11.1**). In contrast to the other genes, the gene encoding VpU was obtained by gene synthesis and the gene for BR had been already available in the vectors pRhotHi-2 [242] and pRhonHi-2 [270] for this work. All genes were cloned in a manner, which upon expression resulted in fusion proteins comprised of the protein of interest and a C-terminal His_6-tag that facilitates immunological detection and affinity purification of the particular protein.

2.3 Influence of membrane protein expression on cell growth

Typically, the growth curve of a batch culture is characterized by an initial phase of adaptation to growth conditions (lag phase) that is succeeded by the logarithmic growth phase, in which cells exhibit constant doubling rates. Enrichment of waste and nutrient depletion leads to a steady state situation, where the growth rate and the death rate are equal (stationary phase). After a period of time the cells die, upon which the growth curve declines (death phase) [271]. Since the shape of a growth curve is characteristic

for a bacterial culture under specified growth conditions, changes in the growth behavior can be indicative of cellular stress. This stress can be induced by a number of different factors, including the production of membrane proteins. The toxicity of membrane proteins is a well-known bottleneck in heterologous membrane protein production and is attributed to the protein´s inherent toxicity or to toxicity mediated by metabolic overload, altered membrane integrity or altered metabolism as described in **I.2.2.2**. Potential bottlenecks such as toxicity and stress behavior need to be comprehended and addressed in order to evaluate a new expression system correctly.

Does expression of therapeutically relevant membrane proteins affect cell growth in *E. coli* and *R. capsulatus*?

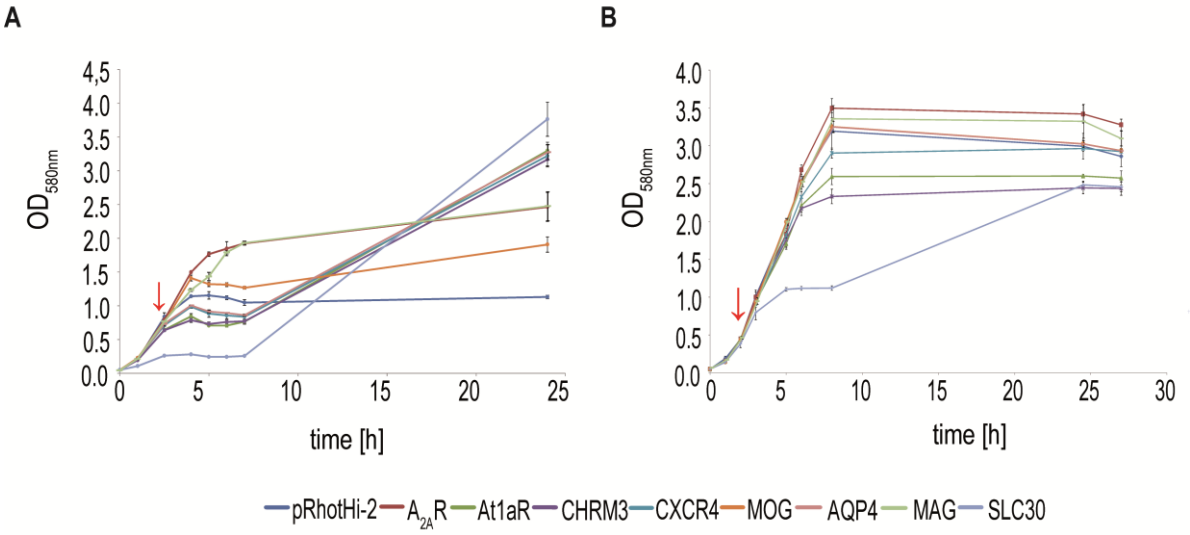
In order to ascertain that production of therapeutically relevant membrane proteins does not represent a potential bottleneck, the impact of membrane protein expression on cell growth was analyzed by classical growth curve experiments in the *E. coli* strains BL21 (DE3) and C43 (DE3), and analogously in the strains *R. capsulatus* B10S-T7, B10S and TD22.

2.3.1 Growth behavior of *E. coli* strains expressing therapeutically relevant membrane proteins

Competent cells of *E. coli* strains BL21 (DE3) and C43 (DE3) were transformed by heat-shock treatment (**II.10.2**) and the resulting expression strains cultivated under selective pressure to maintain plasmids at all times. Main cultures were grown under standardized conditions in LB medium, while target gene expression was induced in cultures upon reaching an $OD_{580\ nm}$ ~ 0.5, by addition of 0.4 mM IPTG. Growth curves were determined by measuring the $OD_{580\ nm}$ over a period of 24 h. The obtained growth curves for *E. coli* cells are illustrated in **Graph III-3**.

The growth curve experiments in *E. coli* demonstrate that induction (red arrow) of membrane protein production affects cell growth differently depending on the expression strain and the target protein. As can be seen in the **Graph III-3A**, upon induction of gene expression in the *E. coli* strain BL21 (DE3), a marked stagnation in cell growth can be noted for all proteins except MAG and $A_{2A}R$. This slow-down in cell growth holds for approximately five hours, after which the culture either recuperates (SLC30,

CXCR4, CHRM3, At1aR, MAG) or goes into a stationary growth phase that is characterized by comparatively lower optical densities after 24 h (MOG, $A_{2A}R$ and pRhotHi-2).



Graph III-3: Growth behavior of *E. coli* BL21 (DE3) and *E. coli* C43 (DE3) strains expressing therapeutically relevant membrane proteins
Illustrated are growth curves of two different *E. coli* strains expressing therapeutically relevant membrane proteins analyzed in this study. The pRhotHi-2 vector containing target genes, as well as an empty vector, were introduced *into E. coli* strain BL21 (DE3) (A) and the strain C43 (DE3) (B). 50 ml LB medium supplemented with kanamycin was inoculated with cells containing plasmids encoding the protein of interest. Cultures were grown aerobically under shaking and gene expression induced by addition of 0.4 mM IPTG (red arrow). Optical density (580 nm) was measured regularly over a period of 24 - 27 h. The growth curve analysis demonstrates major differences with respect to the employed *E. coli* strain and the expressed protein. Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations. MAG = Myelin associated glycoprotein, MOG = Myelin oligodendrocyte glycoprotein, SLC30 = Solute carrier family 30, member 8, AQP4 = Aquaporine 4, $A_{2A}R$ = Adenosine receptor A2A, CHRM3 = Cholinergic receptor, muscarinic 3, At1AR = Angiotensin II receptor type 1, CXCR4 = Chemokine receptor 4. All membrane proteins analyzed in this study were expressed as fusion proteins with a C-terminal His_6-tag.

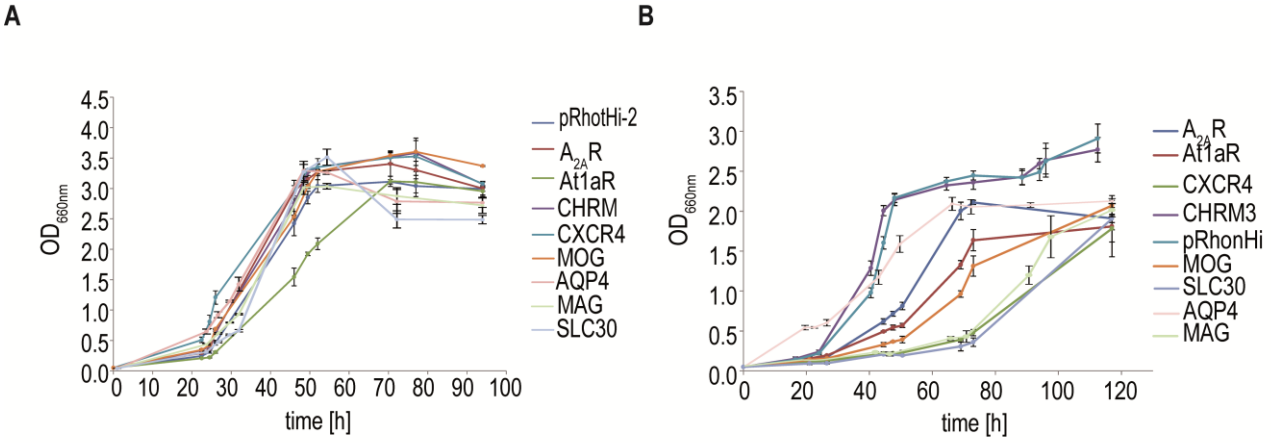
The highest toxicity among analyzed membrane proteins was observed for cultures expressing the SLC30 protein, since they are characterized by a growth phenotype that is inferior to all the other cultures, even before protein production is induced by IPTG. In contrast, cultures producing the $A_{2A}R$ or MAG protein illustrated the least affected growth behavior, demonstrated by the fact that cultures are characterized by a typical growth curve without a stagnating period after IPTG induction. Surprisingly, the empty vector pRhotHi-2 demonstrated toxic effects upon IPTG treatment, which seems unusual considering that the empty vector does not contain a target gene. A closer look into the DNA sequence of the empty vector reveals a coding sequence of approximately 100 bp, which obviously imposes cellular stress upon expression. However, this peptide is not translationally fused or co-produced in vectors that contain a target gene, since molecular cloning removes this particular DNA-fragment.

A different growth behavior could be noted for *E. coli* C43 (DE3) cells expressing membrane proteins (**Graph III-3B**). While in the strain BL21 (DE3) nearly all proteins affected cellular growth upon induction, the reverse could be observed in the C43 (DE3) strain. Here, nearly all cultures exhibited a typical curve progression without any stagnation. This observation was not surprising, considering that this strain was selected in a screening for mutants that are especially suited for coping with toxic effects mediated by membrane proteins [176]. However, as in the strain BL21 (DE3), cultures expressing the protein SLC30 were characterized by a reduced growth behavior. Considering that out of all the analyzed membrane proteins this protein mediated the highest toxicity, even in the C43 (DE3) strain, it is very likely that SLC30 features an inherent toxicity, which is most probably attributed to its physiological function as a zinc-efflux transporter.

In summary, expression of therapeutically relevant membrane proteins affected cell growth negatively in the BL21 (DE3) strain upon induction by IPTG, while the reverse was true for its derivative C43 (DE3). Considering that positive effects of the C43 (DE3) strain are attributed to mutations leading to a reduced transcription of target genes, it can be argued that the reduced growth in the BL21 (DE3) strain is probably caused by a stronger expression of genes encoding membrane proteins.

2.3.2 Growth behavior of *R. capsulatus* strains expressing therapeutically relevant membrane proteins

Membrane protein expression was performed in *R. capsulatus* utilizing the pRhotHi-2 expression vector and the pRhonHi-2 vector to compare the membrane protein expression in *R. capsulatus* with respect to the employed promoter. Since *R. capsulatus* is characterized by a different growth behavior and metabolism than *E. coli*, the growth curve determination was conducted for a longer period of time (95 – 115 h). The pRhotHi-2 vectors containing the target genes were introduced into B10S-T7 cells, whereas the pRhonHi-2 vector was introduced into the wild type strain B10S by conjugation. Precultures were grown under standardized photoheterotrophic conditions in small scale culture vessels (**II.9.1.2**). Target gene expression was induced in the main cultures from the start by addition of 8 mM fructose in the B10S-T7 strain (pRhotHi-2 vector) and by inducing nitrogen-limiting conditions (serine) in the B10S strain (pRhonHi-2 vector). The result of this growth curve determination is depicted in **Graph III-4**.



Graph III-4: Growth behavior of *R. capsulatus* strains B10S and B10S-T7 expressing therapeutically relevant membrane proteins

Membrane proteins were heterologously expressed in *R. capsulatus* strain B10S-T7 by pRhotHi-2 mediated expression (A) and in the strain B10S by employing the pRhonHi-2 vector (B). While gene expression in the strain B10S-T7 was induced by addition of fructose, protein production in the B10S strain was triggered by nitrogen-limiting conditions (serine as the sole nitrogen source). Cultures expressing membrane proteins were grown photoheterotrophically in small scale (Hungates, 10 ml) and the optical density (660 nm) measured in order to obtain the illustrated growth curves. Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations. MAG = Myelin associated glycoprotein, MOG = Myelin oligodendrocyte glycoprotein, SLC30 = Solute carrier family 30, member 8, AQP4 = Aquaporine 4, $A_{2A}R$ = Adenosine receptor A2A, CHRM3 = Cholinergic receptor, muscarinic 3, At1AR = Angiotensin II receptor type 1, CXCR4 = Chemokine receptor 4. All membrane proteins analyzed in this study were expressed as fusion proteins with a C-terminal His_6-tag.

A comparison between the two strains illustrates significant differences in their general growth behavior, when expressing membrane proteins. In the B10S-T7 strain nearly all cultures are characterized by a typical curve progression, without any visible signs of cellular stress (**Graph III-4A**). An exception can be seen in cultures expressing the GPCR At1aR, here a slightly reduced growth can be observed. A completely different growth behavior is reflected in cultures utilizing the pRhonHi-2 vector for membrane protein expression (**Graph III-4B**). Similar to the *E. coli* strain BL21 (DE3) (**Graph III-3A**), a different impact on the cell growth could be noted depending on the target protein. While some proteins did not show any distinct effect on cell growth ($A_{2A}R$, CHRM3, AQP4), the expression of other membrane proteins resulted in a prolonged lag phase with varying degrees of prolongation. The strongest influence was mediated by the proteins CXCR4, SLC30 and MAG, where a stagnation of growth could be observed for nearly 72 h. No noticeable impact on growth behavior could be observed for the *R. capsulatus* B10S-T7 strain. In contrast, just like the *E. coli* strain BL21 (DE3), the *R. capsulatus* strain B10S was characterized by very different curve progression with regard to the expressed membrane protein. This similarity is further emphasized by the observation that the SLC30 protein mediates the highest toxicity, while the protein $A_{2A}R$ conveys the weakest cellular stress. Although there were also differences observed for some proteins (e.g. MAG´s toxicity in B10S), these parallels beg the question, if these discrepancies in growth behavior between the B10S-T7 and B10S strain are attributed to

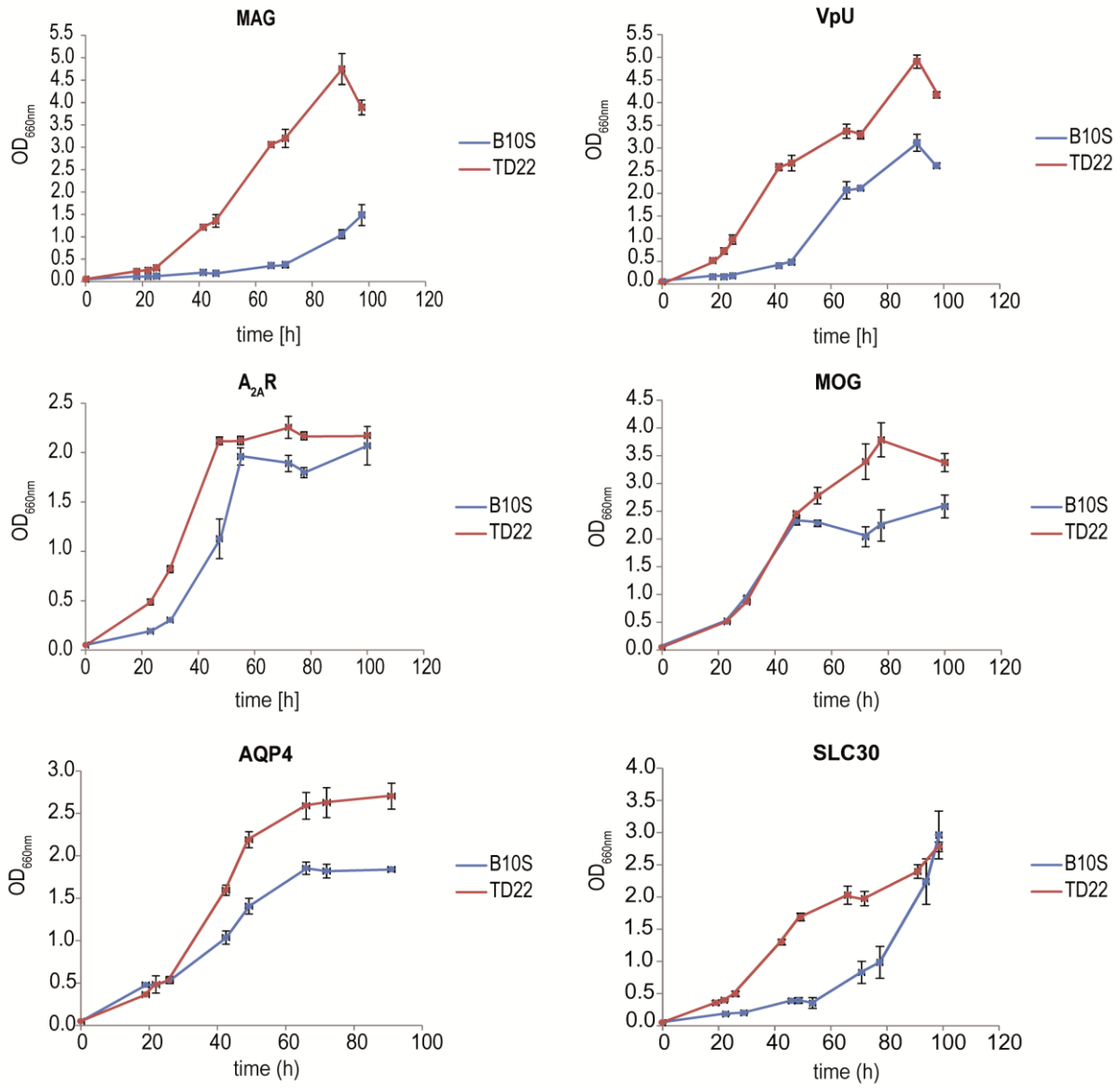
differences in their ability to express membrane proteins or the deployed nitrogen-assimilation metabolism. Since B10S-T7 cells are supplemented with NH_4^+ in the cultivation medium, cultures are growing under nitrogen-sufficient conditions, whereas for the pRhonHi-2 driven expression of membrane proteins cells are cultivated under nitrogen-limiting conditions. In the preceding section, a negative impact of Mo-nitrogenase production on the growth phenotype could already be demonstrated (**Graph III-2B**). Hence, there is a distinct possibility that the observed effect might be attributed to growing the cultures under nitrogen-limiting conditions.

