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

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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), *Escherichia coli* toxin peptide GhoT, cellulose synthase enzyme complex (BcsAB) of *R. sphaeroides* and cytochrome-cy (Cyt-cy) from *Rhodobacter capsulatus*. Titers 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.

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 [20]. Typically, 30% of any genome encodes for MPs [37]. 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 9800 soluble protein classes [19]. 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 [43]. Availability of MPs is also desired for high throughput drug screening [21], biotechnological [29] and biomedical applications [22].

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 [4,29,35]. 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 [17]. *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

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orders of magnitude lower than for soluble proteins [38]. 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 [2].

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 (~4 W/m^2), increase cellular membrane surface area by more than an order of magnitude [3,9]. ICM formation is therefore concomitant with the synthesis of the photosynthetic apparatus [44]. The *Rhodobacter* photosystem is comprised of the photosynthetic reaction center (RC) surrounded by first- and second-tier light harvesting antennae (LH1 and LH2) [36]. 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 [13]. Protein expression in this system is most commonly driven by the *puf* promoter with semi-aerobic induction [18,19,34] 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 [4], 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 [33]. 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 (4 W/m^2, Snowdragon Industrial Co. Ltd, Shenzhen, China). A schematic and picture of the relatively simple experimental apparatus is shown in Fig. 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 [26].

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 OD_{660} measurements within the spectrophotometer [8]. The cultures were grown until the stationary phase by measuring OD_{660} every 3 h. For biomass yield measurements, 5 mL culture was spun down at 15,000×*g*, 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 h in a prepared tube.

Molecular biology and specific constructs

Vector constructs used in this study are listed in Table 1. 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 *R. sphaeroides*, *RsAqpZ* (Genbank id: ABA78939), was included as a positive control for expression level between the constructs [10]. *R. 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 polycystronic gene using in-fusion cloning kit (Clontech Laboratories, Inc., Mountain View, 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 [25]. 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 [10]. The minor modifications employed for purification of BcsAB are described here: BcsAB lysis buffer contained 20 mM Tris at pH = 7.2, 1 mM $MgCl_2$, 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 $MgCl_2$, 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 [10]. The light scattering traces were fit to double