Let in the Light: Towards developing a screening method to tune the absorption spectrum of proteorhodopsin

Abstract

Biologically produced fuel is one approach towards alternative sources of renewable energy. One such method is to use bacteria that can harness solar energy to produce hydrogen gas, which can be directly used as a fuel or combined with carbon dioxide to create methane, a component of natural gas. The protein proteorhodopsin (PR), a light-driven proton pump, can be expressed in *Escherichia coli* to provide energy to synthesize hydrogen. The efficiency of this approach may be increased if the PR protein could harness a wider range of wavelengths in the solar spectrum. Our project works towards developing a screening method for use in directed evolution to change the frequencies of light absorbed by the PR protein. This report details the methods and approaches used during the project, including mutation of the *PR* gene sequence, introducing the *PR* gene into *Escherichia coli*, isolating the PR protein, and developing methods to screen for PR protein variants with enhanced activity.
Introduction

Greenhouse gases from burning conventional fuels increase the temperature of the globe (IPCC 2013). Alternative sources of energy, such as wind and solar power, are able to convert renewable resources into electricity, but their use is currently limited by storage issues. Synthesizing methane, a component of natural gas, is an intriguing alternative to conventional fossil fuels. Hydrogenation of carbon dioxide (combining $H_2$ and $CO_2$) creates methane and other hydrocarbons. This conversion can be done with net neutral carbon emissions if the energy used to combine them is from a renewable source; the same amount of carbon dioxide released when burned is taken out of the atmosphere when methane is synthesized in this way (Centi & Perathoner 2009). Carbon dioxide can be sequestered from industrial smokestacks, but hydrogen is made almost entirely from fossil fuels and thus contributes to greenhouse gas emissions (Ball & Weeda 2015). Carbon-low or carbon-neutral methods of obtaining hydrogen should be pursued.

Some argue that hydrogen is the fuel source of the future, but we currently do not have the infrastructure to run on hydrogen. The energy density per mass of hydrogen gas is better than methane (120 vs. 50 megajoules per kilogram for hydrogen and methane, respectively) (Thomas 2000). Using hydrogen directly would eliminate the steps to convert it to methane. Furthermore, it only releases water upon combustion, as opposed to the carbon dioxide released when hydrocarbons are burned (Ball & Weeda 2015).

Although hydrogen is energy dense per mass, it is the lightest of all elements and therefore is not energy dense per volume (10.7 kilojoules per liter at ambient
pressure and temperature) (Thomas 2000). Possibly the greatest roadblock to a hydrogen economy is that we currently lack the necessary infrastructure. New pipelines and distribution devices for hydrogen in gaseous and liquid form would need to be built, along with a system of refueling stations for hydrogen fuel cell electric vehicles (Ball & Weeda 2015). Gaseous hydrogen is very flammable and requires many safety precautions for storage and transportation (Praxair 2015). On the other hand, liquefying hydrogen requires a lot of energy (12 kWh/kg of hydrogen) because it must be cooled to -253°C (Ball & Weeda 2015). Our current infrastructure is designed for transporting and utilizing natural gas, so developing a carbon-neutral way to synthesize natural gas may serve as an important bridge technology as, or if, we transition to a hydrogen economy.

Biologically produced hydrogen is one potentially carbon-neutral or carbon-low option. Furthermore, the proteins that capture solar energy for an organism can be engineered to increase the total amount of hydrogen produced (Kim, B. Jo, Y. Jo & Cha 2012).

One such protein, proteorhodopsin (PR), exists in the cell membranes of marine planktonic algae (DeLong and Béjà 2010; Bamann, Bamberg, Wachtveitl & Glaubitz 2013). It binds to retinal, an accessory molecule that absorbs light (Bamann et al. 2013). In its relaxed state, the accessory molecule exists as all-trans-retinal, meaning the most important functional groups of the molecule are on opposite corners of a double bond (Kim, Waschuk, Brown & Jung 2008). When irradiated with light, the energy excites its electrons and causes the bonding scheme to switch to 13-cis-retinal (Figure 1) (Kim et al. 2008).
Retinal is embedded within the cyclic structure of the PR protein (Figure 2). 13-cis-retinal pulls at the protein, widening the central channel to allow protons out of the cytoplasm and into the periplasmic space, an area between the cell membrane and cell wall (Bamann et al. 2013). Protons accumulate in the periplasmic space, resulting in a concentration gradient of protons across the cell membrane (Bamann et al. 2013). In an effort to reduce the concentration gradient, protons re-enter the cell through an enzyme called the ATP synthase, providing the energy needed to produce ATP, a molecule that effectively stores solar energy as chemical energy that can be used to drive cellular processes (Walter et al. 2007). Protons can also re-enter the cell through the flagellar rotor, a wheel that turns the flagella (a tail-like mechanism) of the bacterial cell and allows it to swim (Walter, Greenfield,
Bustamante & Liphardt 2007).