Is the observed negative effect on growth in *R. capsulatus* ascribed to the co-production of the Mo-nitrogenase?

In order to address the possible impact of Mo-nitrogenase production on the observed reduced growth phenotype, a comparative analysis with the TD22 strain was conducted (**Graph III-5**).

As can be deduced from the curve shapes, the negative impact on cell growth could be nearly completely abolished in the deletion mutant strain, irrespective of the analyzed membrane protein. TD22 strains expressing membrane proteins illustrate a typical growth curve, which is distinctly different from their counterparts, as it lacks the prolonged lag phase or the reduced growth phenotype. Although the distinct negative growth effect observed in the wild type is not present in the mutant strain, there are still differences in the final $OD_{660\ nm}$ with respect to the analyzed membrane protein. While TD22 cultures expressing proteins VpU or MAG reach $OD_{660\ nm} = 4$, cultures expressing membrane proteins $A_{2A}R$, AQP4 or SLC reach no more than $OD_{660\ nm} = 2 – 2.5$. This leads to the conclusion that the co-production of Mo-nitrogenase has indeed a severe impact on membrane protein mediated stress induction but differently with regard to the protein, since all cultures do not show the same curve progression.

Furthermore, for some proteins even the utilization of the TD22 strain leads to an altered growth phenotype in that that they illustrate a reduced final biomass compared to the B10S strain expressing the same protein, which indicates that these proteins mediate cellular stress that is irrespective of the co-produced Mo-nitrogenase.



Graph III-5: Growth behavior comparison of *R. capsulatus* B10S and TD22 cultures expressing membrane proteins

Illustrated are representative graphs depicting growth curves of B10S and TD22 cultures expressing therapeutically relevant membrane proteins with the pRhonHi-2 vector. Cells were grown photoheterotrophically in RCV minimal medium supplemented with serine as the sole nitrogen source. Comparison of growth curves illustrates the impact of Mo-nitrogenase production on membrane protein expression mediated cellular stress observed in the strain B10S. Values are means of triplicate measurements. Error bars indicate the corresponding standard deviations. MAG = Myelin associated glycoprotein, MOG = Myelin oligodendrocyte glycoprotein, SLC30 = Solute carrier family 30, member 8, AQP4 = Aquaporine 4, $A_{2A}R$ = Adenosine receptor A2A, VpU = viral protein U. All membrane proteins analyzed in this study were expressed as fusion proteins with a C-terminal His_6-tag.

2.4 Optimization of protein sample preparation

For this work, membrane proteins were fused with a C-terminal His_6-tag for immunological detection. To this end, proteins were separated by SDS-PAGE (**II.12.5**) and subsequently transferred to PVDF membranes by Western blotting (**II.12.6**). Afterwards, antibodies raised against the His-epitope were used to specifically detect the target protein on the membranes. In this manner, the immunological detection enables conclusions about the capability of the expression host to express a particular membrane protein. Initial expression studies with whole cell extracts from *E. coli* BL21 (DE3) and C43 (DE3) led to an unusual observation for the membrane proteins SLC30, AQP4 and $A_{2A}R$. As an example, Western blot analyses of the proteins SLC30 and AQP4 are depicted in **Figure III-9**.

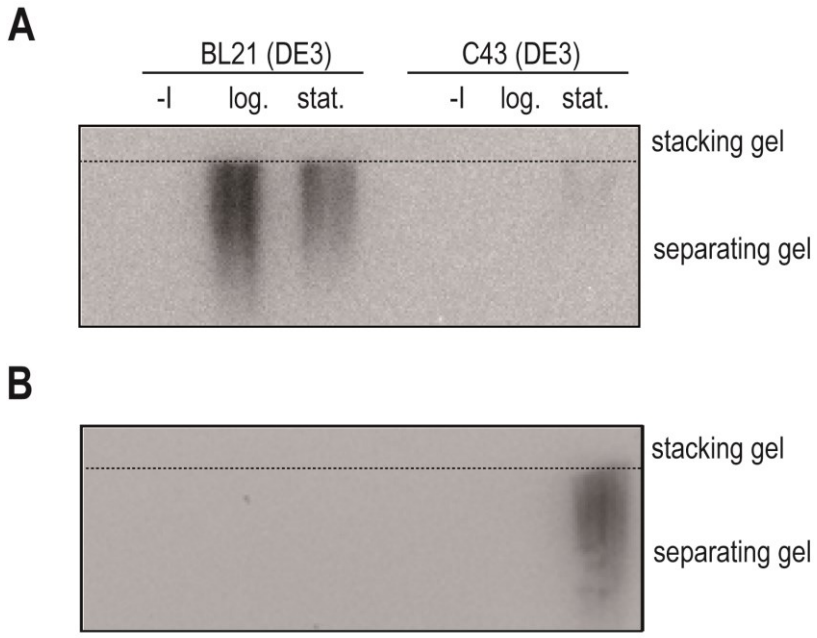


Figure III-9: Protein aggregation observed in SDS gels
Whole cell extracts of *E. coli* strains BL21 (DE3) and C43 (DE3), expressing AQP4 (A) or SLC30 (B), were subjected to SDS-PAGE. Subsequently, proteins were transferred to PVDF membranes by electroblotting and detected by immunological analysis with the help of His-tag specific antibodies. Special attention shall be directed towards fuzzy bands between the interphase of stacking and separating gel. These bands correspond to the protein of interest and indicate protein aggregates that are stuck in the stacking gel. -I = uninduced, log. = logarithmic, stat. = stationary

The illustrations show a fuzzy band at the intersection between the stacking gel and the separating gel, which corresponds to the protein of interest. Since this experiment was repeated several times, the observed effect was deemed genuine and is most probably the result of protein aggregation that leads to the proteins to be stuck in the separating gel.

The aggregation of proteins is a phenomenon already described for some membrane proteins [272], including the membrane protein SARS-CoV [273]. Boiling SARS-CoV membrane protein leads to protein aggregates that are stuck in the stacking gel. Since the problems seem similar, one explanation for the observed effect may be ascribed to thermal aggregation of the analyzed membrane proteins.

Does boiling samples prior to SDS-PAGE lead to protein aggregation?

In order to determine whether boiling of samples causes protein aggregation, membrane fraction of *E. coli* cells expressing the membrane protein of interest was mixed with concentrated Laemmli buffer and either incubated for 10 min at 99 °C or for 30 min at 37 °C. Subsequently, these samples were separated by SDS-PAGE and analyzed by immunological methods. As an example, the impact of heat treatment is illustrated for the GPCR $A_{2A}R$ in **Figure III-10**.

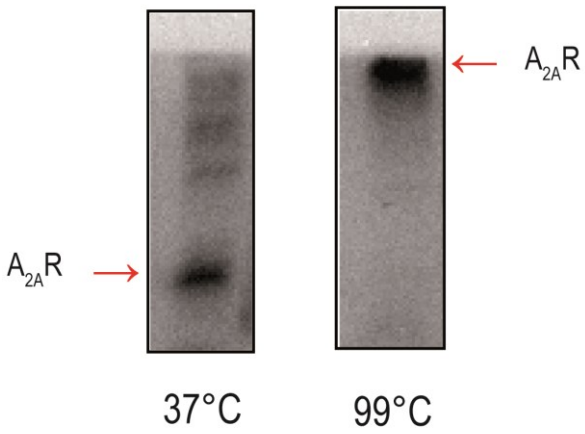


Figure III-10: Thermal aggregation of $A_{2A}R$ proteins
$A_{2A}R$ was heterologously expressed in the bacterial strain *E. coli* BL21 (DE3). Membranes of these cells were mixed with Laemmli buffer and either boiled for 10 min at 99 °C (right panel) or incubated for 30 min at 37 °C (left panel). Subsequently, samples were subjected to SDS-PAGE and proteins transfered to PVDF membranes. Membranes were analyzed with respect to the presence of the His-tag fused to the $A_{2A}R$ protein by employing His-tag specific antibodies.

The Western blot analysis demonstrates very clearly that boiling samples causes aggregation of the protein. Samples incubated at 37 °C give a band corresponding to $A_{2A}R$, whereas boiling samples leads to SDS-resistant aggregates that remain in the stacking gel. In conclusion, boiling treatment was avoided for aggregation prone proteins ($A_{2A}R$, SLC30, AQO4, At1aR, CHRM3, CXCR4), whereas the remaining proteins were routinely subjected to incubation at 99 °C prior to SDS-PAGE.

2.5 Insertion of therapeutically relevant proteins into *R. capsulatus* and *E. coli* membranes employing different expression strains

Many β-barrel membrane proteins can be successfully overproduced in the common expression host *E. coli* as inclusion bodies, from which they can be easily refolded into their native conformation [274]. However, the same is not true for integral membrane proteins of the helix bundle type. These proteins can rarely be refolded from inclusion bodies, which is why they need to be expressed in a manner which leads to proper insertion into the membrane. Seeing that all of the membrane proteins analyzed in this study are α-helical bundle membrane proteins, the evaluation of *R. capsulatus* and *E. coli* was based on the organism's ability to insert membrane proteins into their membrane. To this end, immunological detection of samples was performed exclusively with proteins obtained from membrane fractions of cell extracts, in order to ensure detection of correctly localized membrane proteins.

To exploit the organism´s specialized membrane physiology, the cultivation of *R. capsulatus* expression strains was carried out under bulb light illumination with NH_4^+ as the sole nitrogen source for the T7 expression strain *R. capsulatus* B10S-T7 and serine as the sole nitrogen source for the *R. capsulatus* strains B10S and TD22. Induction of target gene expression was realized by directly inducing nitrogen-limiting conditions in the B10S or TD22 expression strains, whereas induction of T7 RNAP driven expression in *R. capsulatus* B10S-T7 cells was realized by addition of 8 mM fructose into the culture medium. In contrast, target gene expression in *E. coli* cells was not directly induced, but started when expression cultures reached a particular optical density ($OD_{580\ nm}$ = 0.5 – 0.7). To analyze effects of growth phase on membrane protein insertion, samples were taken during the logarithmic and stationary growth phase and subsequently fractionated.

Accumulation profiles of the different proteins were evaluated with respect to the following questions.

Are proteins inserted into the membrane of *E. coli* or *R. capsulatus*?

As a starting point, the proteins were classified according to their tendency to accumulate in the membranes of either *E. coli* or *R. capsulatus*. The result of this classification is illustrated in **Figure III-11**.

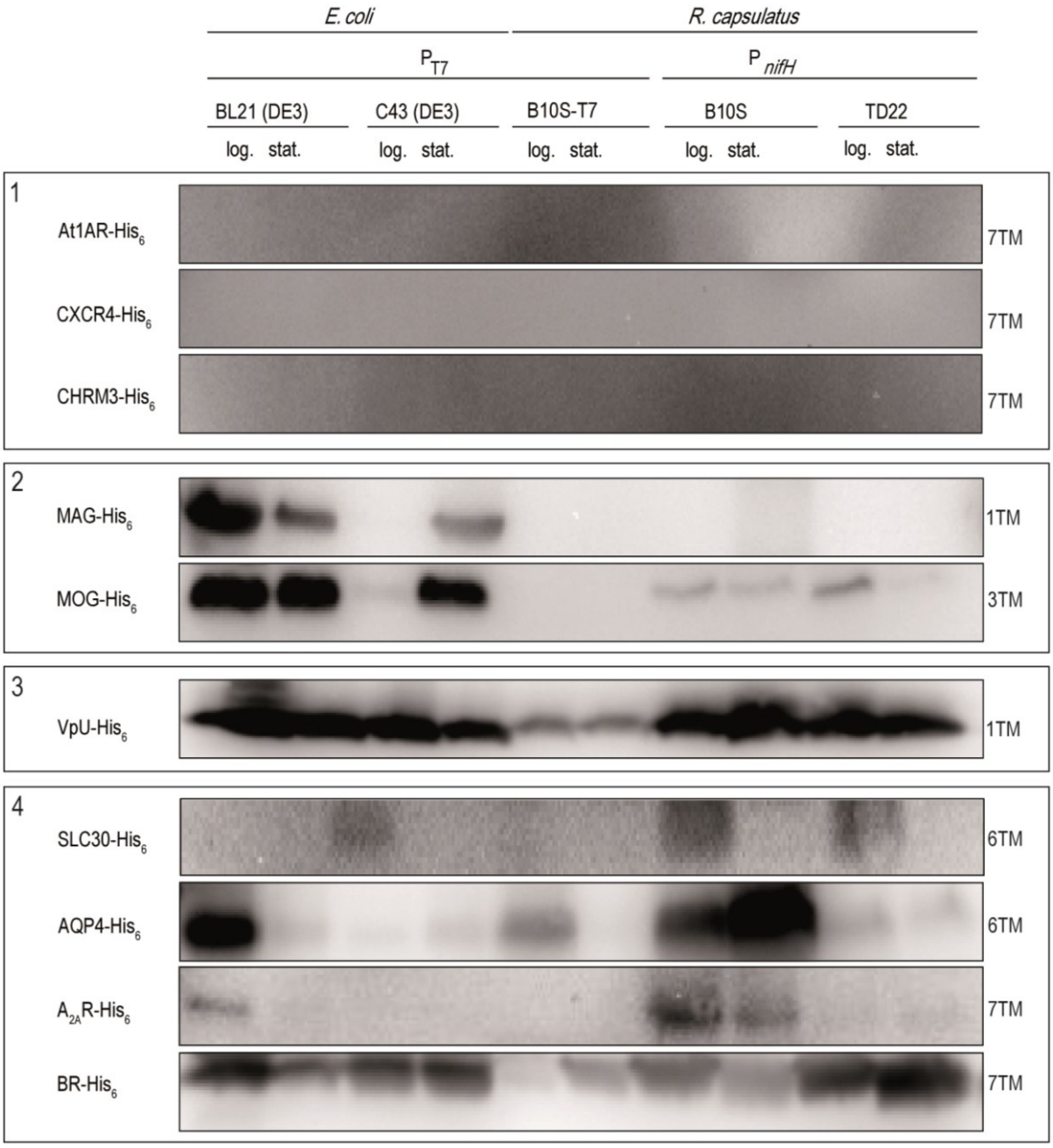


Figure III-11: Immunological detection of therapeutically relevant proteins in the membrane of different *E. coli* and *R. capsulatus* strains

Several human proteins, in addition to a viral (VpU) and an archaeal protein (BR), were expressed in the hosts *E. coli* and *R. capsulatus.* P_{nifH} driven expression was conducted in the *R. capsulatus* wild type strain B10S or the *nifHDK*-deletion mutant TD22, whereas P_{T7} driven expression was conducted in the bacterial strains *R. capsulatus* B10S-T7, *E. coli* BL21 (DE3) and *E. coli* C43 (DE3). Samples were taken from different growth phases, fractionated and membrane fractions subjected to Western blot analysis. According to their accumulation profile, membrane proteins were divided into four groups. Class 1: Membrane proteins that could not be inserted into the membrane of either expression host. Class 2: Membrane proteins which exhibited the highest accumulation in membranes of *E. coli* cell extracts. Class 3: Proteins were equally well accumulated in membranes of *R. capsulatus* and *E. coli* cells. Class 4: Membrane proteins accumulated at higher quantities in *R. capsulatus* membranes. TM = transmembrane helices, log. = logarithmic, stat. = stationary, MAG = Myelin associated glycoprotein, MOG = Myelin oligodendrocyte glycoprotein, SLC30 = Solute carrier family 30, member 8, AQP4 = Aquaporine 4, $A_{2A}R$ = Adenosine receptor A2A, VpU = Viral protein U, BR = Bacteriorhodopsin, CHRM3 = Cholinergic receptor, muscarinic 3, At1AR = Angiotensin II receptor type 1, CXCR4 = Chemokine receptor 4. Proteins were detected by immunological analysis with the help of His-tag specific antibodies.

Class 1 membrane proteins, which are comprised exclusively of GPCRs (CXCR4, CHRM3, At1AR), could not be accumulated in the membrane of either of the tested expression host. Class 2 membrane proteins, which include the human membrane proteins MAG (1TM) and MOG (3TM), are membrane proteins that demonstrated the highest protein accumulation in membranes of the *E. coli* strain BL21 (DE3). The viral protein VpU represents class 3 and was equally well accumulated in the membrane of *E. coli* BL21 (DE3) and *R. capsulatus* (pRhonHi-2 vector). Class 4 membrane proteins were identified by the highest protein accumulation in membranes of *R. capsulatus* cells utilizing the pRhonHi-2 vector. This class includes membrane proteins with a higher number of TMs, such as AQP4 (6TM), SLC30 (7TM), $A_{2A}R$ (7TM) and BR (7TM).

The successful production of membrane proteins can be affected by many factors. One of those factors can be the growth phase, considering that host-specific genes are expressed or inhibited accordingly.

Does the growth phase affect accumulation of membrane inserted proteins?

While the membrane protein VpU showed a membrane insertion behavior that was seemingly unaffected by the growth phase, for all other proteins a growth phase dependent effect in both organisms could be noted. Depending on the expression host and the membrane protein higher quantities of membrane inserted proteins were observed either in the logarithmic growth phase or the stationary growth phase. The proteins $A_{2A}R$, MAG and AQP4 could only be detected in the membranes of logarithmically grown *E. coli* BL21 (DE3) expression cultures, while a signal in the stationary growth phase of the same cultures was reduced or completely abolished. However, for AQP4 this effect seems to be tied to the expression system, since *R. capsulatus* B10S cultures expressing AQP4 with the pRhonHi-2 vector displayed a higher accumulation of the protein in the stationary growth phase. Similarly, the proteins MOG and MAG illustrated a higher protein accumulation in the stationary growth phase, when expressed in *E. coli* C43 (DE3).

