Advancing *Rhodobacter sphaeroides* as a platform for expression of functional membrane proteins

Running title: Functional membrane proteins from *Rhodobacter*

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ABSTRACT

Membrane protein overexpression is often hindered by toxic effects on the expression host, limiting achievable volumetric productivity. Moreover, protein structure and function may be impaired due to inclusion body formation and proteolytic degradation. To address these challenges, we employed the photosynthetic bacterium, *Rhodobacter sphaeroides* for expression of challenging membrane proteins including human aquaporin 9 (hAQP9), human tight junction protein occludin (Occ), *E. coli* toxin peptide GhoT, cellulose synthase enzyme complex (BcsAB) of *R. sphaeroides* and cytochrome-cy (Cyt-cy) from *R. capsulatus*. Titters of 47 mg/L for Cyt-cy, 7.5 mg/L for Occ, 1.5 mg/L for BcsAB and 0.5 mg/L for hAQP9 were achieved from affinity purification. While purification of GhoT was not successful, transformants displayed a distinct growth phenotype that correlated with GhoT expression. We also evaluated the functionality of these proteins by performing water transport studies for hAQP9, peroxidase activity for cytochrome-cy, and *in vitro* cellulose synthesis activity assay for BcsAB. While previous studies with *Rhodobacter* have utilized oxygen-limited semi-aerobic growth for membrane protein expression. Substantial titer improvements are achieved as a result of a 3-fold increase in biomass yield using the anaerobic photoheterotrophic growth regime, which utilizes the strong native *puc* promoter. This versatile platform is shown to enable recovery of a wide variety of difficult-to-express membrane proteins in functional form.

KEYWORDS: *Rhodobacter sphaeroides*, cytochrome, aquaporin, cellulose synthase, toxin-antitoxin, anaerobic photoheterotrophic growth, membrane protein.

INTRODUCTION

Membrane proteins (MPs) play critical physiological roles in all biological cells including selective transport of solutes, signal transduction, energy generation, cell-cell adhesion and motility (Link and Georgiou, 2007). Typically, 30% of any genome encodes for MPs (Stevens and Arkin, 2000). Despite being of crucial importance, MPs are underrepresented in protein structural databases compared to soluble proteins. According to RSCB Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>) nearly 108,000 protein structures are available from different protein families including roughly 9,800 soluble protein classes (Laible et al., 2009). By comparison, the "Membrane proteins of known 3D structure" database (<http://blanco.biomol.uci.edu/mpstruc/>) indicates 539 unique families identified from 1654 total number of MP structures, where the vast majority of these are of bacterial and archaeal origin. Structural data for MPs facilitate more detailed understanding of mechanisms of action, which is crucial to human health, as more than 60% of the FDA approved drugs target MPs (Yildirim et al., 2007). Availability of MPs is also desired for high throughput drug screening (Lundstrom, 2004), biotechnological (Nielsen, 2009) and biomedical applications (Majd et al., 2010).

Most MPs are available in only very limited quantities from natural sources, necessitating recombinant expression for further study. A variety of prokaryotic and eukaryotic expression systems have been employed for recombinant MP expression (Bernaumat et al., 2011; Nielsen, 2009; Sarramegna et al., 2003). While eukaryotic systems employing yeast, insect, or mammalian cells are the inevitable choice for proteins requiring significant post-translational modification; prokaryotic systems offer advantages in ease of genetic manipulation, rapid growth, and inexpensive culturing (Laible et al. 2011). *E. coli* is the most common protein expression system for proteins not undergoing post-translational modifications; however MP

expression titers in *E.coli* remain one to two orders of magnitude lower than for soluble proteins (Tate, 2001). A major challenge to MP expression in *E. coli* is limited membrane area to accommodate overexpressed MPs forcing them into inclusion bodies in which native MP structure and function are lost (Arechaga et al., 2000).

To address these issues, we employed a facultatively anaerobic phototrophic purple bacterium, *Rhodobacter sphaeroides* 2.4.1, for functional expression of challenging MPs. The most striking feature of the *Rhodobacter* species relevant to MP expression is the presence of an extensive network of intracellular photosynthetic membranes (ICMs) that, when induced by reduced oxygen tension (0.5-2%) and low light intensity ($\sim 4 \text{ W/m}^2$), increase cellular membrane surface area by more than an order of magnitude (Adams and Hunter, 2012; Drews and Golecki, 1995). ICM formation is therefore concomitant with the synthesis of the photosynthetic apparatus (Zeilstra-Ryalls et al., 1998). The *Rhodobacter* photosystem is comprised of the photosynthetic reaction center (RC) surrounded by first- and second-tier light harvesting antennae (LH1 and LH2) (Sener et al., 2007). RC and LH1 are expressed from the *puf* operon in response to reduced oxygen tension. LH2 is expressed from the *puc* operon in response to reduced oxygen tension and reduced light intensity.