Introducing the PR gene into *Escherichia coli* supplements their normal energy supplies, which are derived from metabolizing sugars (Jurtshuk 1996). Normal cellular metabolism also relies on a proton concentration gradient to generate ATP, which is established by the electron transport chain that moves protons across the cell membrane (Jurtshuk 1996).

Kim et al. (2012) found that PR activity increased hydrogen yields in bacteria expressing hydrogenase, a hydrogen-producing enzyme. They hypothesized that the excess of protons pushed into the periplasmic space from PR’s pumping activity provided more readily available substrate for hydrogenase to turn into hydrogen gas. The efficiency of converting solar energy to hydrogen in this system was about 3.4%, which is typical for sun-based biologically produced hydrogen (Kim et al. 2012). As a comparison, hydrogen production through water electrolysis is 40-60% efficient when using renewable energy sources for electricity (Zoulias et al. n.d.).

Although this system for hydrogen production is promising, there is a clear need for efficiency improvements. Increased efficiency in the amount of light the PR protein can use to pump protons could make the system more viable for industrial application. PR absorbs light in the blue and green frequencies (490-525 nm) (Delong and Béjà 2010). Light of shorter wavelengths can penetrate further through water, so isoforms of the protein absorb different frequencies depending on their depth in the water column, giving it natural variation (Kim et al. 2008). This range, however, is quite narrow compared to the total solar irradiation that reaches the earth’s surface (Figure 3). We hypothesize that altering PR to absorb various
frequencies of light over a greater range of the solar spectrum could increase the efficiency of converting light energy to hydrogen because more light would be available to do work.

Kim et al. (2008) showed that mutating the $PR$ gene could result in changes to the frequency at which the PR protein absorbs light. To screen absorption changes in the altered proteins, they purified PR protein from bacteria culture. The purified form was a purple color, and the color’s shade changed depending on the wavelength absorbed (Kim et al. 2008). This screen, however, did not optimize the protein’s performance and they found the wild type (WT) PR protein pumped more protons than any of the mutants.

Figure 3. (Astrobiology 2014) Solar irradiance at the top of the atmosphere (grey outline) and at the earth’s surface (colored outline). The strongest light is in the visible spectrum.
Our project is to develop a high-throughput method for tuning the wavelength absorbed by the PR protein and potentially enhancing its performance. The approach to be used is called directed evolution, a cycle in which mutated organisms are screened for a desired phenotype and selected individuals are advanced to the next round, where they are further mutated and screened (Rubin-Pitel et al. 2007). This process continues until the desired trait is reached (Figure 4)(Rubin-Pitel et al., 2007).

The final goal of this project is to use directed evolution to tune the absorption of the PR protein to different wavelengths so that, when multiple bacteria strains expressing different mutations of the PR gene are put together, they will harvest more of the solar spectrum. The critical step in any directed evolution project is the development of an effective screening method. This step was the focus of the summer project.

Figure 4. Schematic of directed evolution (Stone, 2007).
PR Synthesis in *E. coli*

**Introduction**

*Transformations*

The gene encoding PR from marine algae is introduced into *E. coli* in a procedure called a transformation, which relies on *E. coli* whose membranes have been made permeable to DNA. These are called competent cells and they are prepared with a treatment of CaCl₂, which binds to their membranes, making them permeable and masking the negative charge of the DNA, which is normally repelled by the membrane. The foreign DNA is then added to the cells, whose uptake of the DNA is facilitated by exposing the cells to elevated temperatures. The DNA, which is in a circular strand called a plasmid, is then transcribed along with normal DNA. Kim et al. (2008) introduced two plasmids into *E. coli* cells at the same time in what is called a double transformation, so that the bacteria could produce both retinal and PR. Our initial efforts aimed to replicate the double transformation.