Apart from the growth phase, the utilized promoter can have a major impact on successful membrane protein insertion, since the promoter determines the strength and the manner in which a target gene is expressed.

Does the utilized promoter affect production of membrane inserted protein?

The T7 RNAP driven production of membrane proteins in *R. capsulatus* was in most cases not successful, whereas the P_{nifH} driven protein synthesis showed a much higher protein accumulation for most of the proteins (VpU, BR, SLC30, $A_{2A}R$, MOG and AQP4).

For the *E. coli* strains BL21 (DE3) and C43 (DE3) the pRhotHi-2 vector was utilized for membrane protein production. Although in both strains gene expression is regulated by the same promoter, the strength of mediated gene expression is different as described before (**I.2.3.1**). BL21 (DE3) cells express target genes stronger than C43 (DE3) cells. This was also confirmed in the conducted expression study, since nearly all proteins ($A_{2A}R$, AQP4, VpU, MAG and MOG) demonstrated a higher membrane accumulation profile in the strain BL21 (DE3). However, not all membrane proteins are produced successfully with a very strong promoter, especially toxic proteins seem to achieve better results with modest promoters [31]. One example protein that could be better accumulated in the strain C43 (DE3) was the zinc-efflux transporter SLC30. Considering cell growth experiments conducted before (**Graph III-3**), it could be argued that SLC30 features an inherent toxicity that affects the amount of protein which can be produced in *E. coli* cells. Since the protein production rate in BL21 (DE3) cells is potentially higher, and might therefore encourage the formation of inclusion bodies, the observed positive effect for SLC30 in the C43 (DE3) strain could be speculated to be attributed to a modest production of the protein.

The last question addressed in the course of the evaluation was the co-production of Mo-nitrogenase during P_{nifH} dependent expression in *R. capsulatus*, which was shown before to impair cell growth (**III.2.3.2**). The co-production of this enzyme may also pose a potential bottleneck for the production of recombinant membrane proteins and consequently their insertion into the membrane.

Does co-production of Mo-nitrogenase influence accumulation of membrane inserted proteins in *R. capsulatus*?

A comparison between the *R. capsulatus* strain B10S and TD22 reveals that membrane proteins were either equally well accumulated (SLC30), displayed a higher accumulation (MOG, BR, VpU) or a

reduced accumulation (AQP4, $A_{2A}R$) in the membranes of the *nifHDK* deletion strain TD22. These results indicate that for some proteins the eliminated co-production of the Mo-nitrogenase seems to have a positive effect, while for other proteins the physiological changes of the mutant seems to override positive effects gained by the impaired production of the Mo-nitrogenase.

Section Summary

- Expression of the majority of therapeutically relevant membrane proteins mediated cellular stress, demonstrated by a reduced growth, in the *E. coli* strain BL21 (DE3) but not in the strain *E. coli* C43 (DE3)
- While production of therapeutically relevant membrane proteins did not affect *R. capsulatus* B10S-T7 cell growth noticeably, cellular growth was severely affected in the strain B10S utilizing the vector pRhonHi-2
- The impaired growth phenotype in the B10S strain could be abolished for nearly all proteins by utilizing the mutant strain TD22
- Heat treatment of samples prior to SDS-PAGE led to aggregation of the proteins $A_{2A}R$, AQP4 and SLC30. Sample preparation protocols could be optimized for those proteins by low temperature incubation.
- GPCRs At1aR, CXCR4 and CHRM3 could not be accumulated in the membrane of the analyzed expression hosts *E. coli* or *R. capsulatus*
- Membrane proteins with a lower number of TMs (MAG and MOG) could be better or equally well (VpU) inserted into the membranes of *E. coli* BL21 (DE3) as opposed to *R. capsulatus*
- Membrane proteins with a higher number of TMs (AQP4, SLC30, $A_{2A}R$ and BR) could be accumulated in higher quantities in the membranes of *R. capsulatus* cells employing the pRhonHi-2 vector
- Growth phase dependent effects could be observed for all proteins (except VpU) in all analyzed expression systems
- Mo-nitrogenase co-production had a positive impact on accumulation of membrane proteins VpU, MAG and MOG, while it had a negative impact on the accumulation of the proteins AQP4 and $A_{2A}R$ in *R. capsulatus*
- In principle, the membrane protein production was more successful in *R. capsulatus* utilizing the expression plasmid pRhonHi-2 instead of pRhotHi-2

3 Optimization of P_{nifH} based synthesis of membrane proteins in *R. capsulatus*

In the preceding section it could be shown that the new expression platform was particularly successful at inserting membrane proteins with a higher number of TMs. Further optimization of membrane protein production in *R. capsulatus* was attempted by addressing potential bottlenecks such as the light condition, the point of induction of gene expression and the codon usage of selected genes encoding membrane proteins. Since expression of membrane proteins was more productive employing the pRhonHi-2 vector, all following experiments for membrane protein production were conducted with the new expression vector in *R. capsulatus*.

3.1 Optimization of illumination conditions

Phototrophic growth of *R. capsulatus* is influenced by many environmental factors. Foremost among these factors is the energy source of photosynthesis: light. Light quality as well as light quantity is a major concern for phototrophic bacteria, since only specified ranges of the light spectrum can be harnessed for photosynthesis. For *R. capsulatus* these regions are limited to the excitation ranges of carotenoids (420 nm – 520 nm) and bacteriochlorophyll *a* (800 nm – 860 nm), respectively [275]. So far, all phototrophic cultivations have been illuminated routinely by bulb light illumination. However, a recently published work by Kaschner *et al.* [275] showed applicability of LEDs with entirely different spectral properties as alternative light sources for the cultivation of *R. capsulatus* (**Figure III-12**).

Compared to 120 high power infrared LEDs, light bulbs are characterized by a distinctly reduced light intensity in the emission ranges 800 nm – 900 nm (red box). Yet, this range of the light spectrum is of major importance for bacteriochlorophyll *a* excitation and consequently for the photosynthesis metabolism of the organism. Considering the elucidated importance of light, altering the illumination conditions may therefore influence the amount and composition of the photosynthetic apparatus of *R. capsulatus* and consequently the heterologous expression of membrane proteins. Furthermore, light intensity is also a major factor for the nitrogen fixation metabolism [276] and may therefore also have an effect on the *nifHDK* promoter present in the pRhonHi-2 vector.

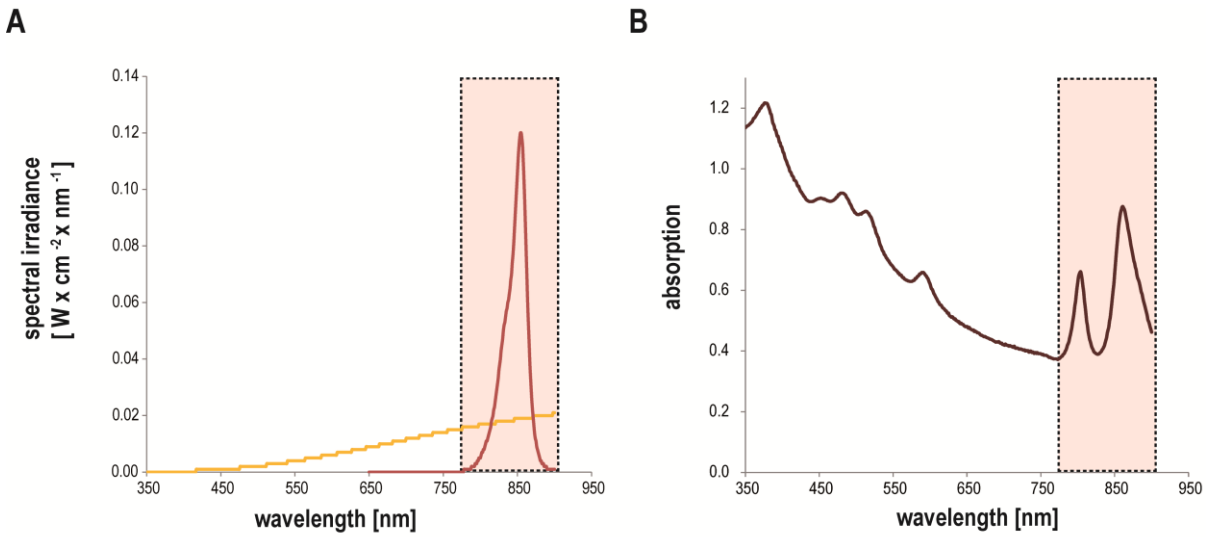


Figure III-12: Spectral characteristics of different light sources employed in this study
(A) Illustrated are spectral irradiance of bulb light panels (yellow line) and infrared-light diode panels (red line) plotted against the wavelength. Bulb light illumination was realized by placing three light bulbs (60 W, Osram, Germany) on each side of the culture vessel at a distance of 25 cm. Likewise, infrared-light diode panels, containing 120 high power infrared LEDs (SFH 4257, Osram, Germany) were placed on both sides of the cultures at a distance of 10 cm for illumination. Spectral irradiance data demonstrates a reduced light intensity of bulb lights compared to infrared-light diodes in the emission range 800 nm - 900 nm. However, an absorption spectrum of *R. capsulatus* B10S cells (B) emphasizes the importance of this portion of the electromagnetic spectrum, since the absorption of whole cells at these wavelengths (red box) is attributed mainly to bacteriochlorophyll α absorption. Source: Kaschner *et al.* [275]

Does altering the illumination condition improve accumulation of membrane inserted proteins in *R. capsulatus*?

To test the effect of altered illumination conditions on production of membrane inserted proteins, a selective assortment of membrane proteins was analyzed. The membrane proteins were chosen based on their originating organism and their topology, so that the impact of different illumination conditions could be tested on a diverse group of membrane proteins. The viral protein VpU, the archaeal protein BR and the human membrane proteins MAG, MOG and $A_{2A}R$ were heterologously expressed in the *R. capsulatus* strains B10S and the mutant strain TD22. To analyze growth dependent effects, samples were taken at different growth phases. Membranes obtained from fractionated cells, grown under bulb light or infrared light LEDs (IR, λ_{max} = 856 nm), were comparatively analyzed by means of immunological detection (**Figure III-13**).

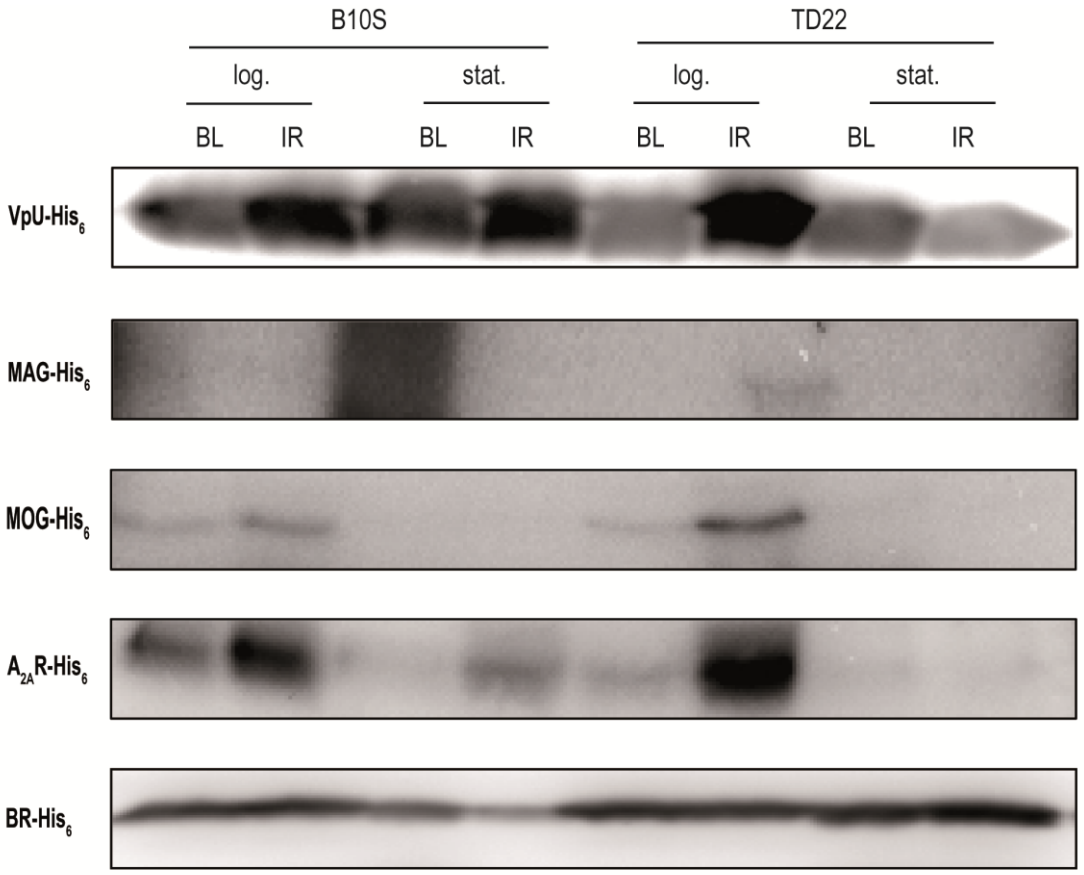


Figure III-13: Immunological detection of membrane inserted proteins from *R. capsulatus* cultures illuminated by different light sources

To improve pRhonHi-2 mediated expression of membrane inserted proteins, a selective assortment of membrane proteins were produced in the *R. capsulatus* strains B10S and TD22. To analyze the impact of altered illumination conditions on membrane protein accumulation, *R. capsulatus* expression cultures were illuminated by either infrared light LEDs (IR) or by bulb light panels (BL). Samples were taken at different growth phases and obtained membrane fractions subjected to western-blot analysis. log. = logarithmic, stat. = stationary, MOG = Myelin oligodendrocyte glycoprotein, BR = bacteriorhodopsin, $A_{2A}R$ = Adenosine receptor A2A, VpU = viral protein U, MAG = Myelin associated glycoprotein

The results revealed a differential effect of infrared LED illumination depending on the analyzed membrane protein. In case of the membrane proteins $A_{2A}R$, MOG, MAG and VpU a positive effect of IR illumination on protein accumulation in the membrane was observed (see lane IR), whereas for the protein BR no significant effect of altered illumination conditions could be noted. A growth phase dependent effect on protein accumulation was observed for the membrane proteins $A_{2A}R$ and MAG, which exhibited a reduced protein accumulation in the stationary growth phase. Remarkably, altering illumination conditions led to the accumulation of MAG in the membrane of *R. capsulatus* TD22 cells, which was not possible in the expression study conducted before (**Figure III-11**).

The observed positive effect was particularly pronounced in the deletion mutant TD22, where a greater accumulation of membrane embedded protein was observed for every protein including the $A_{2A}R$, when cultures were illuminated by infrared light LEDs instead of bulb light. Utilizing infrared light illumination not only abolished the negative effect of the deletion mutant on $A_{2A}R$ production, but enabled a protein accumulation which was greater than in the wild type strain B10S. The only protein which did not illustrate conclusive results was the archaeal proton pump BR. Depending on the expression strain and the growth phase, this protein demonstrated no changes under altered illumination conditions (B10S – log, TD22 – log), reduced accumulation (B10S – stat) or a higher accumulation under infrared light illumination (TD22 – stat). In conclusion, IR illumination led to improved production of membrane inserted protein for the majority of the analyzed membrane proteins.

Aside from the employed light source, the exact moment of induction of target gene expression can be of paramount importance for recombinant protein production [277]. The following section will examine the induction point as a potential factor for membrane protein production in *R. capsulatus*.

3.2 Optimization of the induction point of P_{nifH}-dependent gene expression in *R. capsulatus*

In the preceding sections membrane protein expression and Mo-nitrogenase production could be shown to negatively impact cell growth of *R. capsulatus* B10S cells (**Graph III-5**). Furthermore, the conducted comparative expression study (**Figure III-11**) revealed that some proteins were characterized by a higher protein accumulation in the logarithmic growth phase instead of the stationary growth phase. This, however, can pose a serious drawback for protein production endeavors since logarithmically grown cultures are characterized by a low biomass. To bypass this bottleneck it can be useful to induce protein production at a later growth phase, so that cultures can reach higher optical densities. A delayed start of expression can therefore be particularly beneficial for toxic or stress inducing proteins, which prevent obtaining a high biomass.

Can an auto-induction medium be developed that enables altering the time point of induction?

Can this auto-induction medium be applied to improve pRhonHi-2 mediated production of membrane inserted proteins in *R. capsulatus*?

In order to analyze the effect of the induction point on membrane protein accumulation, the concept of auto-induction medium was introduced into *R. capsulatus*. The pRhonHi-2 expression vector is based on the host specific *nifHDK* promoter, which is strictly repressed by NH_4^+ in the cultivation medium (**III-1**). Therefore, an auto-induction medium can be devised by addition of specifically titrated amounts of NH_4^+. Since target gene expression is induced only after NH_4^+ is completely consumed, increasing concentrations of NH_4^+ should lead to different induction points, so that membrane protein production is initiated at different growth phases of the expression cultures. The impact of auto-induction medium was tested for the same proteins analyzed in the section before, the membrane proteins BR, MOG, MAG, VpU and $A_{2A}R$. The effect of Mo-nitrogenase co-production on utilizing auto-induction medium was tested by comparative expression analysis in the wild type strain B10S and the mutant strain TD22.

Photographic illustrations taken after two days of photoheterotrophic cultivation under IR illumination in RCV minimal medium supplemented with increasing concentrations of NH_4^+ (**Figure III-14)** demonstrate that utilizing auto-induction medium had an impact on growth that was visible with the bare eye.