Previous work has demonstrated improved heterologous MP expression titers by knocking out some or all of the photosynthetic complex proteins, possibly by increasing the available ICM area (Hanson et al., 2009). Protein expression in this system is most commonly driven by the *puf* promoter with semi-aerobic induction (Laible et al., 2004; Laible et al., 2009; Roy et al., 2008) utilization of the *puf* promoter is motivated in part by the ease of implementing ‘semi-aerobic’ growth in oxygen-limited shake flask cultures. Although expression driven by the *puc* promoter in the anaerobic photoheterotrophic growth regime has met with limited success

(Bernaudo et al., 2011), this growth mode has the greatest promise for exploiting the unique physiological properties of *Rhodobacter*. Cellular concentrations of LH2 driven by the *puc* promoter can exceed 5% of dry weight, and are significantly higher than the concentrations of RC-LH1 complexes and ICM under anaerobic photoheterotrophic conditions (Remsen, 1978). Knocking out LH2 frees up membrane surface area in the ICM while retaining the ability to grow photoheterotrophically. In this study, our results substantiate this approach, since anaerobic photoheterotrophic cultures reached 3-4 times higher biomass yield compared to semi-aerobic cultures while experiencing no loss in specific MP productivity. Considering these advantages, we optimized promoter induction under anaerobic photoheterotrophic conditions and demonstrated the versatility of the system for functionally expressing MPs from various sources and levels of complexity.

MATERIALS AND METHODS

Culturing Conditions

Anaerobic photoheterotrophic *Rhodobacter* cultures were grown in 750 mL T-flasks under a bank of infrared LEDs with a peak wavelength of 850 nm (4W/m², Snowdragon Industrial Co. Ltd, Shenzhen, China). A schematic and picture of the relatively simple experimental apparatus is shown in Figure 1. Temperature was maintained by partial submergence in a plastic storage container on a stir plate with a stir-bar inside the completely filled T-flask for mixing. . To maintain consistency across batches with different levels of pigmentation, the optical density was measured via Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale CA) at 660 nm to avoid interference by photosynthetic pigments (Myers et al., 2013).

Measurement of Growth Kinetics and Biomass Yield

8 mL of semi-aerobic cultures were grown in 25 mm culture tubes in gyratory shaker at 32°C, while 8 mL photoheterotrophic cultures were grown in 1 cm diameter screwcap culture tubes mounted in a stellate pattern on a rotator (perpendicular to the 45° rotating access) such that the small gas space would move up and down the tube during rotation at ~15 RPM. Growth in these ~ 1 cm diameter culture tubes enabled OD₆₆₀ measurements within the spectrophotometer (Curtis, 2011). The cultures were grown until the stationary phase by measuring OD₆₆₀ every 3 hours. For biomass yield measurements, 5 mL culture was spun down at 15,000xg, washed twice in 1.6 mL microfuge tubes with tap water instead of deionized water to avoid cell lysis and lyophilized at least for 24 hours in a pretared tube.

Molecular Biology and Specific Constructs

Vector constructs used in this study are listed in Table I. All of the target genes were cloned between unique 5' SpeI and BglII^{3'} enzyme sites in *pRKPLHT1&7* vectors to introduce 7xHis-tag from the vector backbone to the C-termini of target MPs. The native aquaporin of *Rhodobacter sphaeroides*, *RsAqpZ* (Genbank id: ABA78939), was included as a positive control for expression level between the constructs (Erbakan et al., 2014). *Rhodobacter sphaeroides* 2.4.1 codon optimized genes for human aquaporin 9 (Genbank id: NP_066190), as well as transmembrane and C-terminus of human occludin (Genbank id: AAH29886) were purchased from Genscript Inc., Piscataway, NJ. *pET28b-ghoT* vector, kindly provided by Dr. Thomas Wood was used for cloning *ghoT* (Genbank id: BAE78131) into the *pRKPLHT7* vector. BcsAB complex was cloned into *pRKPLHT7* vector as a polycistronic gene using in-fusion cloning kit (Clontech Laboratories, Inc., Mountainview, CA). *bcsA* (Genbank id: ABA79509) and *bcsB* (Genbank id: ABA79508) genes were PCR amplified from genomic DNA with primers having 15 bp overlaps to the vector backbone. A 12xHis-tag sequence and a stop codon was placed in

the downstream *bcsA* gene to generate a C-terminally His-tagged version of BcsA subunit for affinity purification of the protein complex as described previously (Morgan et al., 2013). Start codon of *bcsB* was immediately after *bcsA* stop codon and an additional stop codon was included within the *bcsB* reverse primer to prevent the addition of a vector-encoded 7xHis-tag to its C-terminus.