*Competent Cell Preparation*

Another approach to express both the PR protein and retinal in *E. coli* is to make previously transformed cells competent and then transform them with the remaining plasmid. We made DH5α cells containing the pORANGE plasmid competent and tried transforming them with the pKJ900 plasmid.

*β-carotene Capsules*

Because pKJ900 contains a gene that turns β-carotene into retinal, we experimented putting β-carotene taken from dietary supplements into bacterial cultures. β-carotene is non-polar, while water, the solvent of bacteria cultures, is
polar. Therefore the two are immiscible. We tested methods to solubilize β-carotene and analyzed the effectiveness with thin layer chromatography (TLC). TLC measures the relative amount of various contents of a solution. Samples are spotted on a silica plate and the bottom of the plate is placed in solvent, which slowly rises up the plate and moves the contents of the sample up with it, separating them out by size (Figure 5).

![Figure 5. (University of Colorado at Boulder 2015) Schematic of how a TLC plate works. Contents of a sample move partway up the plate, depending on their size.](image)

External source of Retinal

The most straightforward way to provide *E. coli* with retinal is to add it premade to the cultures. All-trans retinal was purchased for this application.

PR and retinal are not continuously produced; they are under the control of specific promoters, genetic sequences that, when read, initiate the transcription of the following gene. Certain promoters can be turned on (induced) by exposure to specific chemical compounds. A common technique in genetic engineering is to place specific gene sequence(s) (e.g. the PR gene or the retinal-producing gene) under the control of promoters that can be induced by readily available compounds. In this case, the PR gene was placed under the control of a promoter that responds to lactose and its analogues, while the retinal-producing gene was placed under the control of an arabinose-sensitive promoter.
To confirm PR protein production during induction, aliquots were taken out every hour for 6 hours and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which separates proteins on the basis of size so one can determine the presence, relative quantity, and peak production period of the protein of interest.

**Methods**

*Transformations*

Transformations were accomplished using 1 µl of plasmids per 50 µl of competent cells. The transformations were done with BL21 and DH5α *E. coli* from New England Biolabs. The cells were then heat-shocked at 42°C for 1 minute and replaced on ice for 10 minutes. Cells grew for 40 minutes at 32°C in tubes floating in water spun by a stir-bar at 100-200 rpm and were then spread on plates containing a concentration of 50 µg/ml ampicillin and grown at 37°C for 16-24 hours. Double transformations were performed with New England Biolabs BL21 cells following the procedure detailed in iGEM Technische Universiteit Eindhoven (2014).

*Competent Cell Preparation*

DH5α pORANGE cells were made competent following Dr. Melvin’s procedure (Appendix 1).

*β-carotene Capsules*

β-carotene was solubilized in water in the following methods: A) adding 0.7 µl of β-carotene paste extracted from Life Brand Beta-Carotene capsules directly to 15 ml of water to obtain 0.2% retinal, B) heating β-carotene and water in a
microwave for 10 seconds, C) mixing 0.1% Triton x-100 or D) 0.1% DDM with β-carotene and then filling to volume with water.

Samples were spotted on adsorbent silica containing a fluorescent indicator. The plates were placed in a 9:1 mixture of hexane to ethyl acetate and left until the solution had risen to the top of the plate. After drying, the plates were illuminated with UV light to visualize how much β-carotene was in each sample.

External source of Retinal

10 μM all-trans-retinal was added to bacteria culture or plates along with 1 mM IPTG to induce the production of the PR protein (Kim et al. 2008). Cultures were grown to 0.4 OD at 600 nm before induction and grew for 4-6 hours after.

Results and Discussion

Transformations

We obtained the plasmids pORANGE and pKJ900 from Kim et al. (Figure 6)(2008). pORANGE contains a series of genes that make β-carotene (a precursor of retinal) and a gene for chloramphenicol resistance. The plasmid pKJ900 contains the DNA sequence of PR, a gene for an enzyme that converts β-carotene to retinal, and a gene for ampicillin resistance. The antibiotic resistance gene is important for isolating successful transformations; it allows bacteria that have successfully been transformed with the plasmid to grow in that antibiotic while unsuccessful transformations are eliminated.
We transformed the *E. coli* strains DH5α (for optimal plasmid production) and BL21 (for optimal protein production) with pKJ900 and DH5α with pORANGE (Figure 7).

