Bio Circuit Nano Ninjas

Our Project Improves BioCircuits and We Are Nano Ninjas

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Abstract:

This proposal seeks to improve current unsustainable power sources through bacterial conductive proteins, offering a promising alternative. The need for novel energy production and transfer systems is driven by global challenges posed by increasing energy demand and limited rare resources. Our proposal offers a low-cost solution and improved conductive protein polymers in bacteria called pili[1][2]. This proposal aims to engineer the Geopilin domain 1 protein, pilA-N, to improve the conductance of electrons by introducing aromatic residues along key sites of the filament to improve electron-hopping for higher conductance since these alterations have proven effective[3][4][5]. Using a comparative and site-directed mutagenesis approach to introduce aromatic residues will help identify key residues for electron hopping. We will first engineer the pilA-N protein through comparative modeling with known conductive pili to find conserved aromatic residue sites to implement into our pilA protein. We will also apply rational design through visual analysis of the pilA structure to improve the packing of the protein by incorporating more tryptophan and hydrophobic residues, as previous studies show tighter conformation[3][4][5]. Lastly, we will utilize a directed evolution approach by integrating our mutants into *Geobacter sulfurreducens* reiterating variant pilA-N proteins.[6][7][8] Afterward, we will screen mutants by assessing the reductase ability of *G. sulfurreducens* by growing the variants in Fe(III) oxide-rich media. The resulting Fe(II) in the media is a product of *G. sulfurreducens* extracellular electron transport (EET) capable of binding to dye to absorbance measurements. It will be measured with phen green SK (PG SK) fluorescence probe. This probe binds to Fe(II) and increases in fluorescence intensity in correlation to Fe(II) amount, allowing for quantification of Fe(II) levels via Fluorescence-Activated Cell Sorting (FACS), which correlates with the electron transport efficiency of the engineered pilA-N mutants.[24] Mutants with a higher presence of Fe(II) indicate higher conductance, which would be mutant proteins of interest to purify, sequence, and visualize for further iterations of studies.[9] Overall, our proposal will provide a more efficient, accessible, and scalable solution to address increasing global energy demand, resource scarcity, and toxic waste.

Background and Significance:

The rising global energy demand and the depletion of rare earth minerals highlight the need for sustainable circuitry. Several solutions have been developed to provide more sustainable energy sources, such as solar panels and hydroelectric power. Despite new technologies, there has still been a steady increase of 0.2% in carbon emissions every year this past decade[10]. In response to these issues, microbial bioelectrochemical systems have emerged as a promising solution by utilizing bacteria to generate electricity through EET mechanisms. However, the efficiency of these systems is often constrained by the limited conductivity of bacterial nanowires, hindering their potential for large-scale applications.

Looking to bacterial nanowires, such as those found in *G. sulfurreducens* to facilitate electron transport in microbial fuel cells is critical. These structures naturally conduct electricity but their efficiency is restricted by suboptimal aromatic stacking interactions. This project aims to engineer the Geopilin domain 1 protein, or pilA-N, a key structural component of bacterial pili, to enhance its conductivity. By introducing additional aromatic residues, we seek to improve π-stacking networks, reduce spatial constraints, and optimize hydrogen bonding interactions,

ultimately facilitating more efficient conductivity. Furthermore, recent research has highlighted the critical roles of OmcS and OmcZ cytochromes c genes in electron transfer within *G. sulfurreducens*[15]. Cytochrome c genes utilize heme cofactors for redox reactions of Fe(III) oxide, allowing for direct electron transfer to electrodes and contributing to the overall conductivity of microbial fuel cells. These cytochromes play a complementary role to pilA-N in facilitating electron transport, suggesting that improvements in pilA-N could synergize with these native conductive components for enhanced efficiency. The successful development of a high-conductivity pilA-N variant could revolutionize bioelectronic applications, providing a cost-effective, scalable, and environmentally sustainable solution to address the global energy crisis.