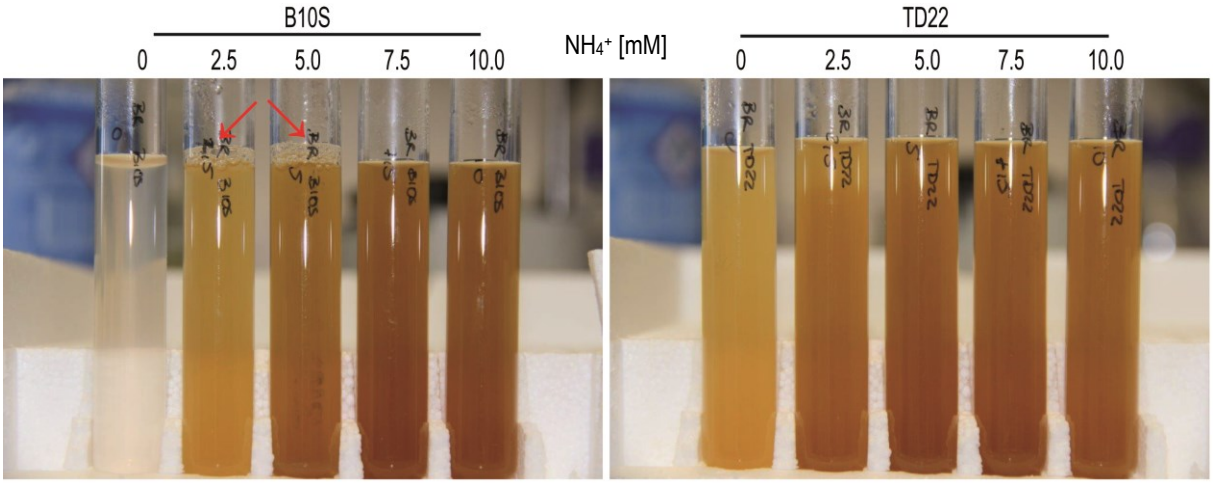


Figure III-14: Photographic illustrations of *R. capsulatus* cultures inducing BR production at different time points
To determine the effect of different points of induction on target gene expression, *R. capsulatus* cultures were treated with 1 mM serine and increasing concentrations of NH_4^+ (0 mM – 10 mM). Since NH_4^+ suppresses target gene expression, induction of BR production was conducted only after complete NH_4^+ consumption, thus achieving an auto-induction effect. Cultures were grown photoheterotrophically for two days under infrared light illumination in RCV-minimal medium and subsequently pictures were taken. Inducing BR expression has a negative impact on cell growth, demonstrated by a reduced turbidity in B10S and TD22 cultures expressing BR in early growth phases (0). Cultures expressing BR are indicated by bubble formation (red arrows), a byproduct of activated *nif*-genes. Pictures were taken by Vera Svensson.

Strongest effects were most noticeable in the wild type strain B10S, since higher pigmentation and higher turbidity is observed with increasing concentration of NH_4^+. Furthermore, the formation of bubbles

in the wild type strain indicates the production of Mo-nitrogenase and consequently the induction of the target protein. The bubble formation is observed only in cultures treated with NH_4^+ concentrations of 2.5 mM or 5 mM, whereas cultures treated without NH_4^+ (0 mM) or with 7.5 mM and 10 mM did not show any bubble formation. Since NH_4^+ inhibits the expression of *nif*-genes, it is not surprising that higher concentrations of NH_4^+, such as 7.5 mM or 10 mM, suppress H_2 production because two days of cultivation are most likely not sufficient to consume these amounts of NH_4^+, so that the Mo-nitrogenase is most probably not produced in these cultures. Bubble formation is not observed in B10S cultures grown under nitrogen-limiting conditions (0 mM), where the highest production of H_2 would be expected. That no bubble formation is observed is most likely attributed to a low biomass of these cultures, resulting in comparatively fewer cells producing hydrogen. The cultures of the mutant strain TD22 seem to show the same trend as B10S cultures, with overall greater turbidity. Indeed, optical density measurements and immunological analysis depicted in **Figure III-15** confirm these observations.

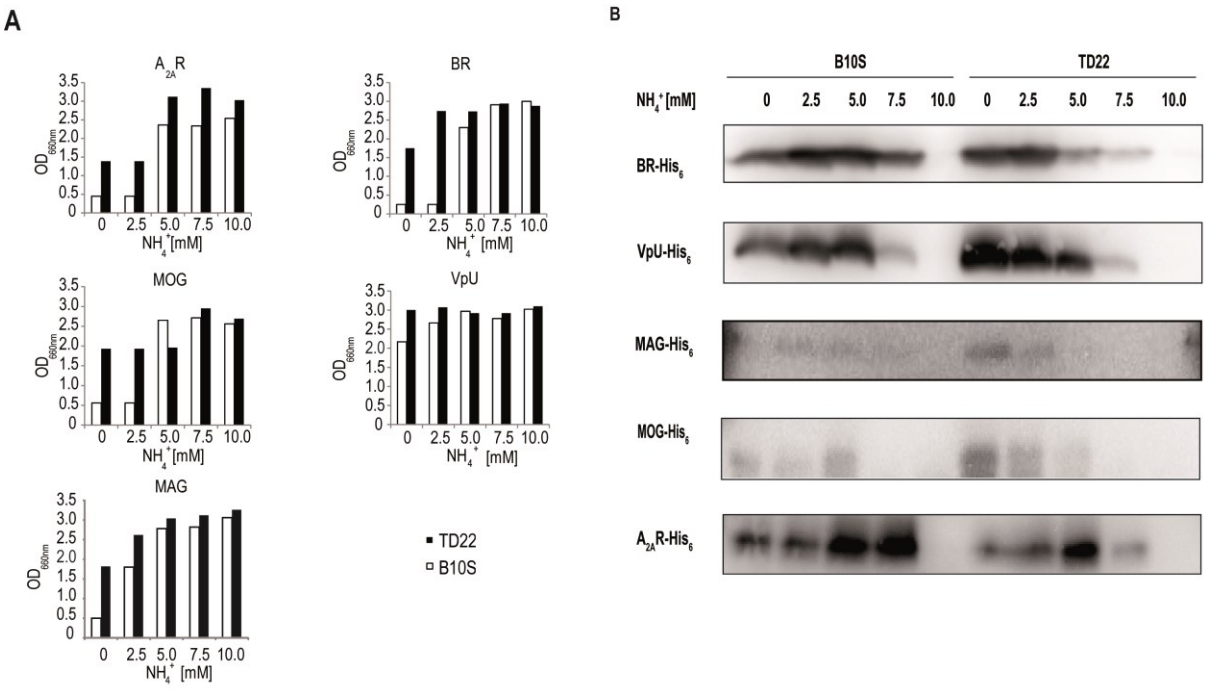


Figure III-15: Influence of auto-induction medium on P_{nifH} mediated production of membrane inserted proteins.

For optimization of the induction point of gene expression, impact of auto-induction point on P_{nifH} driven synthesis of membrane proteins was analyzed under consideration of the optical density (A) and production of membrane inserted proteins of test cultures (B). To determine the effect of different points of induction on target gene expression, cultures were treated with 1 mM serine and increasing concentrations of NH_4^+ (0 mM – 10 mM). Since NH_4^+ suppresses target gene expression, induction of protein production was conducted only after complete NH_4^+ consumption, thus achieving an auto-induction effect. Cultures were grown photoheterotrophically for two days under infrared light illumination and subsequently fractionated. Membrane fractions were subjected to western blot analysis. MOG = Myelin oligodendrocyte glycoprotein, BR = bacteriorhodopsin, $A_{2A}R$ = Adenosine receptor A2A, VpU = viral protein U, MAG = Myelin associated glycoprotein.

Optical density values, measured after two days of cultivation (**Figure III-15A**) of the wild type strain (white bars), illustrate that early induction of target gene expression (0 mM NH_4^+) results in poor optical densities ($OD_{660\ nm}$ < 1), whereas a repression of target gene expression (10 mM NH_4^+) results in distinctly higher optical density values ($OD_{660\ nm}$ > 2.5). An exception to this trend can be seen for the membrane protein VpU, which was characterized by a higher biomass than all the other proteins when gene expression was induced at the beginning of the cultivation ($OD_{660\ nm}$ > 2). A comparison between the wild type strain B10S and the deletion mutant TD22 indicates that for nearly all NH_4^+ concentrations the deletion mutant TD22 displayed higher optical densities than the wild type strain. This difference was particularly pronounced in cultures where membrane protein production was induced in very early growth phases (0 mM NH_4^+ – 2.5 mM NH_4^+), which is in agreement with the growth progression analysis conducted before, where the B10S strain demonstrated slower growth rates than the TD22 strain (**Graph III-5**). Considering protein accumulation of the analyzed membrane proteins (**Figure III-15B**), a very different auto-induction effect was observed depending on the employed *R. capsulatus* strain. In the wild type strain B10S all membrane proteins displayed a higher protein accumulation in culture medium supplemented with at least 2.5 mM NH_4^+ compared to cultivation in medium without NH_4^+.

The highest protein accumulation could be observed for medium supplemented with 5 mM NH_4^+ (MAG, MOG, BR, VpU) or 7.5 mM NH_4^+ ($A_{2A}R$). These findings illustrate that in the strain B10S a positive impact of a delayed protein production can be achieved by supplementing the cultivation medium with at least 5 mM NH_4^+. In contrast, the deletion mutant TD22 demonstrated a very different response to auto-induction medium with respect to membrane protein production. The membrane proteins VpU, MAG and MOG showed the highest protein accumulation in cultures which were not supplemented with ammonium (0 mM NH_4^+), whereas the membrane protein BR exhibited the highest protein accumulation in cultures supplemented without NH_4^+ or with 2.5 mM NH_4^+. However, a very pronounced impact of auto-induction medium could be observed for the receptor $A_{2A}R$, which exhibits the highest protein accumulation in cultures supplemented with 5 mM NH_4^+. Thus, a positive impact of auto-induction medium could be noted for all membrane proteins in the wild type strain B10S, but not for all membrane proteins in the deletion mutant TD22. With regard to potential overall protein yields the findings indicate that for the membrane proteins $A_{2A}R$ and BR, utilizing auto-induction medium would be beneficial for obtaining a higher biomass (BR, $A_{2A}R$) or a higher protein accumulation ($A_{2A}R$). In summary, the concept of auto-induction medium could be established for the new expression system. Utilizing auto-induction medium proved a powerful tool for membrane insertion of several membrane proteins.

3.3 Codon optimization of genes encoding GPCRs

It could be shown that altering illumination conditions and the time point of induction could positively influence the P_{nifH} based accumulation of membrane inserted proteins in *R. capsulatus*. As illustrated in section **III.2**, the GPCRs At1aR, CXCR4, and CHRM3 could not be successfully accumulated into the membranes of *R. capsulatus* nor *E. coli* cells. There can be various reasons for failure to detect these membrane proteins, one of which can be incompatibilities arising from the codon usage bias of *R. capsulatus*, since the organism is characterized by a relatively high GC content (68 %). A codon usage analysis (graphical codon usage analyzer - http://gcua.schoedl.de/) depicted in **Table III-3** reveals great discrepancies between the codons present in the GPCR encoding genes and the codon usage deployed in *R. capsulatus*.

Table III-3: Number of codons present in *GPCR* genes that are used less than 10 % and 20 % in genes annotated in the *R. capsulatus* genome

Gene	Number of codons used less than 10 %	Number of codons used less than 20 %
$A_{2A}R$	47	33
CXCR4	53	34
At1aR	79	44
CHMR3	103	34

These differences in the codon usage can lead to problems during the translation of the mRNA of GPCRs, due to the fact that *R. capsulatus* is characterized by a limited supply of certain tRNA molecules. Aside from an inefficient translation of those mRNAs, the differing codon bias can result in the integration of incorrect amino acids into the nascent polypeptide chain, or lead to the translating ribosome to “slip” and skip nucleotides [278, 279].

Can the accumulation of membrane inserted GPCRs in *R. capsulatus* be improved by codon optimization of gene sequences?

Problems arising from differences in the codon usage bias can sometimes be bypassed by genetically altering the DNA sequence of the gene of interest, so that the transcribed mRNA matches the codon usage bias of the expression host. Therefore, codon optimized variants for all analyzed GPCRs were purchased by gene synthesis (MWG Biotech AG) and inserted into the pRhonHi-2 vector by molecular cloning (*Nde*I/*Xho*I). The codon optimized variants, together with the unmodified variants, were comparatively expressed in the wild type strain B10S and the deletion mutant TD22 under photoheterotrophic conditions (RCV minimal medium supplemented with serine, IR illumination). Membrane fractions of these cultures were subjected to immunological analysis in order to determine the amount of inserted His_6-tagged protein. The corresponding Western blot analysis is depicted in **Figure III-16**.

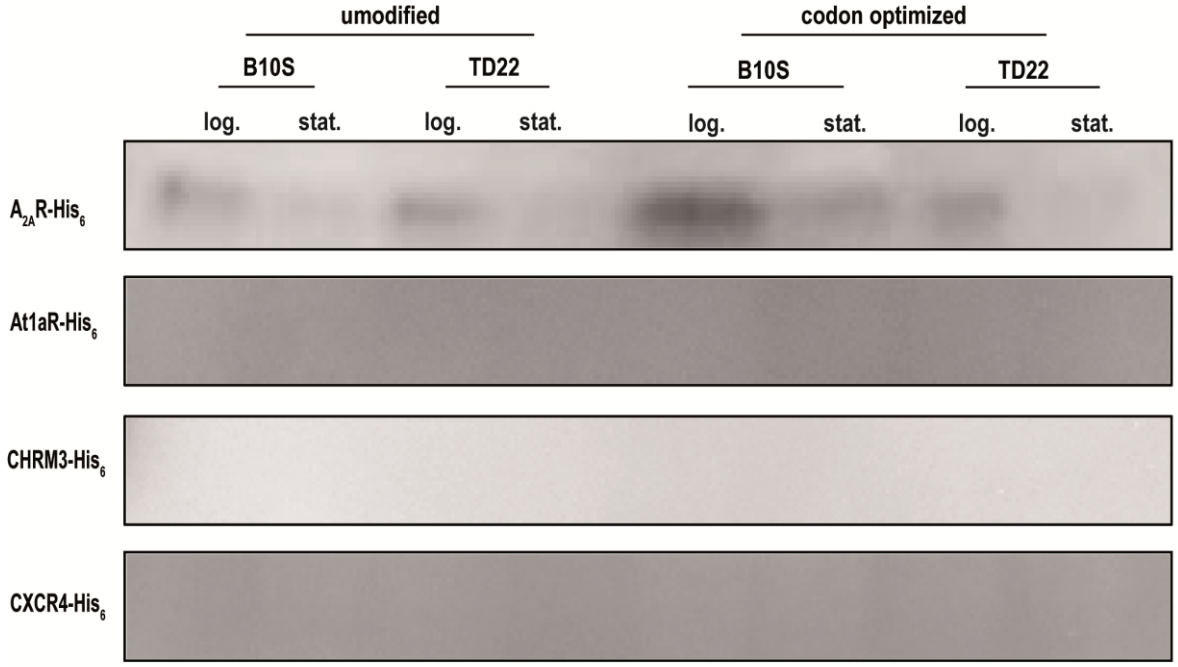


Figure III-16: Effect of codon optimization on GPCR production in *R. capsulatus*
Genes encoding GPCRs analyzed in this study were changed with respect to their DNA sequence in order to account for the codon usage deployed in *R. capsulatus*. To analyze the impact of this codon optimization, genes were comparatively expressed in the strain B10S and TD22. Cells were harvested at the logarithmic (log.) and stationary (stat.) growth phase and fractionated. Membranes obtained from these cultures were subjected to immunological analysis employing His-tag specific antibodies

As the Western blot analysis clearly demonstrates, the codon optimization of genes encoding the GPCRs CHRM3, CXCR4 and At1aR failed to improve *R. capsulatus* ability to insert the proteins into the membrane, since there are no bands observed on the PVDF membranes. While codon optimization failed to improve the membrane insertion of CHRM3, CXCR4 or At1aR, the codon optimization of the $A_{2A}R$ gene led to an improved accumulation of protein into the membrane.

Since membrane protein insertion in case of $A_{2A}R$ could be improved by the codon optimization, the results indicate that successful production or membrane insertion of CHRM3, CXCR4 and At1aR are governed by other factors not yet addressed in *R. capsulatus*.

Section Summary

- Altering illumination conditions, by employing infrared light LEDs instead of bulb light illumination, improved production of membrane inserted proteins VpU, $A_{2A}R$, MAG and MOG
- Auto-induction medium could be applied to pRhonHi-2 regulated gene expression by supplementing RCV minimal-medium with limited amounts of NH_4^+
- The positive impact of auto-induction medium resulted in a higher biomass of the growing culture and/or the improvement of membrane protein insertion
- Positive impact of auto-induction medium could be observed for proteins VpU, $A_{2A}R$, MAG, MOG and BR in the wild type strain B10S by supplementing the medium with at least 5 mM NH_4^+, whereas in the deletion mutant TD22 only the proteins BR and $A_{2A}R$ demonstrated a positive effect when supplemented with NH_4^+
- Codon-optimization of genes encoding CHRM3, CXCR4, At1aR could not improve membrane inserted protein accumulation in *R. capsulatus*, while codon-optimization of the GPCR $A_{2A}R$ led to an improvement of membrane inserted protein

The P_{nifH} based synthesis of membrane proteins could be improved by altering light conditions or the induction point of gene expression.

4 Application of *R. capsulatus* for the production of functional protein

In this last section the basic applicability of the organism to produce functional membrane proteins, which can be isolated chromatographically, will be demonstrated using the example of bacteriorhodopsin. The natural environment of membrane proteins limits the use of biochemical techniques such as IMAC to study basic characteristics of proteins, due to the fact that these processes are primarily suited for proteins in aqueous solutions. Therefore, the protein of interest must be extracted from the membrane in a functional form and converted into a state that makes it "soluble" in aqueous solutions.