Chemical transformations are inefficient; most plasmids do not enter the cells. Therefore, a double transformation is much more inefficient because of the low likelihood that two plasmids will both enter the same cell. No colonies grew from the double transformation attempts, but a single transformation with pKJ900 is more suitable for our purposes. A double transformation introduces the possibility that beneficial *PR* mutations would not be found because a pORANGE
plasmid would not enter the same *E. coli* cell, causing the bacteria to be eliminated by chloramphenicol in the plate.

*Competent Cell Preparation*

The second transformation with pKJ900 on competent DH5α pORANGE *E. coli* failed to produce colonies. We only tried the second transformation with pKJ900, but it would be interesting to try it on the positive control plasmid puc19. This could indicate if the cells are competent and if this method is worth pursuing.

*β-carotene Capsules*

We found that DDM solubilized β-carotene most effectively because it showed the darkest purple spot on the TLC plate upon UV illumination (Figure 8). SDS-PAGE analysis of induced cultures with β-carotene boiled and in DDM did not show significant bands that would indicate PR expression, but they appear promising (Figure 9).

---

Figure 8. TLC plate of the 4 solubility methods. A-control, B-boil, C-Triton x-100, D-DDM. There are faint spots in B and C, and a much more substantial one in D.
We initially resisted this approach because of retinal’s expense. However, our aim was to mutate and assess the performance of PR and we were not concerned with β-carotene production or its conversion to retinal. Therefore, we preferred to perform a single transformation with pKJ900 and add retinal once the bacteria had reached their peak growth rate, which occurs about when the optical density (OD, cloudiness of the culture) is 0.4 at 600 nm.
The cell pellets appeared purple after being induced with retinal and IPTG, grown, and centrifuged, which is highly indicative that the retinal bound to PR because retinal is orange by itself (Figure 10). Kim et al. (2012) observed this same cell color. Furthermore, an SDS-PAGE gel showing hourly aliquots during induction shows a marked darkening of the PR band, suggesting that PR with bound retinal was being synthesized (Figure 11).

Figure 11. Time progression of an induction of BL21 containing pKJ900 and induced with retinal and IPTG. A: before induction, B-G: hour increments during induction, H: ladder. PR production peaks during the fourth hour of induction.
Mutating plasmids

Introduction

To start the directed evolution cycle, we need to mutate the original PR gene. We chose to use error-prone rolling circle amplification (RCA), which both amplifies the number of plasmids and introduces slight mutations (Fujii, Kitaoka & Hayashi 2004). Plasmids are heated to unwind their double strands and then single strands of DNA, called primers, bind to their matching sequences along the plasmid (Fujii et al. 2004). From the primer binding sites, DNA polymerase starts replicating the corresponding strands of DNA (Fujii et al. 2004). Primers will then bind to those strands and start the synthesis again (Fujii et al. 2004). In this way, the plasmid DNA sequence is amplified. To introduce errors, manganese chloride is included, which causes occasional base pair mismatching.

Methods

We used a buffer detailed in Fujii et al. (2004) and added 0.5-5.0 μl puc19, original pKJ900, or pKJ900 plasmids obtained from a miniprep, 4 pmol/μl random primers, and 200 ng/μl BSA. The mixture was heated to 95°C to denature for 3 minutes. Afterwards, 0.2 mM dNTP, 20 mM MnCl₂, and 5 μl taq 2X Master Mix from New England Biolabs RT-PCR kit (for taq-polymerase) was added to the solution and left overnight at 30°C. The enzymes were deactivated at 100°C for 10 minutes and the reaction mixture was cleaned up with a BioBasic EZ-10 Spin Column PCR Product Purification Kit.
To see if the amplification worked, we attempted transformations with 1 μl of the mutated plasmids per 50 μl of DH5α cells from New England Biolabs and analyzed whole plasmids and plasmids that had undergone a restriction digest on an agarose gel. A restriction digest was performed using 10 μl plasmids, 2 μl NEBuffer 3.1, 1 μl of the restriction enzyme Ndel, 2 μl of the restriction enzyme NotI (only for pKJ900), and up to a total volume of 20 μl with RNA-free water. The solution incubated at 37°C for 1 hour. 4 μl of 5X loading dye was added to both cut and whole plasmids. Samples were loaded into a 0.8% agarose gel in SB buffer and run at 300 V until the dye reached the end of the gel (about an hour).