Protein Purification

For purification of hAQP9, Cyt-cy, RsAqpZ, Occludin and GhoT, a detailed protein purification protocol was described elsewhere (Erbakan et al., 2014). The minor modifications employed for purification of BcsAB are described here: BcsAB lysis buffer contained 20 mM Tris at pH=7.2, 1 mM MgCl₂, 5 mM cellobiose, 10 mM imidazole, 1 mM PMSF, 0.1 mg/mL DNase, 0.02 mg/mL RNase, and 1 mg/mL lysozyme. Solubilization buffer used to purify the BcsAB complex had 1% n-dodecyl β -D-maltoside (DDM) (Anatrace, Maumee, OH), and 1% n,n-dimethyldodecylamine n-oxide (LDAO) (Anatrace, Maumee, OH) on 0.5 mL Co-NTA resin (1 mL slurry) (Thermo Fisher Scientific Inc.). Non-specific binding to the column was removed by washing with 30 bed volumes of buffer containing 20 mM Tris at pH=7.2, 0.05 % DDM, 5 mM MgCl₂, 5 mM cellobiose, 300 mM NaCl, 10 % glycerol and imidazole at concentrations ranging between 10-50 mM. For elution of BcsAB complex imidazole concentration in wash buffer was increased to 1 M. Gel filtration chromatography was performed to further purify the eluted fractions from Co-NTA resin with the same buffer devoid of imidazole.

Functional Assays

Water Transport Assay: Water transport activity of hAQP9 was measured in proteoliposomes using stopped-flow light scattering technique as described elsewhere (Erbakan et al., 2014). The light scattering traces were fit to double exponential rise curves to obtain the water transport rate

constant k. Permeability of control liposomes and hAQP9 proteoliposomes were calculated using the following equation:

$$P_f = \frac{k}{(S/V_o) \cdot V_{H_2O} \cdot \Delta\pi} \quad (1)$$

where S/V_0 is the initial surface area to volume ratio of the vesicles, V_{H_2O} the molar volume of water and $\Delta\pi$ is the applied osmotic gradient.

Cytochrome-cy Peroxidase Activity: Cytochrome-cy peroxidase activity was measured using Single Component TMB Peroxidase EIA Substrate Kit (Bio-Rad Laboratories Inc, Hercules, CA) according to the instructions of the manufacturer using Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale CA). The kinetics of the reaction between H_2O_2 and TMB was monitored by shifting of the absorbance maximum of TMB from 280 to 655 nm, which corresponds to the enzymatic activity of Cyt -cy. The extinction coefficient of oxidized TMB ($39000 \text{ M}^{-1} \text{ cm}^{-1}$) was used to calculate the specific activity of Cyt-cy in nmol H_2O_2 reduced/min/ μmol Cyt-cy. Inhibition test of peroxidase activity for Cyt-cy was performed with 1 mM and 5 mM sodium azide, as a radical scavenger (Kim et al., 2004).

BcsAB in-vitro Cellulose Synthesis: The activity of *in vitro* cellulose synthesis by BcsAB complex was evaluated using the UDP- C^{14} -glucose incorporation assay (Brown et al., 2012; Omadjela et al., 2013). The controls were devoid of either BcsAB complex or cyclic di-GMP. For the treatment, 0.3 μmol of purified BcsAB complex was incubated with a reaction mixture of 300 μL containing 0.5 mM Tris at pH=7.5, 1 mM UDP-glucose, 1 μM UDP- C^{14} -glucose, 20 mM $MgCl_2$, 5 mM cellobiose, at 30°C for 30 min. The reaction was stopped by addition of 2% sodium dodecyl sulfate (SDS). The unincorporated UDP- C^{14} -glucose was removed by transferring the reaction mixture onto a glass filter and washing with 15 mL of 0.5 mM Tris

buffer at pH=7.5. The scintillation counts were measured overnight and the results were expressed as nmol UDP-glucose incorporated per minute per mg of BcsAB complex.

GhoT Activity: A phenotype change upon expression of the GhoT toxin was observed growing the *Rhodobacter* YCC-tet1 agar plates under semi-aerobic and photoheterotrophic conditions. *Rhodobacter* strains expressing native and less toxic GhoTF38R mutant (Cheng et al., 2014) from pRKPLHT7 vector were used. Due to protein expression from the puc promoter, which is most active for anaerobic photosynthetic growth, semi-aerobic growth can be used to observe reduced toxicity during lower expression. We streaked empty vector strain (control), pRKPLHT7-GhoTF38R and pRKPLHT7-GhoT strains on YCC-Tet (1mg tet/L) agar plates and grew either semi-aerobically in a 34°C incubator or photoheterotrophically under infrared LEDs in an anaerobic growth chamber.

RESULTS

Choice of Growth Mode

LH2 knockout cells (*PUC705-BA*) with and without expression plasmids for RsAqpZ and Occludin were grown both semi-aerobically and photoheterotrophically as described. Biomass yields for photoheterotrophic cultures were up to four times higher compared to semi-aerobic cultures despite the initial lag phase (Fig. 2A, 2B). Moreover, the specific protein expression level remained comparable for both growth modes, leading to a much higher volumetric productivity for photoheterotrophic cultures. Therefore, this study focused on using a technique to take advantage of photoheterotrophic conditions for protein expression.