Goal and Innovation:

This proposal aims to engineer the Geopilin domain 1 protein, pilA-N, to enhance its electron conductance, providing a solution for improving sustainable energy transfer systems. PilA-N was specifically chosen over other conductive bacterial proteins due to its superior native conductance, structural stability, and well-characterized filament structure, which makes it more amenable to rational design and targeted modifications.[14] Moreover, pilA-N, in conjunction with pilA-C, forms a heterodimer that assembles into filaments with a well-defined architecture. This structural clarity facilitates targeted modifications and rational design strategies.[2] Unlike other pili proteins, pilA-N offers an optimal balance between flexibility and stability, allowing for the strategic incorporation of conductive features without compromising filament integrity. The detailed structural information and extensive research available for pilA-N, including high-resolution cryo-electron microscopy data, provide a robust framework for computational modeling and site-specific mutagenesis. This enables precise engineering of the protein to enhance its conductive properties.[15] PilA-N's structural characteristics and the depth of existing research make it a prime candidate for engineering efforts to improve electron transfer efficiency.

Our modifications are designed to facilitate EET through π-stacking networks, reduced spatial distance between aromatic rings, and optimized hydrogen bonding, all of which collectively strengthen the electron transfer pathway.[7] Optimized hydrogen bonding is expected to stabilize intermediate states and tighten the packing of the hydrophobic core, which minimizes energy loss and creates a more efficient conduction channel.

This proposal is innovative because it combines comparative modeling, rational design, and directed evolution to maximize pilA-N's conductive properties. Other studies build their pili protein in E.coli; in contrast, we aim to use *G. sulfurreducen*s itself. This is because our experimental design relies on *G. sulfurreducen*s reductase's ability, by providing a Fe(III) oxide-rich media. *G. sulfurreducen*s EET reduces Fe(III) oxide to Fe(II), which is measurable by the FACS for high throughput screening when the PG SK assay is added to the media.[24]

Ultimately, this proposal seeks to develop an accessible, cost-effective, and environmentally sustainable method for bio-based energy transfer. By engineering pilA-N to achieve higher electron conductance, we aim to address critical global challenges surrounding renewable energy, resource scarcity, and toxic waste, offering a forward-thinking alternative to conventional energy solutions.

Experimental Approach:

Our experimental approach first uses comparative design techniques, utilizing tools such as UniProt multiple sequence alignments to find conserved aromatic residues from other conductive pili and integrate them into Geopilin domain 1 protein with gene name PilA-N (Fig. 1,2). The UniProt Accession ID: Q74D23 (UniProt) and gene locus name GSU1496 were used. Next, we used rational design by utilizing high-resolution structural data from AlphaFold and PyMol, focusing on enhancing π-stacking interactions and hydrophobic core packing by introducing key residues like tryptophan and tyrosine. Lastly, we use direct evolution using the data collected above to integrate degenerate NNK gene blocks at each site to implement the mutation. This is an iterative approach where we focus on mutating one residue at a time. In Figure 2, we aim to engineer 11 residues, resulting in a predicted 11 iterations of direct evolution. For each iteration, variants will be screened for mutations that may enhance electron transfer efficiency.

To form our knockout library(fig. 3), we'll use a suicide plasmid to integrate our variant PilA-N's into wildtype G. sulfurreducens PCA's genome[20]. To do so, variant sequences will be ordered that contain locus tag (GSU1496), regulatory regions, homology arms, and restriction sites. These ordered sequences would then be inserted into the suicide vector pPLT173 where a Gentamicin cassette will allow for selection. The recombinant plasmid will be linearized before electroporation. After plating in selective media, colonies will undergo screening assays to measure conductance.