Results and Discussion

The transformations from the first attempt did not yield results. Puc19 in Fujii et al.'s buffer was visible on the DNA gel, while puc19 WT was not, indicating puc19 had amplified during RCA (Figure 12).

Figure 12. DNA gel of mutated and original puc19 and pKJ900 plasmids. 1-whole puc19 in Fujii buffer, 5-cut puc19 in Fujii buffer, 7-puc19 WT, 12-ladder.
We therefore thought our initial concentration of \textit{PR WT} plasmid DNA was too low, and amplified it again using a higher concentration (5 μl) of both the original plasmids and plasmids we isolated from bacteria. We also performed RCA with and without MnCl$_2$. Transformations with these plasmids have been unsuccessful.

\textbf{Isolating PR Protein}

\textbf{Introduction}

To determine if mutated PR protein absorbs a different wavelength of light, we must quantify the wavelength it absorbs. This will allow us to see in which direction and by how much mutated PR protein absorption shifts from WT and, therefore, if our directed evolution technique is shifting peak absorption further each round. To measure the wavelength of peak absorbance of PR, we must purify the protein, which involves breaking open (lysing) bacteria cells and, because PR is membrane-bound, dissolving the cell membranes.

The \textit{PR} gene on pKJ900 was modified to contain a histidine tag, that is, an amino acid sequence that facilitates protein purification. When a solution containing PR protein with the histidine tag is applied to a column containing nickel ions, the histidine tag binds to the nickel while the rest of the solution washes through. Afterwards, the PR is detached from the nickel with an elution buffer and collected. Kim et al. (2008) used this method to purify PR and measure the wavelength of maximum absorbance ($\lambda_{\text{max}}$) of different mutations.
Measuring $\lambda_{\text{max}}$ is done in a spectrophotometer, which sends light through a sample and detects the amount of light of each frequency that is absorbed.

**Methods**

We followed the lysing technique detailed in European Molecular Biology Laboratory (2015), but without phenylmethylsulfonyl fluoride (PMSF), which inhibits protease enzymes from degrading proteins. We kept the reaction cold as much as possible to stabilize the proteins. Once we had lysed the cells, we followed the procedure of Kim et al. (2008) to extract PR protein from the membrane and purify it with a nickel column. We used a UV/visible range spectrophotometer and SpectraWiz® software to measure $\lambda_{\text{max}}$.

**Results and Discussion**

During the purification process the cells and solution were purple (Figure 13). The color faded while washing the nickel column and eluting the PR, so the wash and elution samples are only faintly tinted (Figure 14). The color loss is likely due to dilution of the sample with the wash and elution buffers. An SDS-PAGE gel of the purified protein showed that PR came off the nickel column in the wash solution instead of in the elution buffer (Figure 15). This may have been caused by the removal of the histidine tag by protease enzymes.
Figure 13. Stages of lysing induced BL21 pKJ900 cells. A) in lysis buffer, B) lysed cell pellet, C) re-suspended lysed pellet in buffer with DDM.

Figure 14. Samples from PR purification on a nickel column. A) elution 5, B) elution 4, C) elution 3, D) elution 2, E) elution 1, F) wash, and G) sample runoff before buffer was added.

Figure 15. SDS-PAGE gel of purified PR. A) sample runoff, B) wash, C-G) elutions 1-5, and H) ladder. We can tell the induction is working because the PR band is much darker than any other.
While we do not have highly purified PR protein, our samples may be > 90% pure. Therefore, it may still be possible to measure its $\lambda_{\text{max}}$ from the wash solution in the spectrophotometer, although it could be too diluted to read. We used the wash buffer without PR protein as a background reading, and the wash sample containing PR protein as our sample. We hypothesize that, since PR is the most prevalent protein in the wash, it will cause the peak absorption. However, troubles with the SpectraWiz® software of the spectrophotometer has kept us from obtaining absorbency graphs. We also have more induced BL21 pKJ900 cell pellets from which to extract PR protein and purify again, this time with a protease inhibitor such as PMSF.

**Screen**

**Introduction**

*E. coli* transformed with mutated *PR* gene sequences must be screened to select beneficial mutations that will advance to the next round of directed evolution. The screen is based on quantifying the relative motility of transformed bacteria when their normal energy pathway is compromised. The more motile bacteria will most likely contain useful mutations of *PR*, and can therefore be selected to move to the next round of directed evolution.