Protein Expression and Functional Activity Results

Rhodobacter capsulatus Cytochrome-cy

Membrane bound cytochrome-cy from *Rhodobacter capsulatus* is involved in electron transfer during photosynthesis and aerobic respiration (Jenney and Daldal, 1993). Expression levels as high as 50 mg/L have been reported for this protein (Laible et al., 2009), although functionality was not evaluated. SDS-PAGE (Fig. 3A) and Western-Blot (Fig. 3B) analysis demonstrated abundant expression under photoheterotrophic conditions.

Cyt-cy peroxidase activity was measured to evaluate its functionality. The extent of the reaction between hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is monitored by absorbance at 655 nm corresponding to oxidized product; 3,3',5,5'-tetramethylbenzidine diimine. Time-dependent oxidation of TMB was proportional to cyt-cy concentration demonstrating that cyt-cy was active (Fig. 4A). Cyt-cy had a specific activity of 1.18 ± 0.38 nmol H₂O₂/min/ μ mol cyt-cy, which is close to the reported value for soluble c-type cytochromes (Kim et al. 2004) (Fig. 4B). The peroxidase activity was quenched to 25% of the maximum value (0.31 ± 0.07 nmol H₂O₂/min/ μ mol cyt-cy) by 5 mM sodium azide (Fig. 4C), showing that the presence of a free-radical scavenger decreased cytochrome-mediated oxidation of TMB.

Rhodobacter Cellulose Synthase, BcsAB

BcsAB is a membrane-associated cellulose synthase complex composed of BcsA and BcsB subunits. BcsA is an integral MP with 8 transmembrane α -helices and a cytosolic glycosyl transferase domain. The C-terminal α -helix of BcsB associates with transmembrane α -helices of BcsA to form the active complex, while the remaining portion of the protein, which is formed by several α -helix and β -sheet domains, resides in the periplasmic space. In *Rhodobacter sphaeroides*, BcsA and BcsB subunits are expressed as a polycistronic message; we employed

the same strategy in our photosynthetic expression system, with the addition of a 12-His tag to the C-terminus of BcsA to facilitate purification by metal affinity chromatography. BcsAB complex was purified from the membrane fraction of *Rhodobacter* with metal affinity and gel filtration chromatography, which effectively removed smaller contaminating proteins while also resolving a 50kDa band that was identified by mass spectrometry as a S41 type peptidase (uniprot id: Q3IZC0). These purification steps yielded 1.5 mg BcsAB per liter of culture at an OD₆₆₀ of 5, which corresponds to 0.5 mg BcsAB/g cells. In 10% SDS-PAGE gel, oxidized BcsB migrates at 100 kDa while BcsA migrates at 80 kDa as confirmed by LC/MS tandem mass spectroscopy, which is in accordance with the previous reports (Omadjela et al., 2013). Since only BcsA contains the His-tag to bind to the affinity resin, co-purification of BcsA and BcsB subunits at comparable molar ratio as shown in the subsequent SDS-PAGE gel in Fig. 5A indicates the purification of BcsA and BcsB in form of BcsAB complex. Western blotting with His-tag antibody detected a band at 80 kDa corresponding to BcsA-12xHis monomer (Fig. 5A).

In vitro cellulose synthesis activity of BcsAB was evaluated in solution using a UDP-C¹⁴-glucose incorporation assay in the presence or absence of cyclic di-GMP. Consistent with the known regulation of this cellulose synthase (Morgan et al., 2014), cyclic di-GMP controlled enzyme activity in an allosteric manner (Fig. 5B). The background scintillation count in the absence of BcsAB was subtracted from both treatments with and without cyclic di-GMP. The amount of incorporated UDP-C¹⁴-glucose was almost two orders of magnitude higher with cyclic di-GMP treatment, clearly demonstrating a functional BcsAB glucose polymerization activity. The specific activity of BcsAB measured in detergent micelles was 58 nmol/mg BcsAB/min, which was an order of magnitude lower than the published specific activity measured in an alternative of proteoliposomes (Omadjela et al., 2013).

Aquaporin 9

Human aquaporin 9 (hAQP9) belongs to a large family of channel forming porin proteins. It has 6 transmembrane α -helices with two asparagine-proline-alanine (NPA) signature motifs contributing to its hourglass structure. Human aquaporin 9 was overexpressed to generate 0.2 mg/L purified protein. The expression was verified with the presence of monomeric and tetrameric form of the protein at 28 and 60 kDa respectively, which were detected by both SDS-PAGE and Western-blot using a 4-12 % Nu-PAGE Tris-glycine gradient gel (Life Technologies, Grand Island, NY) (Fig. 6). The monomeric form of hAQP9 ran slightly faster on SDS-PAGE gel, which is typical for MPs due to increased SDS binding stoichiometry per amino acid residue (Rath et al., 2009). On the other hand, hAQP9 tetramer showed a significantly increased mobility, which was possibly due to the compact structure of the intact tetrameric form and well documented in the literature for other aquaporins (Borgnia et al. 1999; Erbakan et al. 2014). The water permeability was directly proportional to the quantity of hAQP9 in proteoliposomes (Fig. 7), thereby confirming functional transport of H₂O into vesicles by the heterologously expressed and purified aquaporin.