4.1 Detergent screening

As discussed in the introduction (**I.2.2.5**), detergents are a very diverse class of molecules characterized by a hydrophobic tail and a hydrophilic head group. Detergents may be classified according to their head group into: ionic detergents, zwitterionic detergents and non-ionic detergents. Since solubilization of membrane proteins is highly dependent on the individual membrane protein, a set of different detergents need to be tested empirically for a particular membrane protein.

Which detergent is best suited to extract membrane proteins from *R. capsulatus*?

This question was addressed to a great extent in the scope of a supervised Master thesis by Katrin Pünder [243]. In order to determine the best suited detergent for solubilization of membrane proteins from *R. capsulatus*, a comparative evaluation of five different detergents, belonging to either non-ionic (DDM and OG) or zwitterionic detergents (CHAPS, FC12 and FC16), was performed (**II.12.2**). To this end, six therapeutically relevant membrane proteins were heterologously produced in *R. capsulatus* cells employing the pRhonHi-2 vector under photoheterotrophic conditions. Cells were harvested by centrifugation and the obtained cell pellet stored at -20 °C for one day. Subsequently, cells were mechanically disrupted and the membrane fraction isolated by ultracentrifugation. These membranes were resuspended in defined concentrations of detergent or solubilization buffer (control) and incubated overnight at 4 °C under gentle agitation. Detergent treated membranes were once again subjected to ultracentrifugation and the obtained supernatant (solubilizate) and pellet fraction analyzed with respect to the protein of interest by means of western blotting. The ratio of membrane protein in both fractions

allows for conclusions about the solubilization efficiency of the particular detergent. A very efficient detergent is able to extract nearly all of the protein from the membrane, which would result in the protein to be found predominantly in the supernatant (S). In contrast, a protein which is found mainly in the pellet fraction (P) indicates the detergent´s inefficiency at solubilization. The result of the comparative detergent screening is depicted in **Figure III-17**.

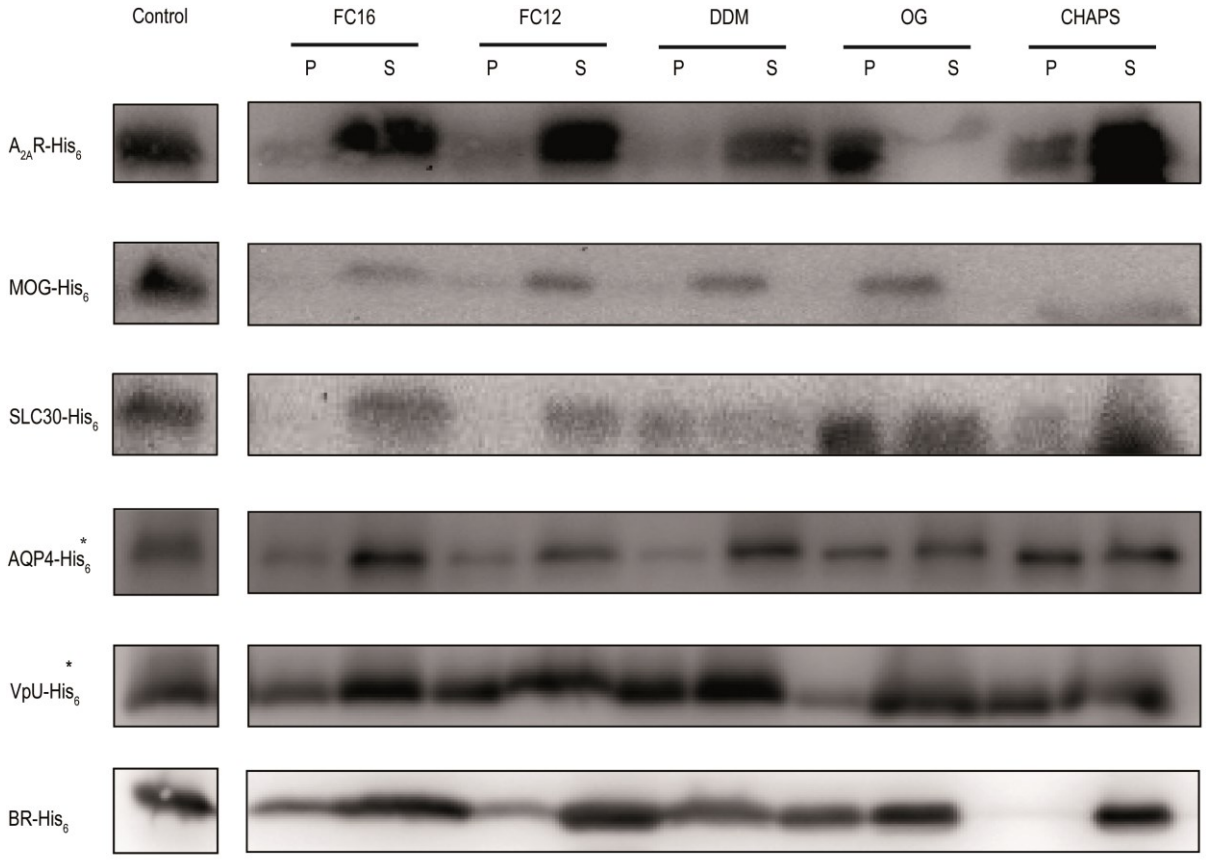


Figure III-17: Comparative evaluation of different detergents for the solubilization of membrane proteins produced in *R. capsulatus* by immunological analysis
R. capsulatus cells expressing membrane proteins MOG, BR, VpU, AQP4, SLC30 and A2AR were grown photoheterotrophically under nitrogen-limiting conditions in RCV minimal medium. Cells were harvested by centrifugation and, following mechanical disruption, subjected to differential centrifugation. Obtained membrane fractions were mixed with detergents FC16, FC12, DDM, OG and CHAPS or buffer (control), in order to determine the best suited detergent for extracting proteins from *R. capsulatus* membranes. Solubilization efficiency of detergents was determined by immunological detection of the pellet fraction (P) and the supernatant (S) fraction. Detergents able to extract proteins efficiently lead to detection of proteins predominantly in the supernatant fraction, whereas detergents not sufficient at solubilizing resulted in proteins to be found mainly in the pellet fraction. * Adopted from the master thesis of Katrin Pünder [243].

The detergent screening illustrates that efficient solubilization was dependent on the detergent and the analyzed membrane protein. While membrane proteins such as MOG could be solubilized by all employed detergents, the proteins BR, $A_{2A}R$, SLC30, AQP4, VpU demonstrated differences with respect to the detergent. The zwitterionic detergents FC16 and FC12 were the most efficient at extracting the proteins from *R. capsulatus* membranes, illustrated by the fact that most of the proteins mixed with these detergents are found nearly completely ($A_{2A}R$, MOG and SLC30) or predominantly (AQP4, VpU, BR) in the supernatant (lane S). The Fos-cholines are closely followed by another zwitterionic detergent, CHAPS. Some proteins could be extracted very efficiently with CHAPS (BR, MOG), while other proteins were characterized by a substantial amount in the pellet (VpU, AQP4). Other proteins treated with CHAPS led to precipitation overnight, which indicates problems in protein stability. As expected, the non-ionic "mild" detergents DDM and OG were not as successful at solubilizing the proteins as were the zwitterionic detergents. Out of these two, DDM is to be named the clear preference detergent, since it could extract proteins very efficiently ($A_{2A}R$, MOG, AQP4) or was able to extract the proteins at least to a high degree out of the membrane. In contrast, the detergent OG illustrated the highest extraction efficiency for only one protein (MOG) and did not show any extracting ability for proteins such as BR or $A_{2A}R$.

4.2 Affinity purification of bacteriorhodopsin produced in *R. capsulatus*

So far, all expression studies have been conducted exclusively with membrane fractions of the cells, to ensure detection of correctly localized proteins. However, correctly localized protein does not necessarily equal correctly folded or functional protein. Out of all the proteins analyzed in this study BR exhibits unique absorption properties, which enable monitoring of the protein´s functional state by means of spectroscopy. The archaeal integral membrane protein BR belongs to the family of retinylidene proteins that utilize retinal as a chromophore for light perception. One molecule of retinal is covalently linked to the amino acid residue Lys216 of the protein through a protonated Schiff base [280, 281]. Upon light illumination, retinal undergoes a photoisomerization from the all-*trans* into the 13-*cis* form, which leads ultimately to the release of a proton from the Schiff base into the extracellular medium. The deprotonated Schiff base accepts a proton from the cytoplasm and after a period of time the retinal isomerizes back to the all-*trans* form [280, 281]. Owing to this chromophore, correctly folded BR is characterized by a typical purple coloration (λ_{max} = 570 nm), which can be monitored when analyzing purified membrane proteins or whole cell extracts [282].

Does *R. capsulatus* produce functional BR?

To investigate whether *R. capsulatus* produces correctly folded and functional proteins, BR was purified by immobilized metal affinity chromatography (IMAC) and analyzed spectroscopically as described in **II-12.3.2**. Shortly, *R. capsulatus* cultures were grown photoheterotrophically for two days under infrared light illumination in flat panel bioreactors supplemented with 15 µM all-*trans* retinal for BR-chromophore assembly, subsequently harvested and processed for purification. Based on the results obtained by the evaluation of different detergents (**Figure III-17**), FC12 was chosen for solubilization as opposed to FC16, since this detergent could be successfully deployed for a number of structural NMR studies so far [283]. Although the detergent CHAPS was more efficient at solubilizing BR, it was not chosen because of problems regarding membrane protein stability. Solubilized membrane proteins were subjected to IMAC and could be successfully purified to high purity as demonstrated by the Coomassie Blue stained gel illustration (**Figure III-18A**). However, spectroscopic analysis (**Figure III-18B**) of the purified membrane protein revealed a yellow coloration (λ = 380 nm) instead of the typical purple color.

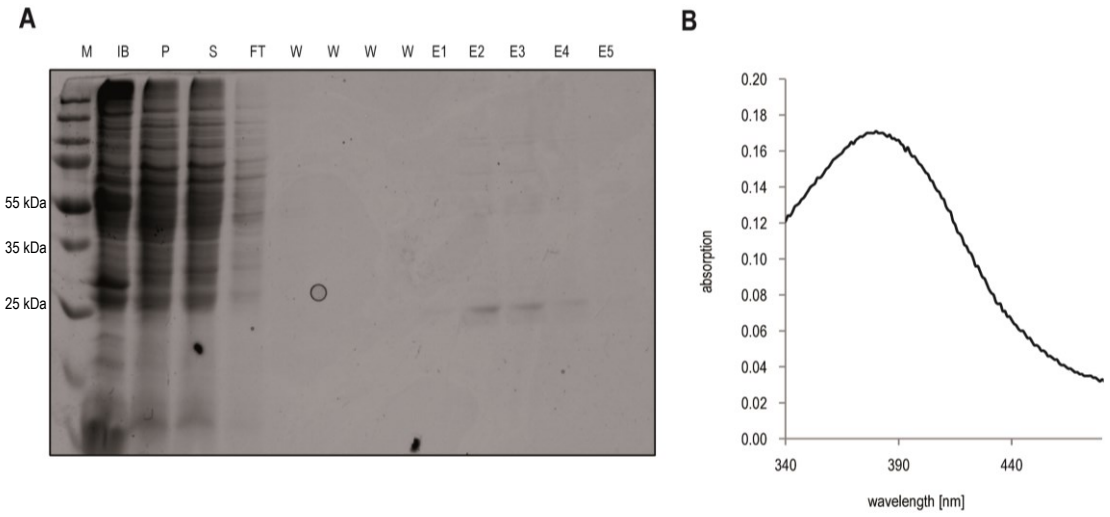


Figure III-18: Affinity purification and spectral characteristcs of BR-His$_6$ solubilized by FC12
R. capsulatus cells expressing BR-His$_6$ were cultivated photoheterotrophically for two days under infrared light illumination in RCV minimal medium supplemented with 15 µM all-trans retinal. 1 l of culture was harvested by centrifugation and membrane fractions isolated by differential centrifugation. Proteins were solubilized by incubation with FC12 at 4 °C over night under gentle agitation. Solubilized BR-His$_6$ was then used for affinity purification by IMAC. Different steps of the purification process are depicted in form of a Coomassie stained SDS-gel on the left side (A). Eluted protein was subjected to buffer exchange and concentrated to a volume of 0.5 ml by use of concentrators. Concentrated BR-His$_6$ was analyzed by spectroscopry, which is illustrated on the right side (B). Although proteins could be purified to near homogeneity by IMAC, spectral characterization of the protein revealed a yellow coloration instead of the typical purple color. M = marker, IB = inclusion body fraction, P = pellet, S = solubilizate, W = wash 10, E = elution.

As a zwitterionic detergent FC12 has a net charge of zero, but still contains charged groups that can affect the chromophore assembly or the folding of the BR protein.

Can DDM solubilize BR in a functional state?

In order to examine if the utilized detergent for solubilization caused the observed problems, a mild detergent was used as an alternative. The experiment was repeated, and this time DDM solubilized membranes were subjected to IMAC, where BR could be purified with a yield of 1 mg protein/l culture. To test the folding and functional state of the isolated proteins, purified BR proteins were spectroscopically analyzed (**Figure III-19).**

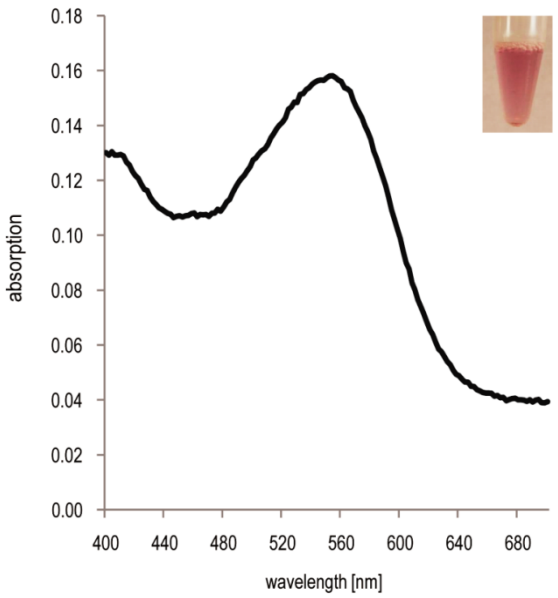


Figure III-19: Absorption spectrum of BR-His_6 solubilized by DDM

R. capsulatus TD22 cells were cultivated photoheterotrophically under infrared light illumination in RCV minimal medium supplemented with 15 µM all-trans retinal. BR-His_6 was produced recombinantly employing the pRhonHi-2 expression plasmid. 1l *R. capsulatus* cultures were harvested by centrifugation, subsequently mechanically disrupted and the resulting cell extract utilized for membrane fractionation. Membrane proteins were solubilized by 20 mM DDM and subjected to IMAC. Spectroscopic analysis of purified protein revealed the proteins to be identified by the typical purple coloration (photographic illustration) with an absorption maximum at 550 nm and yields of 1 mg protein per liter of culture. Picture was taken by Achim Heck.

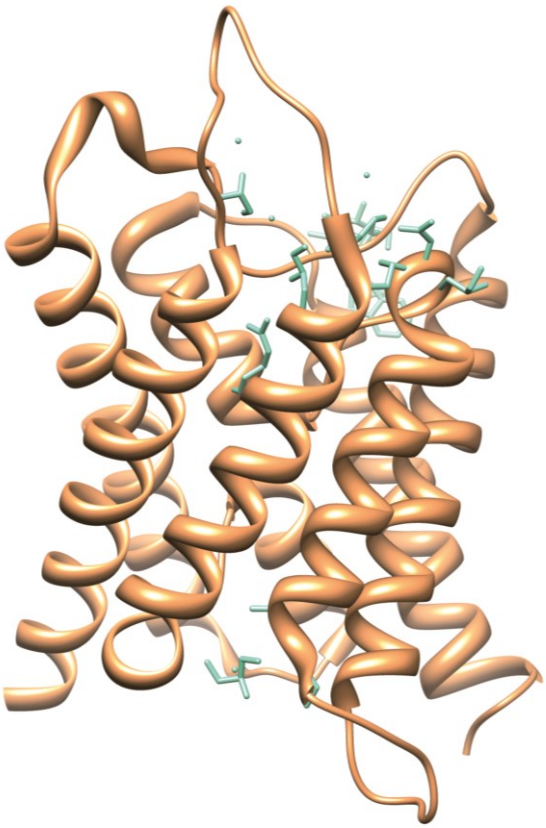
In contrast to FC12 solubilized proteins DDM solubilized BR proteins were identified by a typical purple coloration. In accordance with literature, a slight shift of the absorption maximum of DDM solubilized BR was observed (λ_{max} = 550 nm), which is caused by a lack of specific lipids that are present in the natural purple membrane of the originating organism [88]. Since *in-vivo* assembly into functional proteins is facilitated by simple addition of *all-trans* retinal into the culture medium, complicated and time consuming *in-vitro* assembly procedures, which may influence folding as well as obtained protein yield, are prevented. In summary, the results demonstrate that the newly developed expression system is basically applicable to produce functional membrane proteins.

Section Summary

- Proteins VpU, AQP4, SLC30, $A_{2A}R$, MOG and BR could be solubilized with differing efficiencies by employment of the detergents FC12, FC16, DDM, OG and CHAPS
- The zwitterionic detergents FC12 and FC16 were the most successful at extracting proteins from the membrane, followed by the detergents CHAPS, DDM and OG
- FC12 solubilized BR could be purified to homogeneity by IMAC, but was characterized by the wrong spectral attributes, indicating a disassembly of the protein
- DDM solubilization of membranes led to correctly assembled BR, which was characterized by a typical purple coloration

In conclusion, this section demonstrated the basic applicability of *R. capsulatus* for the production of functional proteins, which can be solubilized by detergents and purified to homogeneity by means of IMAC.