The motility assay is as follows: bacteria are transferred into a soft gel that allows partial motility. The gel contains triphenyltetrazolium chloride (TTC) salt that turns red when oxidized by bacteria. This red color will show where bacteria have been. The gel also contains sodium azide, which Walter et al. (2007) found
obstructed the metabolic pathway of *E. coli*. Azide inhibits the last enzyme in the electron transport chain of the metabolic pathway (cytochrome oxidase), which transfers protons across the cell membrane (Walter et al. 2007). They found azide inhibited *E. coli*’s movement, presumably because the degradation of the proton gradient prevented the spinning of the flagellar rotor. *E. coli* expressing the PR protein, however, were able to move when exposed to light, even in the presence of azide, strongly suggesting PR was able to replace the metabolic pathway to generate a proton gradient (Walter et al. 2007).

Within the main principles of the motility assay we explored two main methods, the 96 well plate system and the layered agar system.

**Methods**

**96 Well Plates**

96 well plates were sterilized in ethyl alcohol and filled to 400 μl per well with a soft gel mix. The soft gel mix contained 4 parts tryptone, 2 parts NaCl, 2 parts yeast extract, and 1 part agar (based on weight) with a total concentration of 2.25 g per 100 ml of solution. 50 mg/ml ampicillin, 0.9-50 mM sodium azide, and 0.01% TTC were added after autoclaving the soft gel solution. Wells were filled using a Scilogex multi-channel pippetor. Once the soft gel had solidified, wells were inoculated with stab cultures of induced bacteria or individual colonies. Parafilm sealed the top. Plates were placed in either a light or dark location and allowed to grow for several days.

*Replica Plating*
Filter paper was cut to the shape and size of petri dishes and overlaid on plates of transformed colonies. The paper was then transferred to a petri dish containing the soft agar mixture. After a minute, the paper was removed and the plates were grown in dark or light environments.

*Layered Agar*

After cooling the soft gel solution so that it was only slightly warm to the touch, 20 ml was poured on top of transformation plates of induced bacteria. The plates were placed in the light or dark and monitored for a few days.

**Results and Discussion**

*96 Well Plates*

The initial idea was to put the soft gel into 96 well plates and then transfer every colony from a transformation of mutated *PR* plasmids into separate wells. We believed that wells that turned red first, had the biggest spots, or were the deepest color could indicate the highest activity of the PR protein. The 96 well plates have not yet shown greater motility in PR-expressing bacteria. At 50 mM sodium azide, stab cultures do not turn red. Without sodium azide, the stab line turns red but the bacteria do not seem to move in their wells (Figure 16).

With a concentration of 0.9 mM of sodium azide bacteria were able to grow. It seems likely this is because the concentration is not enough to inhibit normal metabolic processes. Bacteria expressing PR should be able to move in the presence of 50 mM sodium azide according to Walter et al. (2007). It is unclear why the induced bacteria are not turning their wells red.
While the 96 well method has its merits, a major benefit of creating the screen in petri dishes is that it eliminates the process of transferring individual colonies one at a time. We initially tried replica plating transformed plates. Replica plating is where cells from one petri dish are transferred with a sterile overlaying material to a new petri dish in order to copy the exact layout of the colonies on the original plate. The transfer took the colonies off the original plate, but we were able to see where they had been and compare it to the new soft agar petri dish. In some
instances the colonies matched up, but often the colonies would be dragged around the plate (Figure 17).

![Figure 17. Replica plating DH5α pORANGE. Colonies were taken off the original plate (left) by filter paper (the location of the removed colonies is indicated by the black circles), but transferred well onto the soft agar plate.](image)

Layered Agar

We then decided to try pouring the soft agar right on top of existing plates to eliminate the transfer process. This has worked the best so far. One challenge to overcome is how to induce the transformed colonies while they remain in their original petri dish. First we tried pouring a solution of the inducing ingredients onto the plate, but this tended to dislodge the colonies. We then spread 40 μl of the inducing solution on the plate before plating bacteria and dried it for 30 minutes. However, colonies on these plates and non-induced plates with different
concentrations of sodium azide and light conditions all appeared similar (Figure 18).

Figure 18. Layered agar plates with the inducing solution spread on the agar before cultured and then grown for 30 hours. The sodium azide concentration is at 0.9 mM. A) Light treated plates, B) Dark treated plates. Rows 1 and 4) not induced, Rows 2 and 3) induced.