Occludin

Occludin is a tight junction protein with 4 transmembrane α -helices forming two extracellular and one intracellular loop. Extracellular loops from adjacent cells bind strongly to contribute to formation of the tight junction barrier. A codon-optimized human occludin gene corresponding to last 474 amino acids of the protein encompassing the transmembrane and C-terminal region was expressed in *Rhodobacter* at 7.5 mg/L as evidenced by SDS-PAGE and Western-blotting (Fig. 8). Unlike other target proteins in this study, occludin lacks an enzymatic

or transport activity, which makes it difficult to assess the functionality of the protein. An attempt at quantifying occludin-mediated proteoliposome aggregation by dynamic light scattering was not successful.

***Escherichia coli* Toxin, GhoT**

GhoT forms a type V toxin/anti-toxin system with GhoS protein in *E.coli*, in which GhoS protein degrades *ghoT* mRNA to ensure survival of the cell (Wang et al., 2012). Upon repression of *ghoS* gene transcription, *ghoT* mRNA is translated resulting in a ghost cell phenotype, where GhoT toxin disrupts plasma membrane, thus leading to cell death. We expressed GhoT in *Rhodobacter* under both semi-aerobic and anaerobic photoheterotrophic conditions. Since the *puc* promoter has limited activity during semi-aerobic growth, the GhoT expression level is expected to remain low. Consistent with low expression, there was not a noticeable phenotype change for the semi-aerobically grown colonies (Fig. 9A). However, under anaerobic photoheterotrophic conditions, where *puc* promoter was highly active, a distinct phenotype was observed that was characterized by excessive biofilm formation, consistent with GhoT imposed toxicity (Fig. 9B). To provide the further evidence for functional GhoT expression, the strain expressing less toxic GhoT-F38R mutant was tested under both growth conditions, as well. The stressed biofilm phenotype was nearly completely attenuated for both semi-aerobic and anaerobic photoheterotrophic growth consistent with a greatly reduced toxicity for the mutant toxin form. The observation of general stress but not death due to GhoT expression suggests that high levels of intracellular membranes in *Rhodobacter* could buffer the toxic effects. The purification of 7-Histidine tagged version of GhoT using cobalt or nickel affinity resins was not

successful possibly due to its small size (7.7 kDa) and extremely high isoelectric point (pI~10.7) as estimated by isoelectric point calculator (Kozlowski, 2013).

DISCUSSION

Our results suggest that functional expression was achieved for at least four out of the five of the diverse MPs tested. The purified protein levels, as summarized in Table II, were superior to alternative expression hosts, where comparisons were available. More importantly, the goal of maintaining functionality was confirmed for four of these proteins, where a functional test was available. We attribute the successful expression to the high levels of intracellular membranes in *Rhodobacter* as has been shown to improve membrane protein expression in PAH1 mutants of the yeast, *Yarrowia lipolytica*, that display excess proliferation of endoplasmic reticulum membranes (Guerfal et al., 2013). While *Rhodobacter* has been recognized for its utility for MP expression, the photoheterotrophic growth mode has received minimal attention because it is more difficult to implement. At the same time, the additional parameters of light intensity, light quality, temperature and dissolved oxygen present a tremendous opportunity to improve and optimize this system. Optimization was not addressed in this work in favor of initially establishing a greater breadth of functional testing. Light driven degradation of antibiotic selection marker, tetracycline, was not an issue in our expression studies, as we avoided this problem using infrared LEDs as light source. Modification of the expression vector selectable marker to avoid this problem has been demonstrated (Mather et al., 1995), but adoption of that approach for this expression platform remains to be addressed in future studies. The individual MP targets are discussed further below.

Cyt-cy was chosen as a model protein known to be expressed at high levels reaching 50 mg/mL (Laible et al., 2009). In this work, the expression titers were improved and functionality of this protein was evaluated by measuring its peroxidase activity using a chromogenic peroxidase substrate TMB. The measured activity was as 1.18 ± 0.38 nmol H₂O₂/min/μmol Cyt-cy, which was comparable to the other reported values in the literature for P450-type cytochromes (Kim et al., 2004).