CHAPTER IV: DISCUSSION



Human AQP4 monomer (PDB file 3GD8)

The class of membrane proteins represents one of the most important physiological targets of drugs, owing this to the fact that major biological processes are regulated by these proteins. However, the presence of hydrophobic domains and the intracellular localization of membrane proteins hamper their structural and functional studies. In addition, problems encountered during recombinant production are oftentimes rooted in membrane protein biogenesis, due to the fact that these proteins need to be properly targeted, inserted and folded in the membrane of the expression host. Despite strain engineering and new expression tools, common expression hosts are limited in their membrane space as well as their capacity to produce great amounts of membrane protein. For this reason, organisms which are specialized in producing membrane proteins (e.g. higher membrane biogenesis capacity and greater membrane space) have been employed in recent years. The success of this approach was demonstrated with both eukaryotic and prokaryotic expression platforms.

The purple phototrophic bacterium *R. capsulatus* offers a highly adapted physiology for the production of membrane proteins in high quantities [215, 221]. Considering the importance of membrane proteins and the problems encountered in common expression hosts, the presented thesis was concerned with the evaluation of an alternative platform organism for the heterologous expression of therapeutically relevant membrane proteins. Given the novelty and early stage in establishment of *R. capsulatus* as a microbial platform for membrane protein production, the thesis had two major focal points. While the construction and characterization of a new expression vector for recombinant protein production in *R. capsulatus* represented one aspect, the actual evaluation of the bacterium for producing therapeutically relevant membrane inserted proteins stood for another. Therefore, the following discussion will conform to the appraisal of these two key aspects.

1 The novel pRhonHi-2 expression vector enables tightly controlled gene expression in *R. capsulatus*

In order to appraise the capability of a new expression platform properly, one needs to ascertain that basic molecular biological tools are available. This was reflected very strongly in a study conducted by Bernaudat & co-workers, where 20 different membrane proteins were comparatively expressed in six different expression hosts [162]. In addition to different prokaryotic and eukaryotic organisms, the phototrophic bacterium *Rhodobacter sphaeroides* was evaluated as an expression platform for the production of membrane proteins. Surprisingly, the bacterium was capable of expressing only 20 % of the analyzed membrane protein targets. The failure to express even a homologous protein (NapC) in this expression host led to the critical examination of the deployed methodology. And thus, expressing the homologous protein with an alternative expression plasmid, instead of a Gateway-based vector, resulted in the production of functionally active protein. This example emphasizes the importance of the deployed expression vector for the proper evaluation of a new expression host, since in the study by Bernaudat the lack of proper molecular biological tools resulted in the inadequate evaluation of the expression platform. By the same rationale, before evaluating *R. capsulatus* for the heterologous expression of membrane proteins, a new expression vector based on the host-specific *nifHDK* promoter (pRhonHi-2) was constructed.

1.1 Architecture of the *R. capsulatus nifHDK* promoter region

Prior to this thesis, the idea to implement the *nifHDK* promoter in the pRho-vector series was already established [240, 242, 248], resulting in four variants of the pRhonHi-2 vector. These constructs differ with respect to the upstream sections of the *nifH* genomic region implemented into the pRho vector, as illustrated in **Figure III-2**. Expression studies with a reporter gene revealed no significant differences in target gene expression between the constructs, leading to the construction of the pRhonHi-2 vector variant 5. A comparative study between all pRhonHi-2 vectors (**III.1.2**) demonstrates great differences between the newly constructed vector and the pRhonHi-2 variants 1 - 4, with regard to mediated YFP expression (factor 40). Considering that the other pRhonHi-2 vectors all illustrate a strict regulation of gene expression by the presence of NH_4^+ in the cultivation medium, the observed problems seem to be associated with the positive regulation of P_{nifH} driven transcription rather than the negative regulation by ammonium. Since the $pRhon_5Hi$-2 vector differs mainly with respect to a downstream region of the *nifH* gene, the obtained results raise the question if this genomic region contains regulative elements that enhance the transcription of target genes. Although a positive regulation by elements present in the

coding region of a gene seems rather unusual, they are not unheard of. On the contrary, in the diazotrophic bacterium *Azotobacter vinelandii* a similar form of positive regulation was observed for the nitrogen fixation operon *nifLA*, which is characterized by a regulatory element in the coding region of the operon as well [284]. Deletion of this regulative element leads to the complete loss of promoter activity, which is different from the results observed in the conducted expression studies (**III.1.2**), seeing that promoter activity can still be detected in the pRhonHi-2 variants 1 - 4. A closer look into the cloning strategies applied to construct the pRhonHi-2 vectors revealed subtle differences among the different expression vectors with regard to the DNA sequence of the implemented genomic DNA that are depicted in **Figure IV-I**.

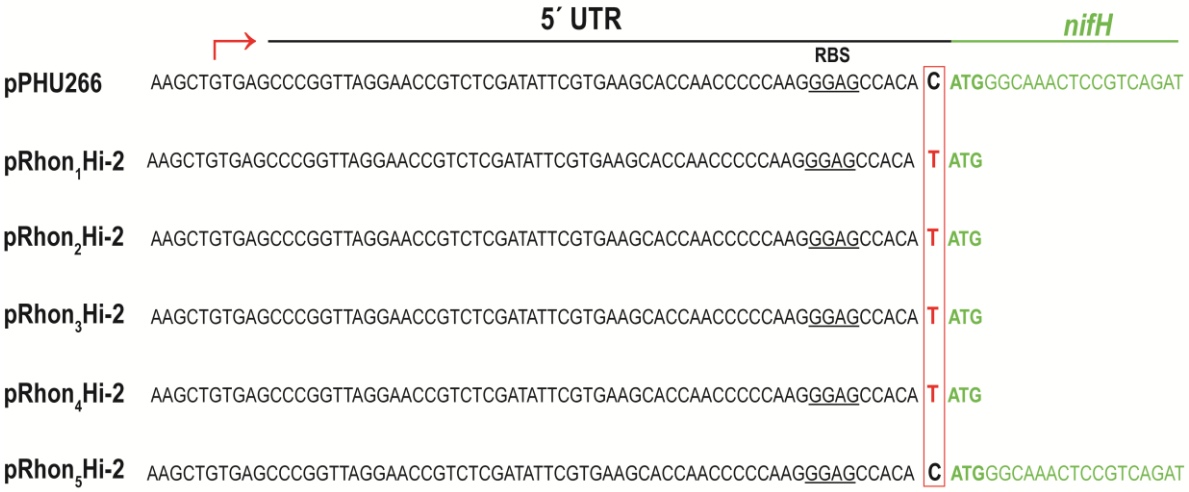


Figure IV-1: Sequence specific differences of pRhonHi-2 vectors
The pPHU266 vector provides a reference sequence for a successfully implemented *nifHDK* promoter region [249].The red arrow marks the transcription starting point, while the underlined sequence signifies the ribosome binding site (RBS). Sequence analysis of this genomic region reveals a point mutation (red box) in the 5´ UTR region of the pRhonHi-2 vectors 1 - 4, which was inserted by means of molecular cloning. The sequence in green letters conforms to the coding sequence of the *nifH* gene. UTR = untranslated region.

Figure IV-1 demonstrates a minor difference in the DNA sequence found among the different pRhonHi-2 vectors. In the course of cloning, a point mutation was inserted right before the first codon of the *nifH* gene (green letters) in the pRhonHi-2 variants 1 - 4, so that instead of the original sequence (CAC ATG) the recognition site for the enzyme *Nde*I (CAT ATG) was obtained [240]. Although this change in the DNA sequence seems minor, it is not known how it affects the secondary structure of the resulting mRNA molecule. Mutations in the 5´ UTR region of mRNA molecules can have severe repercussions for stability as well as translation initiation of the mRNA molecule, considering the regulative significance of

this region (**I.2.1.2** and **I.2.1.3**). It might very well be that poor performances of the preceding pRhonHi-2 vectors are attributed to this very point mutation, which potentially leads to a weaker mRNA stability or an inefficient translation initiation. In order to determine whether positive regulative elements in the downstream region of *nifH* have an enhancing effect on gene expression, the evaluation of an additional construct, containing no downstream region and no point mutation, may allow for reliable conclusions.

1.2 Light intensity regulation of pRhonHi-2 mediated expression of target genes

In the scope of this thesis the pRhonHi-2 vector was thoroughly characterized as an expression plasmid for recombinant protein production in *R. capsulatus*. As illustrated in chapter **III.1.3**, differences were noted in pRhonHi-2 mediated YFP production with respect to the employed culture vessel. Results obtained in small scale cultures grown in Hungates were not in accordance with protein yields achieved in large scale cultures grown in Schott flasks. The discrepancy in protein yields however was too great to reduce them solely to differences caused by problems associated with upscaling of cultures, since similar results were not observed for cultures expressing genes by T7 RNAP driven transcription (pRhotHi-2 vector). A potential limitation of the employed pRhonHi-2 vector was found to be associated with the prevailing light conditions, since Schott flasks are characterized by thicker glass walls and smaller surface-to-volume ratio than Hungates. To address this bottleneck, the purification study was repeated in optimized large scale culture vessels identified as flat panels [213]. Flat panels are characterized by thinner glass walls and greater surface-to-volume ratios than Schott flasks, which presumably results in higher light permeability and consequently in higher light intensities. Although the production was not in complete agreement with results observed in Hungates, a distinct improvement over Schott flasks was apparent when flat panels were employed (**Figure III-8**), emphasizing a modulation of the P_{nifH} mediated protein production depending on prevailing light conditions.

It is known that the nitrogen fixation is governed by many environmental factors, among them the light intensity [188]. One explanation for the observed discrepancy in the production yields could be attributed to the transcription regulator HvrA. This protein is known to positively modulate the expression of photosynthesis genes such as *puf* and *puh* in response to alterations in light intensity [285], whereas it could also be shown to bind selected *nif* promoters and negatively modulate the expression of the *nifH* gene in particular [286]. Dr. Drepper [287] and Dr. Raabe [288] could provide evidence in their doctoral theses that demonstrates a correlation between the light intensity and HvrA accumulation within the cell.

Based on this information, a putative interdependence depicted in **Figure IV-2** might be assumed for the regulation of pRhonHi-2 mediated target gene expression in response to the light intensity.

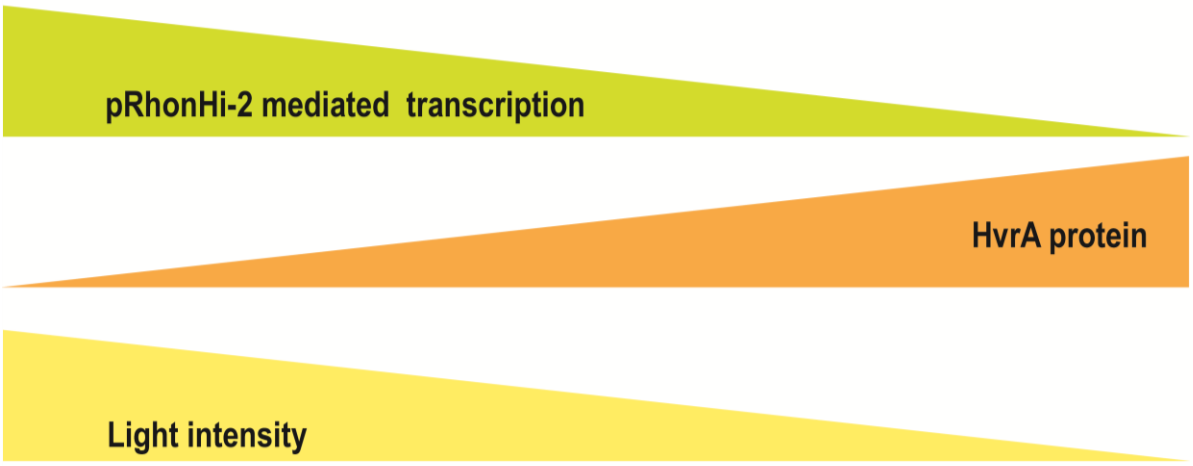


Figure IV-2: Proposed interdependence of light intensity and regulation of pRhonHi-2 mediated gene expression
Here depicted is the hypothetical relationship between HvrA regulation of pRhonHi-2 mediated target gene expression in response to light intensity. Among other target promoters, the trans-activator HvrA is known to inhibit the transcription of the *nifH* gene in *R. capsulatus*. Preceding works of Dr. Drepper and Dr. Raabe could illustrate that the activtiy and the accumulation of HvrA correlates with the light intensity. While high light intensity leads to low production of HvrA, high amounts of HvrA can be found in cells cultivated with low light intensity. Further details can be found in the text.

This model proposes that high light intensity leads to a reduced expression of the transcription regulator HvrA, whereas low light intensity results in higher expression levels. Consequently, in Hungates where the light intensity is assumed to be much higher than in Schott flasks, due to thinner glass walls and lower self-shading effects, HvrA production might be reduced. In contrast, cultivation conditions leading to lower light intensities (presumably prevailing conditions in Schott flasks), HvrA might be accumulated at higher concentrations within the cell, resulting in stronger repression of the *nifHDK* promoter driven transcription of target genes. To verify this model, future experiments could evaluate target gene expression in response to specified alterations in light intensity, under consideration of *hvrA* expression.

1.3 pRhonHi-2: a universal expression plasmid for purple bacteria?

The requirements for an ideal expression plasmid have been thoroughly discussed in the introduction (**I.2.2**). Based on this information, the presented pRhonHi-2 vector is one of the most convenient and effective vehicles constructed for the *Rhodobacter* species so far. Apart from being one of the strongest promoters in *R. capsulatus*, the *nifHDK* promoter offers the benefit of strictly regulated expression of genes, as could be demonstrated in this work (**III.1.2**). Compared to other promoters designed for *Rhodobacter* plasmids, such as the promoter of the *dor* genes [235], the promoter for photosynthesis genes *puf*Q [289] or *pucB* [290], the fructose uptake promoter [291] and even the T7 promoter [213], the pRhonHi-2 vector offers the possibility of convenient modulation of gene expression by cost-effective and simple alteration of the nitrogen source in the cultivation medium [292]. Furthermore, the promoter provides a huge advantage over most commonly employed promoters by being compatible with auto-induction medium. As could be shown in chapter **III.3.2**, the use of auto-induction medium offers the advantage of reaching high cell densities before inducing target gene expression autonomously. Although promoters initiating transcription of photosynthesis genes are also auto-inducible (e.g. by the oxygen tension) it is not possible to achieve gene expression by convenient use of auto-induction medium. Despite the fact that promoters of photosynthesis genes have been frequently employed in different purple bacteria [220, 221, 289, 290] inducing protein production by environmental factors such as oxygen or light intensity can pose a serious drawback in phototrophic organisms. Although the pRhonHi-2 mediated expression is also affected by these factors, the main difference between the *nifHDK* promoter regulated transcription of genes and the photosynthesis promoters is that with the pRhonHi-2 vector physical stimuli are not used as a means to induce protein production, this is accomplished by the nitrogen source in the cultivation medium. Light intensity or oxygen tension as an inducer can be problematic for precise control and modulation of recombinant protein production, due to the fact that these factors affect the deployed energy metabolism of the organism [293]. Since heterologous expression resorts to the cells own energy supply, choosing a physical stimuli that governs the formation of ATP prevents efficient production of protein. In addition, co-production of photosynthesis gene products would also exhaust the organism's own cell resources, which could be potentially harnessed for recombinant protein production. Bypassing this bottleneck by deletion of co-induced genes may have consequences for the energy metabolism or in the worst case be lethal, when the bacterium is grown under phototrophic conditions. In contrast, in *R. capsulatus* the co-induction of *nif*-genes is not necessary for survival and can be easily deleted, as demonstrated in this work by employing the deletion mutant TD22 [239]. Deletion of the Mo-nitrogenase in *R. capsulatus* increased the YFP production in cells and led to improved growth rates, demonstrating the versatile applicability of the pRhonHi-2 vector (**Figure III-8**).

Although the pRhonHi-2 vector illustrates remarkable properties, it nevertheless demonstrates limitations like any expression vector. One of those limitations is the aforementioned light intensity regulation of the *nifHDK* promoter, which hampers the upscaling of cultures expressing genes with the pRhonHi-2 vectors. This however, is not an insuperable bottleneck if the proposed model of light intensity regulation (**Figure IV-2**) is de facto governed solely by the HvrA protein. Positive effects gained by optimized culture vessels could be demonstrated in this work (Schott flasks vs. flat panels), these results suggest that further optimization can be performed on culture vessels to achieve illumination conditions that minimize self-shading effects further and result in cultures exposed to higher light intensities. Likewise, alternative light sources characterized by higher light intensity illumination can be employed, as demonstrated with the LEDs panels in this study (**III.3.1**). A different approach could target the HvrA mediated light regulation directly, so that the protein is accumulated at low concentrations irrespective of the prevailing light conditions. Deletion of the *hvra* gene however is not advisable, since modifications of this nature abolish the ammonium and oxygen control of *nif*-gene expression in *R. capsulatus* [194].

A study by Butzin *et al.* [294] illustrates that the *R. capsulatus puc* promoter could be utilized for the heterologous expression of genes in the related purple bacterium *Rhodospirillum rubrum*. This indicates that promoters which are regulated by conserved mechanisms are in principle interchangeable between different expression hosts. Considering that nitrogen fixation is a very common metabolic feature among purple nonsulfur bacteria, and that all nitrogen fixation bacteria contain Mo-nitrogenase [276], it would be interesting to see if the pRhonHi-2 vector is also applicable in other purple bacteria.