This could be for several reasons. The inducing solution may diffuse into the agar and become less available for the bacteria. Also, the concentration of sodium azide may be too low. When we optimized the sodium azide concentration, we were
growing bacteria in the solution. Here the cells are already grown, we only need to
limit motility. Walter et al. (2007) found increasing the sodium azide concentration
exhibited greater differences between the velocity of light-illuminated and non-
illuminated recombinant PR bacteria, reaching the greatest difference in velocity at
around 80 mM. For these two reasons, we increased the sodium azide concentration
to 50 mM and added the inducing ingredients directly to the hard agar before
pouring plates (Figure 19).

Figure 19. Layered agar plates with the inducing ingredients in the hard agar and the sodium
azide concentration at 50 mM in light and dark conditions for 11 hours (A and B respectively)
and 36 hours (C and D respectively). A1) induced without sodium azide, A2) induced with
sodium azide, A3) not induced without sodium azide, A4) not induced with sodium azide. B1)
induced without sodium azide, B2) not induced without sodium azide, B3) induced with
sodium azide, B4) not induced with sodium azide. C1) induced without sodium azide, C2)
induced with sodium azide, C3) not induced with sodium azide. D1) induced without sodium
azide, D2) not induced without sodium azide, D3) induced with sodium azide, D4) not
induced with sodium azide.
These new plates took longer to turn red beyond the bounds of their colonies, which could be due to the low air temperature of their locations (about 20°C). We therefore set up a light and dark incubator at 37°C and transferred the plates to them (Figure 20). The plates without sodium azide turned red within two hours. On the plates containing sodium azide, the colonies turned red but did not spread, indicating they are alive but not moving. Therefore, the concentration of sodium azide needs further analysis. Furthermore, the red color cannot be traced back to a specific colony, which may mean the 96 well plates could more clearly show which colonies are the most motile.

![Figure 20](image-url)

Figure 20. Light (A) and dark (B) plates in incubator overnight. 1) without sodium azide, 2) with sodium azide. The columns i and iii are not induced while the columns ii and iv are induced.
Conclusion

The system to use directed evolution to tune the absorption of the PR protein is nearly ready. Our method to mutate the PR gene has worked on a control plasmid, but needs optimization on the plasmid containing the PR gene. We have a system in which E. coli expresses the PR protein and the accessory molecule, retinal, is added. The purple color during PR purification strongly suggests the purification is working, and adding PMSF during lysing will likely keep PR on the nickel column until the elution buffer is poured through. The screening method is in its final stages of experimentation. Further tests on the 96 well plate and layered agar methods are needed in the light and dark incubators. Once these parts are addressed, the whole system is ready for directed evolution and one could use the screening method to tune different mutations of the PR gene to different frequencies of light. With a group of those mutated plasmids, one could have several strains of bacteria that, together, absorb a greater range of the solar spectrum.

Applications of our project fall in the realm of biologically produced solar fuel. One could modify the strains of bacteria containing mutated PR plasmids to produce hydrogen fuel, potentially more efficiently. Methane could also be produced through the hydrogenation of carbon dioxide, where catalysts combine hydrogen and carbon dioxide (Centi & Parathoner 2009).

Further steps for this project after the screening method and directed evolution system have been worked out are to quantify the efficiency of such a system to determine its viability as a source of energy, as well as test the hypothesis that increasing the range of light wavelengths used increases the efficiency of
converting solar energy to fuel. One could also engineer the prototype of how the system would actually work, such as the location, media type, sunlight exposure, methods for capturing the gas, etc.
References


Appendix 1: Competent cell preparation method written by Dr. Neal Melvin

CaCl2 method

The following protocol describes a protocol for producing a large batch of “pre-competent” cells. In principle, this will allow you to simply thaw a tube of cells (on ice!), add your plasmid, and proceed immediately to heat shock. I have only tested this method with HB101s, DH5alphas, and BL21(DE3)s. You should try a few different times for other strains, as they could grow at different rates. For HB101s and DH5alphas, 3.5 hours a good time to harvest. For BL21(DE3)s, take them at 2.5 hours.

To begin with, make some wash/storage solution that contains a final concentration of 50 mM CaCl2 and 20% glycerol. To make 30 mL (the maximum volume you can autoclave in a 50 mL Falcon tube), add 1.5 mL of a 1M CaCl2 solution, then water up to about the 20 mL mark on the tube. I then visually add 6 mL of glycerol, and vortex so that the glycerol becomes dispersed in the solution. Top up to the 30 mL mark with water, and autoclave the solution. Keep this at 4C.