BcsAB is the first cellulose synthase enzyme complex for which the high-resolution crystal structure has been determined. Both BcsA and BcsB proteins were reported to be necessary for the enzymatic activity (Morgan et al., 2013). We expressed a C-terminally 12-His-tagged version of BcsA and BcsB from a single *puc* promoter and purified with a yield reaching 1.5 mg/L culture, which significantly reduces the expression volume of 36 L reported for the *E. coli* expression system which yielded 0.15 mg BcsAB per liter of culture. The complex was active as evidenced by *in vitro* cellulose synthesis assay using UDP-C¹⁴-glucose. The specific activity measured for this work in detergent micelles was 58 nmol /mg BcsAB/min. Although this is significantly lower than previously reported BcsAB activity that was measured in proteoliposomes using an enzyme-coupled assay (Omadjela et al., 2013), the results reported here demonstrate a reduced activity by an order of magnitude in the absence of cyclic di-GMP, the allosteric effector of BcsAB, as a demonstration of functionality that is consistent with other previous reports (Morgan et al., 2014). The expression of the cellulose synthase represents an increased challenge for *Rhodobacter* platform, since BcsAB is a much more complex multi-subunit MP. Somewhat ironically, a large effort to express this important class of proteins from more than 20 different species resulted in *Rhodobacter sphaeroides* BcsAB emerging as the major expression success including the structure determination (Morgan et al., 2013). Although

this is a homologous MP candidate, it represents considerable significance due to the ongoing efforts to understand plant cell walls – including their use as a biofuel feedstock (Abramson et al., 2010). It would be interesting to screen the expression of cellulose synthases from other model organisms such as the plant *Arabidopsis thaliana*. The purified BcsAB amount was almost an order of magnitude less compared to RsAqpZ, a homologous aquaporin water channel protein (Erbakan et al., 2014), possibly due to the challenge of functional assembly of the multimeric protein. Such assembly likely benefitted from expression in a homologous host as well as our polycistronic construct, which would provide stoichiometric balance of the co-translated subunits.

Human aquaporin 9 (hAQP9) is an aquaglyceroporin, which has permeability to water and several other small-uncharged molecules (Tsukaguchi et al., 1999). Here, we expressed a *Rhodobacter* codon-optimized hAQP9 gene under anaerobic photoheterotrophic conditions with a yield around 0.2 mg/L as estimated by SDS-PAGE and Western-blotting. This level of purified protein is 2-fold higher than Sf9 insect cell expression system (Viadiu et al., 2007). Functionality was assessed by measuring the osmotic water permeability in phospholipid vesicles embedded with purified hAQP9. The water permeability was directly proportional to the protein mass fraction in liposomes indicating significant water transport by heterologously expressed hAQP9. An assumption of 100% incorporation and functionality of the expressed hAQP9 provides a single channel permeability of $6.8 \times 10^{-14} \text{ cm}^3/\text{s}$, which is roughly 2-fold lower than the original report for *E. coli* AqpZ (Borgnia et al., 1999). However, as we have shown using fluorescently tagged *Rhodobacter* AqpZ by means of fluorescence correlation spectroscopy, the reconstitution efficiency of aquaporins in liposomes is severely reduced at higher protein to lipid ratios, which results in significant underestimation of the actual water permeability (Erbakan et al. 2014). The

success in achieving functional heterologous expression of a human aquaporin in *Rhodobacter* is a significant accomplishment for the *Rhodobacter* expression platform and opens the opportunity for further study of this exciting class of proteins. Although codon optimization of the hAQP9 gene may have contributed to reaching the highest heterologous expression level yet reported, the titer remained significantly lower compared to structurally similar homologous aquaporin, RsAqpZ. This emphasizes the utility of developing a breadth of MP expression platforms to study this important class of proteins.

Occludin is a structural MP localizing in tight junctions of epithelial and endothelial cells with many implications in breast, prostate and brain cancers (Feldman et al., 2005). Therefore, understanding its function and structural properties is useful towards design of new cancer therapeutics. A high level expression of occludin construct composed of transmembrane and C-terminal domains is observed in our *Rhodobacter* system. The focus on transmembrane and C-terminal domains was chosen because they have been reported to represent the critical functional domains in tight junction formation and signaling through binding partners, respectively (Feldman et al. 2005). Functional characterization of occludin is hampered by a lack of known enzymatic or transport activity for this protein. Thus, a radio-ligand binding or complementation assay remains to be performed for proven functionality.

GhoT is a small microbial toxin, responsible for ghost cell phenotype in *E. coli* (Wang et al., 2013). When expressed in *E. coli* using the T7 expression system, it causes sudden death of the cells precluding use of this host for production studies. The expression of GhoT in *Rhodobacter* system was undertaken in part to test the hypothesis that the large membrane content of *Rhodobacter* during photoheterotrophic growth might reduce the toxic effects of GhoT. Indeed, *Rhodobacter* showed a higher level of tolerance to GhoT expression reaching

OD₆₆₀ ~3.0. Although the biomass yield remained lower compared to the strains expressing other target MPs, GhoT expression strain survived and was associated with an expression-dependent mucoid phenotype. The subsequent efforts on expression quantification and purification were problematic due to high isoelectric point (~10.7) and small molecular weight (~7.7 kDa) of the protein.

The work with GhoT and occludin is an important reminder that achieving expression is only an initial step in the study of MPs, as their individual characteristics require extensive expertise in purification and subsequent functional characterization. In this study, we show not only versatility of *Rhodobacter* expression system but also functionality of the expressed proteins, which is critically important for studies requiring function such as biomimetic devices, high-throughput drug screening and drug delivery applications.