2 Evaluation of *R. capsulatus* for the heterologous expression of therapeutically relevant membrane proteins

The heterologous expression of membrane proteins is a delicate process of finding the right balance between protein synthesis and protein insertion into the membrane, so that an overload of the host's protein translocation capacity is prevented. A study by Bonander *et al.* demonstrates that optimized cultivation conditions allowing fast growth rates do not correlate with optimized conditions for expressing membrane proteins [295]. Monitoring mRNA levels and expression of a model protein from yeast in response to alterations of different cultivation parameters, including the pH, the temperature and the expression duration, Bonander *et al.* could further conclude that monitoring membrane protein expression by means of whole cell extracts was an inefficient indicator for functionally active membrane proteins, since whole cell extract contain misfolded proteins as well. Likewise, mRNA levels did not correlate with the obtained yield of corresponding protein, emphasizing the importance in the approach to membrane protein detection for reliable conclusions about functional membrane protein production.

2.1 Expression conditions leading to improved production of membrane inserted proteins in *R. capsulatus*

Evaluation of *R. capsulatus* for the heterologous expression of therapeutically relevant membrane proteins was conducted in a similar manner to the one performed in the study by Bonander & colleagues. A set of different expression parameters, including the expression duration, the induction time-point, the utilized promoter and the employed illumination conditions was tested in order to determine a generic guideline for conditions leading to optimal production of membrane inserted proteins. In this manner, the current potential of the organism was assessed for the production of therapeutically relevant proteins.

2.1.1 The pRhonHi-2 vector enables higher membrane protein production than the pRhotHi-2 vector

The presented work demonstrates that for membrane protein production in *R. capsulatus* the pRhonHi-2 vector seems to be the preferable expression plasmid, since T7 RNAP (pRhotHi-2) driven protein production was less efficient or in most cases not successful at all. The reason for these distinctive differences between P_{T7} driven expression and the P_{nifH} driven expression of genes can be most likely attributed to two factors.

As the comparative expression study with different pRho vectors has demonstrated, the pRhotHi-2 vector differs by a factor of 10 with regard to mediated YFP expression from the newly constructed pRhonHi-2 vector. So, one reason for the observed differences is most likely associated with the comparatively weaker performance of the pRhotHi-2 vector. However, since whole cell extracts were not analyzed, the exact reason for the observed differences cannot be reduced to this aspect alone.

In *E. coli*, membrane insertion of the assumed toxic protein SLC30 could only be observed in the C43 (DE3) strain but not in the strain BL21 (DE3). Since both strains differ with respect to mediated transcription strength [177], this might indicate that a weaker production of the protein can be beneficial for the efficient insertion of the protein into the membrane. By this assumption, a weaker expression of this protein in *R. capsulatus* may also have resulted in higher protein accumulation levels, since the toxicity of the protein was noted in this host as well (**Graph III-4**). But this outcome was not observed, as with all proteins analyzed in this study, the SLC30 protein was accumulated at comparatively lower levels in the membrane when the pRhotHi-2 vector was employed. This might indicate a problem at a different step of T7 RNAP driven membrane protein assembly in *R. capsulatus*.

Membrane protein biogenesis of eukaryotic proteins in bacteria is a known bottleneck, since polypeptide elongation and protein folding rates are considerably different in pro- and eukaryotic organisms, with eukaryotic organisms illustrating slower translation and protein folding rates [136]. So, another reason for these differences could possibly be attributed to problems caused by a too high processivity of the T7 RNAP driven transcription. In bacteria, transcription and translation occur at virtually the same time therefore it is a distinct possibility that for eukaryotic membrane proteins the folding and translocation of membrane proteins does not comply with the rate the mRNA is produced by T7 RNAP. Translation is not always a continuous process, but rather a discontinuous act, where intermediate pauses of the ribosome facilitate proper folding of the nascent polypeptide chain [296]. This mechanism emphasizes a very intricate interplay of transcription and translation for individual proteins, which would be severely disturbed in a system where a viral RNA polymerase does not act in concert with the host specific ribosome, even more so for co-translationally translocated proteins. A high transcription rate of the T7 polymerase may also result in the accumulation of “free” mRNA molecules that are not associated with the ribosome. While in eukaryotic organisms this is a natural process, in bacteria mRNA molecules not associated with the ribosome are usually instable and get degraded [297]. This could be another explanation for the performance of the pRhotHi-2 vector in *R. capsulatus*.

2.1.2 *R. capsulatus* TD22 is a complement to the B10S strain for pRhonHi-2 driven membrane protein production

Growing cultures under nitrogen-limiting conditions leads to the co-induction of all *nif*-genes in *R. capsulatus* [193]. In order to determine the impact of Mo-nitrogenase production on recombinant membrane protein insertion, the *nifHDK* deletion mutant TD22 [239] was employed. While the membrane insertion of some of the tested proteins could be improved (BR, MOG, VpU) in the *nifHDK* deletion mutant TD22, the protein accumulation of other proteins ($A_{2A}R$, AQP4) was decreased compared to the wild type strain. Although the deletion of the *nifHDK* operon should presumably provide more cell resources and a better energy supply for recombinant protein production, physiological changes induced by this modification were obviously not beneficial for some of the tested proteins. Considering auto-induction experiments and growth behavior studies (**Graph III-5**), it is evident that Mo-nitrogenase production imposes a serious burden on the organism, which results in reduced growth rates in the *R. capsulatus* wild type strain B10S. As already described before, cultivation conditions leading to faster growth rates do not always correlate with higher accumulation of membrane inserted protein. Considering the interdependence of growth rate and gene expression in bacteria [299], a slower growth rate may be beneficial for protein folding and membrane insertion of some proteins. Membrane proteins with a more complex structure, which need more time for proper folding, may therefore be better expressed in the wild type strain rather than the deletion mutant TD22.

2.1.2 Using auto-induction medium improves accumulation of selected proteins and enables reaching high cell densities

A clear preference for the optimal growth phase in *R. capsulatus* could not be discerned, since some proteins displayed equal accumulation rates in the logarithmic and stationary growth phase (e.g. VpU), while others displayed a higher protein accumulation in the logarithmic growth phase and a reduced or abolished protein accumulation in the stationary growth phase (e.g. $A_{2A}R$, MOG). There can be many reasons for a reduced protein accumulation in the stationary growth phase, one of which can be due to proteolytic degradation or effects caused by stress induction mediated by membrane protein expression [133, 298]. Since these effects are not observed for all proteins, and therefore cannot be predicted, growing the expression cultures to the stationary growth phase remains risky for membrane protein production in *R. capsulatus*. On the other hand, harvesting the cultures in the logarithmic growth phase will be problematic as well, since the obtained biomass will be very low. However, the pRhonHi-2 vector provides the means to overcome this bottleneck by introducing the concept of auto-induction medium in *R. capsulatus* (**III.3.2**).

Using auto-induction medium in *R. capsulatus* cultures expressing genes with the pRhonHi-2 vector enables reaching high cell densities before protein production is induced. Interestingly, auto-induction medium had a different impact with regard to the employed expression strain. While a positive effect of auto-induction medium could be observed for all of the tested membrane proteins in the wild type strain B10S, in the strain TD22 only two of the tested proteins ($A_{2A}R$ and BR) demonstrated a positive impact. This might indicate that the positive effect observed in the strain B10S is most probably affected by two different factors.

It can be presumed that one major factor contributing to the positive impact observed in the strain B10S is associated with the production of the Mo-nitrogenase itself, since this is the main difference between the deletion mutant and the wild type strain. The production of the Mo-nitrogenase as well as the catalytic activity of the enzyme exhausts a lot of energy and cell resources, so a delayed production of this highly energy demanding enzyme could possibly lead to higher levels of membrane inserted proteins. The other major factor contributing to the positive effect of auto-induction medium is presumably tied to the protein´s individual characteristics.

Recalling the optical density values of the expression strains (**Figure III-16A**), it is evident that some proteins have a negative effect on cell growth. As can be seen for the membrane protein BR, the optical density values of TD22 cultures supplemented with 2.5 mM NH_4^+ and without NH_4^+ differ distinctly, yet the western blot analysis (**Figure III-16B**) illustrates equal protein accumulation in the membrane. Thus, in this case using auto-induction medium is beneficial to obtain a higher biomass and thus higher yields of protein. Another representative protein which illustrated a pronounced effect of auto-induction medium was the GPCR $A_{2A}R$. Compared to BR, this protein displayed an improved protein accumulation and a higher biomass in response to the NH_4^+ concentration in both the wild type and the mutant strain. Considering that this protein was shown to illustrate a protease susceptible C-terminal tail [157, 289], a positive effect of an induction at a later time point would be observed for both bacterial strains, simply because of the expression duration rather than the exact moment of induction. Therefore, utilizing auto-induction medium can be a very powerful tool for proteins which, i) are better expressed in the wild type strain ii) have a negative impact on cell growth or iii) are protease susceptible proteins like $A_{2A}R$.

2.1.3 Employing infrared LEDs improves membrane protein production in *R. capsulatus*

As a phototrophic organism, *R. capsulatus* is highly responsive to changes in the light conditions. Light quality as well as light quantity influences the photosynthesis metabolism of the organism [293, 299]. The results from this study indicate that cultures illuminated by infrared light LED panels demonstrate a positive impact on membrane protein insertion for the majority of the tested proteins (**Figure III-14**). The mechanism governing the positive effect of infrared LED illumination on pRhonHi-2 mediated expression of membrane proteins remains hypothetical at this point. Spectral characteristics between common bulb lights and the LED panels (**Figure III-13**) underline that both light sources differ with regard to light intensity and also light quality. While bulb lights encompass a broader range of the electromagnetic spectrum, LED panels demonstrate a higher intensity illumination at a particular wavelength.

One explanation for the improvement could be attributed to changes in the photosynthetic capacity of the bacterium, since a higher light intensity or a different light quality are environmental factors that can alter the formation of vesicles as well as their composition [293, 299]. This in turn would enhance energy resources available for cell growth and protein biosynthesis. Another explanation for the observed positive effect may be that the promoter activity of the employed expression plasmid was directly affected by the altered illumination conditions. An effect of light intensity on the *nifHDK* promoter was already proposed in the preceding section (**IV.1.2**), so a higher light intensity emitted by LED panels may result in lower production of the HvrA protein and consequently in a higher promoter activity.

Previous works conducted in the research group “Bacterial Photobiotechnology” headed by Dr. Drepper discovered a potential blue-light regulation of the *nifHDK* promoter in *R. capsulatus*. Results obtained by Dr. Bergmann [248] and Philipp Hanisch [300] indicate that *R. capsulatus* might be repressing the expression of the *nifHDK* operon by an unknown mechanism in response to blue light, but only in the logarithmic growth phase. Furthermore, Dr. Malach could demonstrate in her doctoral thesis that pRhonHi-2 mediated target gene expression is completely repressed under blue light illumination in *R. capsulatus* [270]. Considering that bulb lights emit blue light as well, it stands to reason if the pRhonHi-2 mediated target gene expression is at least partially negatively affected by bulb light illumination. Thus, cultures illuminated exclusively by infrared LEDs might not be exposed to this modulation and therefore illustrate a higher promoter activity.

2.2 Can *R. capsulatus* be regarded as an alternative platform organism to *E. coli* for the heterologous expression of therapeutically relevant membrane proteins?

The presented thesis was concerned with the evaluation of the phototrophic bacterium *R. capsulatus* for the heterologous expression of therapeutically relevant membrane proteins. Owing to a special membrane physiology the bacterium is characterized by a highly enlarged membrane space under phototrophic growth conditions, a specialized lipid composition and presumably illustrates a high folding capacity which might be beneficial for producing membrane proteins. In the scope of this work ten therapeutically relevant membrane proteins were comparatively expressed in *R. capsulatus* and *E. coli* strains, employing the vector pRhotHi-2 for T7 RNAP driven expression and the vector pRhonHi-2 for P_{nifH} regulated expression of membrane proteins. The comparative analysis (**Figure III-12**) demonstrates that successful production of membrane inserted protein depends mainly on the analyzed membrane protein and by the parameters discussed in the preceding section. However, the obtained results illustrate very clearly that the photosynthetic bacterium can compete with the well-established prokaryotic platform *E. coli* with respect to production of multi-spanning polytopic membrane proteins.

If *R. capsulatus* is compared to the well-established *E. coli* strain BL21 (DE3) or C43 (DE3), it would appear that *R. capsulatus* is the preferable expression host for membrane proteins with a higher number of transmembrane helices (AQP4, SLC30, $A_{2A}R$, BR), whereas proteins with a low number of transmembrane helices seem to be equally well (VpU) or better (MAG, MOG) accumulated in the membranes of the *E. coli* strain BL21 (DE3). The successful performance of *R. capsulatus* indicates the general suitability of the bacterium for membrane protein production. However, the obtained results do not allow a throughout evaluation of the full potential of the organism yet. One has to consider that the comparative expression study reflects initial studies of *R. capsulatus*, performed under standardized conditions. Furthermore, a codon usage analysis depicted in **Table IV-1** reveals that nearly all analyzed genes contain a significant amount of codons that are rarely used in *R. capsulatus*, whereas in *E. coli* the codon usage bias illustrates no incompatibilities.

Table IV-1: Number of codons present in analyzed genes that are used less than 10 % in *E. coli* and *R. capsulatus*

	E. coli	*R. capsulatus*
MAG	0	64
MOG	0	81
AQP4	0	84
SLC30	0	66
BR	0	23
CHRM3	0	103
$A_{2A}R$	0	47
CXCR4	0	53
At1aR	0	79
VpU	**0**	**0**

As illustrated in **Table IV-1**, *VpU* is the only gene which does not show any incompatibilities with the codon usage deployed in *R. capsulatus*. Considering that VpU was the only protein that was characterized by equal accumulation in both *E. coli* and *R. capsulatus*, the codon usage discrepancies in the remaining genes raise the question how severely the limited supply of certain tRNA molecules affected the efficient translation of mRNA molecules in *R. capsulatus*. In addition, optimization studies performed in this work resulted in distinct improvement of membrane protein production in *R. capsulatus*. While an improvement of P_{nifH} regulated membrane protein production could be achieved by employing infrared LED panels, the growth-dependent decrease of proteins such as $A_{2A}R$ could be completely abolished by utilizing auto-induction medium. In addition, proteins such as MAG could only be detected when cultures were grown with altered illumination conditions. Therefore, the obtained results emphasize all the more the great potential of *R. capsulatus* as an expression host, since even with disadvantaged conditions the phototrophic bacterium could compete with an expression host such as *E. coli* that is genetically optimized for protein production (e.g. deletion of lon and OmpT protease).

2.2.1 *R. capsulatus:* an expression platform for slow folding membrane proteins?

R. capsulatus proved particularly successful at inserting membrane proteins with a higher number of TMs, whereas *E. coli* illustrated problems with regard to the amount (e.g. $A_{2A}R$) as well as the growth phase (e.g. AQP4, only detectable in the logarithmic growth phase) at which these proteins could be accumulated in the membrane. One possible explanation for these results can be attributed to differences in the polypeptide elongation and protein folding rates between both organisms. From the standpoint of translocation duration, it can be assumed that multi-spanning polytopic proteins take usually more time for assembly than single-spanning membrane proteins, since more domains need to be translocated across and into the membrane. In *E. coli*, a direct correlation between the growth rate and the translation rate could already be demonstrated [301, 302]. Considering that *R. capsulatus* is characterized by slower growth rates than *E. coli* (**Graph III-3** and **Graph III-4**), there is a possibility that the phototrophic bacterium might exhibit slower polypeptide elongation rates than *E. coli*. Based on the assumption that multi-spanning proteins need more time for proper assembly, *R. capsulatus* might demonstrate a superior ability to insert complicatedly folded membrane proteins, due to the fact that it provides more time for protein folding. Taken together with the fact that in eukaryotic organisms the translation and protein folding occurs at slower rates than in bacteria [136], this could mean that *R. capsulatus* could potentially be the better host for eukaryotic proteins.

2.2.2 *E. coli* BL21 (DE3) illustrates a better performance than *E. coli* C43 (DE3)

Aside from differences between *E. coli* and *R. capsulatus*, the study revealed that between the two *E. coli* strains the C43 (DE3) strain proved inferior to BL21 (DE3) with regard to production of membrane inserted proteins. The mutant strain C43 (DE3) is a BL21 derivative that was discovered in a screen designed for a mutant strain with improved characteristics in coping with toxic effects caused by the expression of membrane and soluble proteins [176]. Beneficial effects of this strain for toxic membrane proteins are contributed to a specific mutation in the *lac* promoter, which effectively leads to a lower production of T7 RNAP. Considering that too much protein production can lead to the saturation of the Sec-translocon capacity, a reduced transcription can be beneficial for some genes. The characteristics of these Walker strains lead to the engineering of the strain Lemo21 (DE3) [177]. By utilizing the natural inhibitor of the T7 RNAP (T7 lysozyme), this strain allows the effective tuning of the transcription rate, to create an optimal equilibrium between the transcription of a gene and the Sec-translocon capacity of the cell. However, the saturation of the Sec-translocon cannot be the only factor governing successful synthesis of a membrane protein, otherwise the Walker strains, which work on a similar principle, would show improved yields for all membrane proteins tested. This was not the case in this study and is in

agreement with observations from literature [176, 177]. Considering that a recent work by Lee *et al.* [303] revealed no real differences in production yields for 10 membrane proteins produced in the expression strains *E. coli* Lemo21 (DE3) and *E. coli* C43 (DE3), when cultivated in LB medium, this work emphasizes the superiority of *R. capsulatus* for the production of polytopic multi-spanning membrane proteins.

