ABSTRACT

Geobacter species are abundant dissimilatory metal reducing bacteria present in various subsurface environments. These microbes reduce metal oxides and hence perform an important biogeochemical process of anaerobic bioremediation of groundwater contaminated with metal or organic contaminants. *Geobacter sulfurreducens* (GS) has served as a primary model species for elucidating the physiology of *Geobacter* species. Type IVa pili (T4aP) in *G. sulfurreducens* are highly specialized as they aid respiration by mediating direct long range extracellular electron transfer to metal oxides in oxygen limiting conditions. These "bacterial nanowires" have metallic like electricity conducting properties and act as electronic conduits to transfer electrons to the metal oxides, thus extending the electron transfer capabilities of the cell well beyond the outer surface. The gene cluster of Type IVa pilin consists of conserved genes *pilB-pilT4-pilC-pilS-pilR-pilA-N-PilA-C.* These genes encode proteins necessary for biogenesis/assembly/function of this highly specialized pilus. PilA is the structural subunit of the mature pilus that aids electron transfer. The expression of *pilA*, which encodes the pili structural protein, is directly regulated by a two-component regulatory system in which PilR functions as an RpoN-dependent enhancer binding protein and PilS functions as a sensor histidine kinase. Unlike other PilA proteins, *G. sulfurreducens* pilin lacks C-terminal globular domain, present in longer Type IVa pilin from *Neisseria gonorrhea* and *Pseudomonas aeruginosa.* The gene *pilA-C* present downstream of *pilA-N* is predicted to form mostly β-strands and the function of this protein in *G. sulfurreducens* is not known yet. PilB and PilT4 are one of the most powerful ATP driven molecular motors that helps in the extension and retraction of the pilus, respectively. Though PilA-N alone can transport electrons, cytochromes OmcS and OmcZ have also been reported to play an important role in mediating extracellular electron transport. When the project was initiated there were no structural details available for any proteins of Type IVa pilus machinery involved in electron transfer from the model organism *G. sulfurreducens*. Even there were two distinct proposed models known for the rotation of PilB and PilT4 extension and retraction motors. So, this project was aimed at taking a structural biology approach to dissect the molecular mechanism of PilB motor and to characterize other proteins involved in the assembly/functioning of this highly specialized pilA.

To purify the target proteins for structural and biophysical studies we adopted three different approaches. In first approach, recombinant proteins were overexpressed using

Escherichia coli as an expression host. For this, we cloned all the genes present in the predicted *G. sulfurreducens* PilA operon in *E. coli* based expression vectors. These proteins were purified for structural, biophysical and biochemical studies. To prepare samples suitable for structural studies and to achieve protein expression in soluble form several deletion constructs were created for each target. The expression conditions and protein purification parameters were further optimized to purify target proteins suitable for structural/biophysical/biochemical studies. However, the key protein PilA-N, was extremely difficult to purify due to its intrinsic tendency to form higher order structure. We could manage to purify recombinant 6xHis tagged PilA from inclusion bodies using denaturing conditions. To restrict protein oligomerization and express protein in soluble form we created an engineered MBP-PilA fusion protein which could be purified with ease from the soluble fraction. We determined the low resolution solution structure of MBP-fusion of PilA-N using Synchrotron based small-angle X-ray scattering (SAXS) experiments. Analytical ultracentrifugation, size exclusion and SAXS studies suggest that MBP-PilA self-associate to form a defined globular assembly mediated likely by the self-association of PilA-N. The second strategy was to purify PilA and cytochromes from the native host *i.e. G. sulfurreducens*. Due to low expression of pili per cell, slow growth rate, requirement of strict anaerobic conditions and lack of selection marker resulted in poor yield of protein samples even after using lab scale fermenters. The third approach was to use engineered vectors (pCD342 and pMMB206) suitable for overexpression of proteins in *G. sulfurreducens*. We successfully cloned PilA and PilR in these vectors but the preparation of electro-competent cells turned out to be a challenge. Despite investing lots of time and resources the last two strategies could not be used successfully to purify target proteins.

PilB, PilS, PilC and PilR could be purified suitable for structural studies and extensive crystallization trials were setup. We were successful in crystallizing three proteins and solved structure of PilB. We were successful in solving the first unliganded crystal structure of core ATPase domain of PilB from *G. sulfurreducens* at 3.1 Å resolution. Crystal structure revealed that PilB hexamer is a dimer of trimers. Further structural analysis revealed three distinct conformations i.e., open, closed, and open' in PilB which were previously proposed to be mediated by ATP/ADP binding. We have annotated open' conformation for the first time. We determined the binding affinities for the ATP, ADP and cyclic di-GMP (cdG) using isothermal titration calorimetry. We show that PilB hexamer has two high affinity binding sites for ATP and four high affinity sites for ADP. This correlates well with the crystal

structure as the unliganded PilB protein contains two molecules in the open state that could be responsible for ATP binding while the other 4 molecules would be in an ADP bound conformation. We propose "symmetric rotary model" for PilB motor where binding of ATP first to the high affinity binding sites and then cycling through the sites, enacting conformational changes through ATP hydrolysis and then nucleotide release drives clockwise rotation in the motor. Using thermofluor and circular dichroism studies we also show that binding of these ligands provide thermal stability to the protein and brings about significant changes in the tertiary structure. ATPase assays revealed slow ATPase activity in PilB and is not significantly affected by the presence of cdG. We also describe the structural and mechanistic similarities and differences in the molecular mechanisms of the extension ATPase motor. Our comparative structural analysis further suggests that extension and retraction motors rotate either clockwise or counterclockwise to facilitate assembly of the right-handed or left-handed pilus.

Non-toxic nature, stable in aqueous environments, high flexibility, uniform thickness and metallic-like conductivity makes *G. sulfurreducens* pilA a highly specialized protein with immense applications. The fundamental understanding of this phenomenon can also aid design of better conducting biological/synthetic materials. Therefore, a comprehensive understanding and control of electron transport in nanowires could have great implications for the construction of bioelectrochemical systems, and the development of the next generation of electronics.