CONCLUSIONS

This preliminary success of the photoheterotrophic *Rhodobacter* MP expression platform invites additional improvements and optimizations. Besides optimization of light and dissolved oxygen, the expression vectors themselves were originally designed for semi-aerobic growth in the dark and warrant improvement. Light-sensitive tetracycline selectable marker and the requirement of conjugal plasmid transfer from *E. coli* are few of the cumbersome aspects of the system that could be readily improved. Even in the current unoptimized form, the tools developed in this work allow for rapid assessment of the expression level and the ease of purification; where assays are available for functional testing, the production of a particular protein in *Rhodobacter* deserves consideration.

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Tables

Table I. Vector constructs used in this study

Construct	Description	Reference
<i>pRKPLHT1</i> -cyt cy	Semi-aerobic expression of <i>Rhodobacter capsulatus</i> Cytochrome-cy under control of <i>puf</i> promoter	(Laible et al., 2009)
<i>pRKPLHT7</i> -cyt cy	Photoheterotrophic expression of <i>Rhodobacter capsulatus</i> Cytochrome-cy under control of <i>puc</i> promoter	(Laible et al., 2011)
<i>pRKPLHT7</i> -RsAqpZ	Photoheterotrophic expression of <i>Rhodobacter sphaeroides</i> under control of <i>puc</i> promoter	(Erbakan et al., 2014)
<i>pRKPLHT7</i> -ghoT	Photoheterotrophic expression of <i>E. coli</i> GhoT peptide under control of <i>puc</i> promoter <i>ghoT</i> _F*: TAAAGTACTAGTGGAGGCCATTCATGGCACTATTCTCT <i>ghoT</i> _R*: GCCGCCAGATCTAAAGAGAGAAAAAAGTAATGCCAC	This study
<i>pRKPLHT7</i> -occludin	Photoheterotrophic expression of transmembrane and C-terminus of human occludin under control of <i>puc</i> promoter	This study
<i>pRKPLHT7</i> -human AQP9	Photoheterotrophic expression of human aquaporin 9 under control of <i>puc</i> promoter	This study
<i>pRKPLHT7</i> -bcsAB	Photoheterotrophic expression of <i>Rhodobacter sphaeroides</i> 2.4.1. cellulose synthase complex under control of <i>puc</i> promoter <i>bcsA</i> _F:GCATGTGGCGACTAGTGGAGGATTCCTAATGACCGTTCGAGCCAAGGC <i>bcsA</i> _R:GTCCATCAGTGGTGATGATGGTGATGGTGGTGATGGTGATGGTTTCGACCCCCAGGCGACCGTGTT <i>bcsB</i> _F: ATCACCCTGATGGACATGCGACTGCTGCCTTTC <i>bcsB</i> _R:GGTGGTGGTGAGATCCTTAAGTCATTTCAGGCCCTTCCTCCG	This study

449 **Table II. Membrane proteins expressed in *Rhodobacter sphaeroides* in the present study**

Protein	Class	Origin	Topology	Expression (mg/L)	Active?
Cyt-cy	Heme protein, electron transfer	<i>Rhodobacter capsulatus</i> (<i>R. cap.</i>)	1 TM	50 in <i>R. sph.</i> (Laible et al., 2009) 47 in <i>R. sph. Purified</i> (This study)	Yes
BcsAB	Membrane bound cellulose synthase	<i>Rhodobacter sphaeroides</i> (<i>R. sph.</i>)	BcsA:8TM BcsB:1TM	0.2 in <i>E. coli</i> (Morgan et al., 2013) 1.5 in <i>R. sph. Purified</i> (This study)	Yes
AQP9	Porin; water & glycerol transporter	<i>Homo sapiens</i>	6 TM	Not detectable (Oberg et al., 2009) 0.1 in <i>Sf9</i> cells (Viadiu et al., 2007) 0.5 in <i>R. sph. Purified</i> (This study)	Yes
Occ	Tight junction protein	<i>Homo sapiens</i>	4 TM	Not determined (Neophytou et al., 2007) 7.5 in <i>R. sph. Purified</i> (This study)	ND
GhoT	Microbial toxin	<i>Escherichia coli</i> (<i>E. coli</i>)	2 TM	ND	P*

450 TM: Transmembrane, ND: Not determined, P*: Phenotype change upon expression

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Figure 1. Growth kinetics and biomass yield of *Rhodobacter* cultured under semi-aerobic and photoheterotrophic conditions. A: Growth kinetics depicted a lagtime for photoheterotrophic cultures (open symbols) possibly due to transition of the growth mode from semi-aerobic to photoheterotrophic. B: Photoheterotrophic growth yielded 4-fold higher biomass.