Our conductance screening assay(fig. 3) allows for the rapid comparison of different pilA-N variants by measuring Fe(II) production. This can be done by culturing G. *sulfurreducen*s mutants in lactase, hydrogen, and Fe(III) oxide-rich media and measuring Fe(II) levels at various time points. After culturing, PG SK will be added to the media to detect Fe(II). PG SK binds to Fe(II), and upon binding, the fluorescence intensity of the probe increases. This change in fluorescence will be quantified using FACS, allowing for a rapid comparison of Fe(II) production across the pilA-N variants. Higher fluorescence intensity will indicate greater Fe(II) production, correlating to enhanced electron transfer efficiency by the engineered pilA-N variants and allowing a quantitative assessment of each pilA-N variant's conductance.

After the quantification and iteration through multiple cycles of direct evolution, the highest conductors will be purified to characterize our variant pilA-N better possibly using multiphoton microscopy. The cells will first be plated on a biofilm of graphite electrodes, which act as electron acceptors for the acetate oxidation. These cells will then be scraped, collected, and washed in a series of wash buffers. This will be completed before shearing the cell pellet with a special solution. A final resuspension with ethanolamine buffer ends purification.[22] To verify, SDS-PAGE and Western Blot will read an expected size of ~6.5 kDa. Atomic force microscopy provides a high-resolution image to visualize the spatial arrangement of aromatic residues in the variants.

Alternative Approaches and Anticipated Hurdles

Exploring the synergy of the pilA-N and cytochrome c proteins—OmcS and OmcZ through our approach could engineer a more conductive nanowire since they have been shown to contribute to EET[13][22]. The selection marker for homologous recombination could be inefficient, so counter-selection like sacB could be used to increase confidence[17][21]. Pili purification could be impeded by mechanical shearing, which may not selectively isolate *pila-N* filaments, as it could also release other surface-associated proteins or cell debris. This could result in a less pure sample, complicating downstream analyses like SDS-PAGE and Western blot. Additionally, the use of ammonium sulfate precipitation might not fully separate *pila-N* from other similarly soluble proteins, leading to the co-purification of contaminants. To address this problem, we can

incorporate a Glycine-Serine (GS) linker sequence between the pila-N protein and 6x His tags, since they provide flexibility and reduce steric hindrance to the polymer structure, ensuring that any added fusion tag does not interfere with the protein's native function or structure. Combined with an affinity tag like 6x His tags, it allows for more targeted isolation, which ensures that only tagged pilA-N are pulled down and minimizes contamination from other surface proteins. In addition, we can further isolate the pilA-N protein using ion exchange chromatography by choosing an ion-exchange resin based on the isoelectric point (pI) of pilA-N. For pilA-N, a cation or anion-exchange resin is appropriate if the protein is charged at the working pH.

Potential Long-Term Pitfalls

Our engineered protein addresses conductivity in G. *sulfurreducens* for future use as a nanowire. While we might obtain increased conductance, polymer stability is critical to consider polymer length. Additionally, control over the direction of polymerization is key for targeted connections. The next steps would address these issues to better understand the characteristics of PilA-N to discover its niche in future applications. We would consider site-directed mutagenesis at the subunit-subunit interface to encourage linear stacking and reduce aggregation off-pathway. Additionally, there is the possibility of integrating click chemistry like copper(I) catalyzed azide-alkyne cycloaddition reaction(CuAAC) for its polymerization application[23]. Although, using a rare metal like copper is contrary towards our focus on reducing rare-earth mineral use. There are many pitfalls in designing a protein for such lofty goals as we are only scratching the surface, but the challenge would be for the better good.