2.2.3 Limitations of *R. capsulatus* as an expression platform for therapeutically relevant membrane proteins

Although a number of therapeutically relevant proteins could be accumulated in the membrane of both *E. coli* and *R. capsulatus*, both microbial platforms demonstrated severe problems when expressing GPCRs. Four different GPCRs were analyzed in this study (A_{2A}R, CHRM3, At1aR and CXCR4), out of these four only the GPCR A_{2A}R could be accumulated at considerable amounts in the membrane of *R. capsulatus* cultures. Despite addressing bottlenecks such as the illumination condition, the growth phase, the induction point, the co-production of Mo-nitrogenase or even the codon usage, the successful production of these GPCRs remained elusive in *R. capsulatus*. There can be a number of reasons for failure to express these proteins, since the process of membrane protein assembly encompasses many steps, with each one representing one bottleneck, but the most likely explanation might be connected to missing post-translational modification of these proteins. Many GPCRs are glycosylated [304] or characterized by disulphide-bonds [305-308] which are usually missing in prokaryotic platforms or can be realized only with the utmost difficulty. Since some GPCRs could be functionally expressed in *E. coli* [157, 309] and the phototrophic bacterium *R. sphaeroides* [289] this indicates that while for some GPCRs these post-translational modifications are seemingly unnecessary, for others they can be of paramount importance for assembly as well as stability. Therefore, membrane proteins depending on post-translational modifications for functional expression might be better expressed in eukaryotic platforms such as yeast, mammalian or insect cells.

2.3 Alternative expression hosts for membrane protein production

The problems associated with recombinant production of membrane proteins led to the development and exploitation of several expression systems in the recent years. Each expression platform provides certain advantages and disadvantages over the others, yet all of them are used for various applications, ranging from functional studies to challenging purification and crystallization trials. Although specialized expression hosts such as *Xenopus* oocytes [310], zebra fish [311], *Caenorhabditis elegans* [312] or the

eyes of the fruit fly [313] have been employed, the following section will discuss expression hosts that have emerged as the most commonly used platform organisms for membrane protein production.

2.3.1 Bacteria

A genuine alternative to *E. coli* has presented itself in the last years with the Gram-positive bacterium *L. lactis*. This food degradable bacterium is characterized by only one membrane, does not show any formation of inclusion bodies, features low protease activity and does not produce any endotoxins like *E. coli* does [314]. A genetically robust system such as the nisin inducible controlled gene expression [315] allows for efficient production of membrane proteins. Furthermore, the suitability for the production of eukaryotic membrane proteins could be demonstrated by the overproduction of mitochondrial transport proteins from yeast, receptors from *Homo sapiens* as well as different proteins from plants in a functional state [316, 317]. However, as prokaryotic platforms both *E. coli* and *L. lactis* are usually characterized by limitations with regard to membrane proteins that depend on post-translational modifications such as glycosylation.

2.3.2 Yeast

The yeast strains *S. cerevisiae* and *P. pastoris* have emerged as popular eukaryotic expression hosts that combine properties of prokaryotic cells with regard to easy handling and easy upscaling, while simultaneously providing post-translational modifications observed in higher eukaryotes. The very first atomic structure of a mammalian membrane protein recombinantly produced was achieved by heterologous expression in *S. cerevisiae* [318]. Several strains and many expression plasmids are available in this eukaryotic expression host, relying on both constitutive (e.g. *PMA1* promoter) and inducible expression of target genes (e.g. *GAL1* promoter) [319]. Despite the many advantages *S. cerevisiae* offers, hyperglycosylation has been observed for many membrane proteins [320] and the problem of reaching high cell densities, due to the production of ethanol (byproduct of fermentation). Although the methylotrophic yeast *P. pastoris* is regarded as one of the most employed eukaryotic platform for membrane protein production [321], it nevertheless illustrates severe problems with regard to large scale applications. The most commonly employed expression manner in this host relies on the utilization of methanol as a carbon source and as a means to induce gene expression by the very strong *AOX1* promoter [322]. Despite methanol being a cheap inductor it has the disadvantage of being toxic to humans, which makes it suboptimal for large scale cultivation in the industry.

2.3.3 Higher eukaryotes

In addition to yeast strains, cell lines from higher eukaryotes, including insect and mammalians, have been employed extensively for recombinant membrane protein production [165]. Both expression platforms offer certain advantages and disadvantages. Introducing DNA into eukaryotic cell lines relies most commonly on either viral (transduction) or non-viral based methods (usually referred to as transfection). Typically, two different manners of introducing DNA into eukaryotic cell lines can be distinguished: transient transfection and stable transfection. Transiently transfected genes are not integrated into the genome of the expression host. Taken with the fact that the employed DNA vehicle is usually not able to self-replicate in cell cultures, the target gene is diluted with each cell division or gets degraded with time, resulting in a "transient" expression of recombinant proteins. In contrast, with stably transfected cells the gene of interest is integrated into the genome, so that despite cell division daughter cells contain the recombinant gene and are therefore able to produce the protein of interest at any time [323]. Although stable transfection provides a reproducibility which lacks in transiently transfected cells, the problem is the time required to establish a stable cell line (6 to 12 months [324]). Another problem that many cell lines face are difficulties associated with upscaling. Not every cell line is able to grow in suspension like bacterial cells. Many cell lines grow anchorage-dependent on the solid surface of a culture vessel (adherent cell culture). This type of cultivation hampers reaching high cell densities in an easy manner because cell-proliferation is directly limited by the surface of the culture vessel itself. To achieve high cell densities, a great culture surface and high quantities of expensive cultivation medium is required. The cost and technical requirements of mammalian cell cultures has led to the increased employment of insect cell lines for the production of mammalian membrane proteins, since insect cells provide a lipid environment and protein processing machinery akin to that of mammalians with the advantage of up scaling. Several mammalian receptors, including GPCRs were successfully produced in insect cell lines and used for crystallographic studies [325, 98]. One of the most common expression system deployed in insect cell cultures is the baculovirus expression system [326]. This expression system is based on generating recombinant baculoviruses by integrating the gene of interest into the viral genome through homologous recombination or site-specific transposition. After purification and amplification, the virus containing the target gene is used as a means to transfer DNA into insect cells (usually the cell line *Spodoptera frugiperda - Sf9*) by infection [326, 327]. However, as can be deduced from these steps, not only are the processes involved very time consuming (up to 50 days - https://tools.lifetechnologies.com/content/sfs/manuals/bevtest.pdf), as with mammalian cell cultures, the technical requirements and the costs are major factors encouraging the exploration of alternative expression systems.

2.4 Future directions

Expression of membrane proteins remains a challenging task. In this work a new expression system for the production of therapeutically relevant membrane proteins, based on the purple photosynthetic bacterium *Rhodobacter capsulatus*, was presented. By introducing the new expression plasmid pRhonHi-2, the organism's physiology could be exploited for the production of therapeutically relevant membrane proteins. The results demonstrate that future applications of *R. capsulatus* could potentially focus on further downstream processes connected to successfully produced membrane proteins, as depicted in **Figure IV-3**.

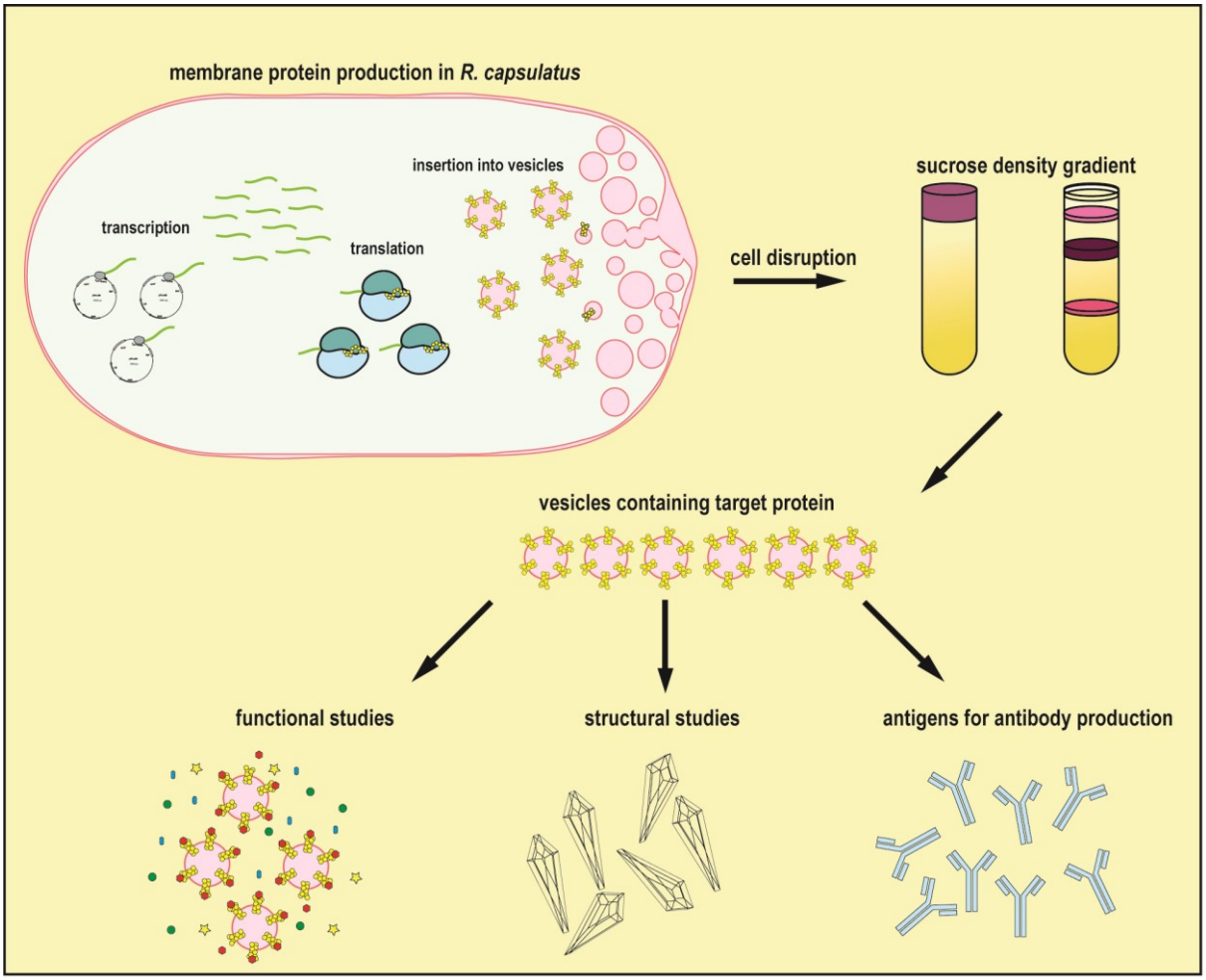


Figure IV-3: Future applications of *R. capsulatus* as a platform organism for vesicle-embedded membrane proteins
A large membrane space, a special lipid composition and highly efficient membrane insertion machineries distinguish the phototrophic bacterium *R. capsulatus* as an expression platform for membrane protein production. Heterologously expressed membrane proteins are inserted into host specific membrane vesicles, which can be easily isolated by sucrose gradient centrifugation as inside-out vesicles. Depending on the application, these vesicles can then be used directly for functional studies or utilized for further downstream processing. Target proteins solubilized by detergents can be purified by means of chromatography, while purified membrane proteins may be utilized for structural studies or serve as antigens for generating antibodies.

Future experiments could also exploit the membrane vesicles of *R. capsulatus* as potential bioreactors. Dr. Malach [270] and Dr. Heck [328] could demonstrate in their doctoral theses that membrane proteins expressed in *R. capsulatus* are targeted and inserted into vesicles of the ICM. As could be shown in this thesis, the expression system is basically applicable for functional production of membrane proteins. Vesicles of phototrophically grown cells can be isolated very easily by differential centrifugation and could potentially be utilized for ligand screenings of recombinant receptors. Since vesicles are composed only of lipids and the enzymes of the photosynthetic apparatus, ligand screenings could be performed without interference from metabolites and proteins of whole cells. Considering that the analyzed proteins in this study had a therapeutic background, *R. capsulatus* could potentially be employed for providing antigens, which can be utilized for generating antibodies against membrane proteins important for cancer and autoimmune disorders. This approach could prove very valuable for future applications, because antibodies raised against antigens from frequently employed expression hosts such as *E. coli* or *L. lactis* may show high cross-reactivity in humans, since both organisms occur naturally in the human body. Furthermore, incorporation of selenomethionine into recombinantly expressed proteins in *R. capsulatus* [329] illustrates the possibility of employing purple bacteria for production of recombinant proteins for X-ray crystallography.

Taken together, these findings make *R. capsulatus* a genuine alternative platform organism, not only for membrane proteins but for recombinant proteins in general. Considering limitations of presently available expression vectors for purple bacteria, the presented pRhonHi-2 expression plasmid may prove an ideal tool for recombinant protein production in *R. capsulatus* but also in related organisms.

Summary

The biotechnological production of membrane proteins by genetically modified organisms plays a pivotal role for medical and pharmaceutical research. Around 50 % - 70 % of all available drugs on the market are either directly or indirectly targeting membrane proteins. Despite this huge interest in this protein class, there is still fundamental knowledge lacking about the structure and function of these proteins. Common expression systems and common expression hosts such as *E. coli* are optimized for the production of soluble proteins, but not for the heterologous expression of membrane proteins. For this reason, apart from exploring new expression systems it is important to exploit alternative expression hosts, which are naturally adapted to produce membrane proteins due to their specialized physiology.

The presented thesis was concerned with the evaluation of the phototrophic bacterium *Rhodobacter capsulatus* for the heterologous expression of therapeutically relevant membrane proteins. To this end, a new expression vector (pRhonHi-2) based on the host specific *nifHDK* promoter was first constructed. Expression studies conducted with the help of a reporter gene illustrated that genes placed under the control of the *nifHDK* promoter can easily and very strictly be regulated by the nitrogen source in the cultivation medium. Furthermore, deploying alternative cultivation vessels as well as a deletion mutant could improve pRhonHi-2 mediated target gene expression significantly, so that an ideal basis for the efficient evaluation of the bacterium was established. For the actual evaluation, 10 therapeutically relevant membrane proteins characterized by different topologies and physiological functions were comparatively expressed in *R. capsulatus* and the established platform organism *Escherichia coli*. The evaluation of *R. capsulatus* was conducted under consideration of the following parameters: i) the employed promoter ii) the expression duration iii) the employed *R. capsulatus* strain. The comparative expression analysis illustrated that successful insertion of proteins into the membrane of an expression host is affected by all these factors. Furthermore, the results led to the conclusion that membrane proteins exhibiting a higher number of transmembrane domains were accumulated at higher levels in the membrane of *R. capsulatus*, whereas membrane proteins exhibiting a lower number of transmembrane domains illustrated equal or better membrane insertion in the expression host *E. coli* BL21 (DE3). Subsequent experiments could further improve the new expression system by alteration of cultivation conditions with regard to illumination and medium composition. Finally, the applicability of *R. capsulatus* for the production of correctly folded membrane proteins could be verified using the example of the proton pump bacteriorhodopsin.

Zusammenfassung

Die biotechnologische Produktion von Membranproteinen mit Hilfe von gentechnisch veränderten Organismen nimmt eine zentrale Rolle für die medizinische und pharmakologische Forschung ein. 50 % - 70 % aller auf dem Markt erhältlichen Medikamente sind direkt oder indirekt gegen Membranproteine gerichtet. Trotz großer Nachfrage fehlen immer noch fundamentale Informationen über die Struktur und Funktion dieser Proteine. Viele bekannte Expressionssysteme und Expressionswirte wie z.B. *E. coli* sind für die Produktion von löslichen Proteinen, nicht jedoch für die heterologe Expression von Membranproteinen optimiert. Aus diesem Grund ist es wichtig, nicht nur alternative Expressionssysteme zu erforschen, sondern auch alternative Expressionswirte, die aufgrund ihrer speziellen Physiologie natürlicherweise an die Produktion von Membranproteinen adaptiert sind.

Im Rahmen der vorliegenden Arbeit wurde das fakultativ phototrophe Bakterium *Rhodobacter capsulatus* für die heterologe Expression von therapeutisch relevanten Membranproteinen evaluiert. Hierzu wurde zunächst ein neuer Expressionsvektor (pRhonHi-2) basierend auf dem wirtseigenen *nifHDK* Promoter konstruiert. Expressionsstudien mit einem Reportergen haben gezeigt, dass die Expression des Zielgens unter der Kontrolle des *nifHDK* Promotors sehr strikt und einfach über die verwendete Stickstoffquelle im Kultivierungsmedium gesteuert werden kann. Ferner konnte durch Verwendung von alternativen Kultivierungsgefäßen und einer Deletionsmutante die Expression gesteigert werden, so dass eine ideale Basis für die effiziente Evaluierung des Bakteriums geschaffen wurde. Für die Evaluierung wurden 10 therapeutisch relevante Membranproteine, die alle unterschiedliche physiologische Funktionen und Topologien aufweisen, vergleichend in *R. capsulatus* und der etablierten mikrobiologischen Plattform *Escherichia coli* exprimiert. Hierbei wurde die heterologe Expression in *R. capsulatus* unter Berücksichtigung folgender Paramater evaluiert: i) verwendeter Promoter ii) Expressionsdauer iii) verwendeter *R. capsulatus* Stamm. Die vergleichende Expressionsstudie hat ergeben, dass die erfolgreiche Insertion von Proteinen in die Membran von *R. capsulatus* von all diesen Faktoren abhängt. Ferner konnten die Ergebnisse aufzeigen, dass Membranproteine mit einer hohen Anzahl an Transmembrandomänen in höheren Mengen in der Membran von *R. capsulatus* akkumulierten, wohingegen Membranproteine mit einer geringen Anzahl an Transmembrandomänen vergleichbar oder effizienter in die Membran von *E. coli* BL21 (DE3) inseriert werden konnten. In nachfolgenden Studien konnte das neue Expressionssystem durch Veränderung der Kultivierungsbedingungen hinsichtlich der Beleuchtung und des verwendeten Kultivierungsmediums weiter optimiert werden. Abschließend konnte die Anwendbarkeit von *R. capsulatus* für die Herstellung von korrekt gefalteten Membranproteinen am Beispiel der Protonenpumpe Bakteriorhodopsin gezeigt werden.

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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(Unterschrift)