Figure 2. Growth kinetics and biomass yield of *Rhodobacter* cultured under semi-aerobic and photoheterotrophic conditions. A: Growth kinetics depicted a lagtime for photoheterotrophic cultures (open symbols) possibly due to transition of the growth mode from semi-aerobic to photoheterotrophic. B: Photoheterotrophic growth yielded 4-fold higher biomass

Figure 3. Cytochrome-cy (Cyt-cy) expression in *Rhodobacter sphaeroides*. A: 10 % SDS PAGE gel of Cyt-cy purified via Ni-NTA column; SM: Solubilized total membrane fraction, FT: Flow-through from Ni-NTA column, 10-50: Wash fractions with imidazole concentrations between 10-50 mM; E: Elution with 500 mM imidazole; M: Protein ladder. Cyt-cy is identified as two relevant bands at 25 and 35 kDa, corresponding to its two different conformations in SDS-PAGE gel, as reported in other studies (Jenney and Daldal, 1993; Myllykallio et al., 1997); B: Western-blot analysis of Cyt-cy detected by His-tag antibody in total cell (TC) and purified protein (E1).

Figure 4. Peroxidase activity of Cyt-cy. A: Hydrogen peroxide reduction to water monitored by absorbance shift of chromogenic peroxide substrate TMB from 280 nm to 655 nm at different concentrations of Cyt-cy and radical scavenger sodium azide, NaN_3 . The conversion of TMB is

severely affected by the increasing concentrations of azide. **B:** Peroxidase activity of 1 μ M Cyt-
cy was reduced to 40 and 25 per cent in the presence of 1 mM and 5 mM NaN_3 , respectively. **C:**
Photograph showing the progression of the green color formation due to reduction of TMB. The
delay in the presence of increasing NaN_3 is observed.

Figure 5. SDS-PAGE and Western-blot analysis of complex and *in vitro* cellulose synthesis assay. **A:** BcsAB purification via metal affinity chromatography was monitored via SDS-PAGE. Lanes M: Protein marker, SM: Solubilized membrane, FT: Flow-through from Co-NTA column, 20-50: Wash fractions with imidazole concentrations between 10-50 mM, E1&E2: Elution with 500 mM imidazole. Oxidized BcsB and BcsA are indicated around 100 kDa and 80 kDa, respectively. To the right, a Western-blot using His-tag antibody directed to C-terminal 12-His-tag of BcsA confirmed that the faster migrating band was BcsA. **B:** BcsAB activity was measured by UDP- C^{14} -glucose incorporation assay. The scintillation count for the control devoid of BcsAB complex is marked with black column. Cyclic di-GMP, allosteric effector of BcsAB complex, had a large effect on the enzyme activity, as two orders of magnitude higher scintillation count was measured in the presence of 12 μ M cyclic di-GMP.

Figure 6. SDS-PAGE and Western-blot analysis of human AQP9. Lanes correspond to the samples collected from different stages of purification. FT: Flow-through from Co-NTA column; 0, 25, 50: wash fractions with corresponding imidazole concentrations 0-50 mM; M: Novex-sharp prestained protein ladder; E: Elution with 500 mM imidazole and WB is Western-blot image of the elution detected using a polyclonal histag antibody.

Figure 7. Water permeability of human AQP9 proteoliposomes. **A:** Functionality of the purified hAQP9 was tested in proteoliposomes via stopped-flow light scattering. Liposomes were rapidly mixed with an outward directed osmolyte, which results in water influx and increase in scattered light at 90°. **B:** 3 to 5-fold increase was observed in water permeability for hAQP9 proteoliposomes compared to control liposomes devoid of hAQP9 at LPR 250 and 100, respectively.

Figure 8. SDS-PAGE and Western-Blot analysis of Occludin. Lanes M: Protein marker; FT: Flow-through from Co-NTA column; 10-50: Wash fractions with imidazole concentrations of 10-50 mM that were collected from Co-NTA column, one column volume at a time totaling 6 column volumes for each imidazole concentration; E1&E2: Elutions with 500 mM imidazole collected from Co-NTA column one column volume at a time and WB is Western-blot image of E1.

Figure 9. Phenotype test. *R. sphaeroides* strains harboring empty vector control, a mutated version of the protein (GhoTF38R) and intact GhoT were grown on YCC agar plates supplemented with 1 µg/mL tetracycline under semi-aerobic and photoheterotrophic conditions. **A:** Semi-aerobic chemoheterotrophic growth, where *puc* promoter driving the protein expression has a low baseline level activity. No significant phenotype change is noted among Control, GhoTF38R and GhoT. Although GhoT had some clear merged colonies possibly due to leaky expression. **B:** Anaerobic photoheterotrophic growth conditions induce *puc* promoter for

663 elevated GhoT expression. Control and GhoTF38R harboring strains display no phenotype;
664 however, a dramatic change in production of extracellular polymeric substances was observed
665 for GhoT expression strain.

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667

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Figure 7

Figure 8

A Semi-aerobic Chemoheterotrophic

Control

GhoTF38R

GhoT

B Anaerobic Photoheterotrophic