Figures:

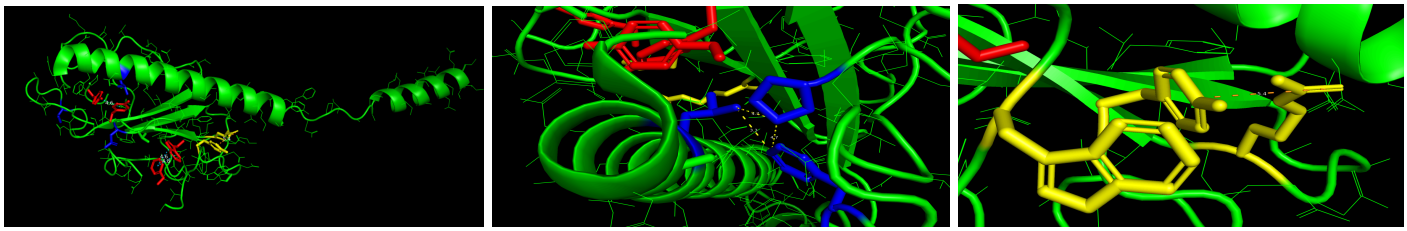

Fig. 1: PilA-N from Geopilin domain|monomer|PDB: 7TGG made with PyMol. Red = pi-pi interactions, Yellow = pi-cation interaction which can be altered for pi-pi interactions Blue = target residues for aromatic alteration

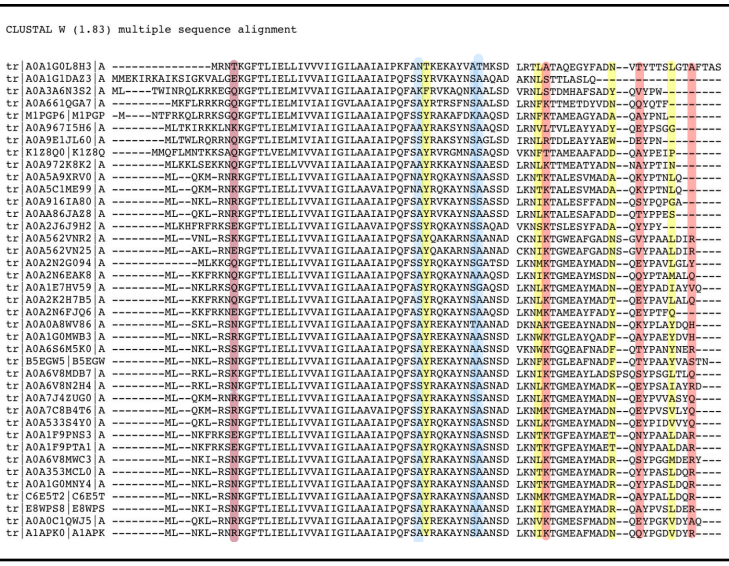

Fig. 2: Residues for further investigation under altered state. Made by UniProt MSAAromatic residues: Position 33 (T to Y), 48 (L to F), 60(N to Y), 67 (L to Y).
Are in the transmembrane domain.Charged residues: Position 4 (T to), 50 (A to), 61 (T to)
69 (A to)Hydrophobic residues: Position 4, 14. Are in the transmembrane domain.
Position 32, 41 not in transmembrane domain A) 1st half of compared Pilus assembly
protein sequence B) 2nd half of compared Pilus assembly protein sequence
Uniprot:
https://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-R20250221-210418-0208-50446466-p1m/aln-clustal_num

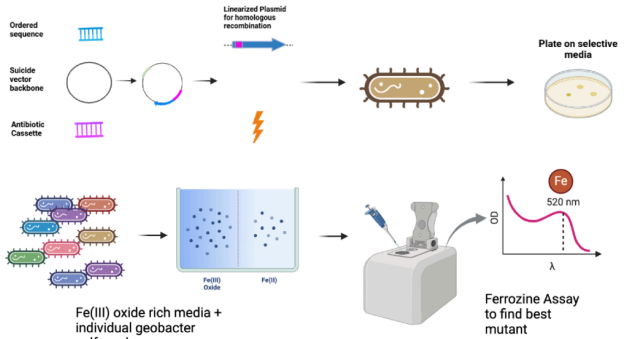

Fig. 3: Experimental design & workflow made with BioRender
Create mutant strain - create library - introduce variant pilA-N into mutant - test with Fe(III) oxide rich medium - measure Fe(II) using FACS with PG SK

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