The day before you want to freeze your cells, grow a 2 mL liquid culture of your chosen strain in LB. Take from a single colony, a stab culture, or a frozen stock. Grow this to “saturation” (maximum growth), which is about 20 hours or so at 37C. You can also prepare your larger 20 mL culture flask to autoclave today too. This can then be left in the autoclave overnight, and then it’s ready to go the next day.

Prepare a 20 mL volume of LB in a 100 or 200 mL Erlenmeyer flask, including a stir bar. Add the required amount of LB powder (400 mg) directly to the flask, and add 20 mL of water. Place aluminum foil over the top of the flask, and autoclave this (stir bar and all!) for 15 minutes. It’s a good idea to autoclave your CaCl2/glycerol solution now too. After it’s done autoclaving, wait for the CaCl2/glycerol solution to cool some at room temp, then throw it in the fridge or in ice to get it good and cold.

Once the LB solution has cooled down enough, add a clean thermometer to the flask. You’ll need this to be at 37C to grow your cells, so try not to let it cool below 37C in the first place. Place the flask on a hot plate and monitor the temperature to get to 37C; have the stirring function set at around 300 rpm as well. Place a number of labelled 1.5 mL centrifuge tubes into an ice bucket or in the freezer at this point, as these are the tubes you’ll be using to centrifuge your cells in and it will be important to keep the cells cold as much as possible during the process.
Once your flask is at 37°C, add a 1:100 dilution of your 2 mL overnight culture to it. If you’re growing 20 mL of cells, add 200 uL of your saturated culture directly to the flask. You will want to harvest your cells when they’re in their log phase of growth. Normally, this is done by monitoring the optical density of the culture with a spectrophotometer using 600 nm light. However, I’ve found that about 4 hours of incubation in this larger volume works well. The culture should be reasonably cloudy by that point.

At the appropriate time point for your strain, (see above), take the flask off the hot plate and put on ice for 10 minutes to halt growth. Put the flask back onto a stir plate with no heat and get it mixing again.

Pipette 1.5-2 mL volumes directly into your pre-chilled tubes. I don’t measure this, but merely use a plastic transfer pipette and fill each tube to near the top with culture. Centrifuge the cells at 8,000 x g for 2 minutes to collect the cells. Pour off the LB medium from each tube, and place the tube back on ice after you’ve poured off. Add 500 uL of the 50 mM CaCl2 and 20% glycerol solution to each tube and vortex to disperse the pellet into the solution. It is unlikely that vortexing will completely disperse the pellet on its own. One trick I use is to rake the tube across a 1.5 mL centrifuge tube rack a few times. Be sure to visually confirm that the pellet is gone before proceeding, but keeping the tubes on ice as much as possible.

Once dispersed, spin the cells again at 8,000 x g for 2 minutes. Pour off the solution, and add 220 uL of the 50 mM CaCl2 and 20% glycerol solution and put back on ice for 20 minutes. You will use 50 uL for any given transformation, so keeping these at 220 uL means that you’ll have 4 possible transformation reactions per tube, with a bit of a buffer to allow for pipettor error.

After 20 minutes on ice, I’d strongly recommend taking one of the tubes and trying a transformation right away to test your batch before freezing it. In this case, add your plasmid to 50 uL of competent cells (depending on the plasmid amounts, anywhere from 1-4 uL; we use a 1:25 dilution of a typical pUC19 mini-prep as a + transformation control). Proceed with the usual heat shock/outgrowth protocol as per usual (42°C for 60 seconds, then back on ice for 5-10 minutes before adding 50 uL of LB and incubating at 37°C for 60 minutes before plating). Put the rest of the tubes containing 200 uL of competent cells in the -80°C freezer, but not in the pre-competent cells box until you’ve confirmed that these are, in fact, transformable!
If you’re going to use these pre-competent cells for a transformation from a tested source of plasmid (i.e., one that has already been confirmed to be good in some way), then plate all 100 µL of this transformation per plate. If you’re using them for a likely low efficiency transformation (e.g., from a ligation reaction), I’d suggest plating up to 200 µL on one plate to increase your odds of getting a successful transformant to harvest.