Role of LapD gene from Pseudomonas sp., in initial attachment and biofilm formation

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Abstract
Magnanimous bacteria have the ability to form surface-attached complex, surface-associated multicellular communities that consist of cells attached to a surface, and cohered to each other. The species of Pseudomonas form compact biofilm on both abiotic and biotic surfaces with truncated accessibility of nutrients. There are various factors such as flagella, type IV pili, cup fimbriae, sad genes and adhesions such as lapA, lapD and also regulatory domains such as GGDEF and EAL mandatory for the preliminary attachment for the formation of biofilm. In that lapD, a gene sited contiguous to the lapA gene, plays a chief role in biofilm formation in most of the Pseudomonas sp., Pseudomonas sp., is known to respond rapidly to the root exudates in soils, congregating at root colonization sites and establishing stable biofilms contains the lapD gene for the initial and stable biofilm.

Introduction
A biofilm is an organizational community of bacteria adherent to a surface and encompassed in a self-fashioned exopolysaccharide matrix. Biofilm are complex communities of microorganisms that develop on surfaces in diverse environments. Bacteria in biofilms have an exclusive multicellular architecture and augmented resistance to host fortifications and antimicrobials. Biofilm was not new but it was very old when the father of Microbiology Antonie van Leeuwenhoek perceived that the “animalcules” on living and dead matter (Percival et al., 2011). Bacteria attaching to the surface were demonstrated by Zobell (1984). He was the first to demonstrate it by keeping the slide in seawater for long time was able to form a biofilm by attachment. Further Marshall et al., 1971 revealed that Achromobacter and Pseudomonas undergo an immediate reversible attachment followed by a time-dependent irreversible attachment.

Lawrence (1987) confirmed there were two distinct stages of early adhesion in Pseudomonas fluorescens. “Micro niche” term first used by Costerton et al., (1994) and commencement of Biofilm is hypothesized to facilitate the development of a protects bacteria against various physical and chemical stresses and confers resistance to deleterious agents such as antibiotics and detergents. Many bacteria form biofilm, complex, surface-associated multicellular communities that consist of cells attached to a surface, and cohered to each other. An outbreak of research activity over the last two decades has led to a more detailed and nuanced understanding of the process of biofilm formation for diverse microorganisms (Haussler and Parsek, 2010). Bacteria in nature typically exist as members of structurally complex, surface attached communities known as biofilms, and this fact has led to great interest over the last two decades.

Biofilms have been studied the most in medically important bacterial pathogens like Pseudomonas aeruginosa. These same pathogens may also form biofilms on various medical devices leading to serious infection. These pathogens frequently colonize and form biofilms inside or on the external surface of the patients and result in very dangerous chronic infections. P. aeruginosa is known to exist as biofilms in lungs of cystic fibrosis patients (Parsek and Singh, 2003). Dental plaques create biofilms of many species of bacteria existing together and can cause vascular diseases when parts of biofilm enter the blood stream (Okuda et al., 2004). The initial attachment and irreversible formation is more important in the biofilm formation and stabilization. So the initial attachment and the gene involved in the initial formation of the biofilm in beneficial bacteria towards plants became much of concern.

Species of Pseudomonas form impenetrable biofilms on both abiotic and biotic surfaces, and are a primary model in biofilms research (Fuqua, 2004). Pseudomonas putida can respond rapidly to the presence of root exudates in soils, converging at root
colonization sites and establishing stable biofilms (Urgel et al., 2002). The plant-growth-promoting Pseudomonads have been reported to discontinuously colonize the root surface, developing as small biofilms along epidermal fissures. The release of plant exudates into soils can stimulate rapid mobilization and chemo taxis of Pseudomonads towards root systems (Urgel et al., 2002). The formation of the biofilm is beneficial in the Plant growth Promoting Rhizobacteria (PGPR). Several Pseudomonas species and derivatives are effective plant growth promoting rhizobacteria, and some are biocontrol agents (Lugtenberg et al., 2002).

The LapA cell surface protein identified in P. fluorescens is required for the transition from reversible to irreversible attachment on abiotic surfaces (Hinsa et al., 2003). Roots are colonized along surface fissures when inoculated with plant growth-promoting pseudomonads, but this has not been commonly observed for natural populations, and thus may be a consequence of the inoculation strategy (Bloemberg et al., 2000). Bacterial adherence to seeds is a process that strongly influences rhizosphere colonization. Suppliers often deliberately coat their seed stocks with microbial biofilms to inoculate the developing rhizosphere. P. putida adheres effectively to seeds and will subsequently colonize the rhizosphere (Urgel et al., 2000). Several P. putida mutants, including the one in lapA homologue of P. fluorescens, are deficient in seed adhesion and biofilm formation on inert surfaces, emphasizing the overlap between these activities (Urgel et al., 2000). There are prolific impacts responsible for biofilm formation of that lapD gene is most important for Pseudomonas sp., for its initial attachment and there are different stages in biofilm formation.

**Attachment:**

Pseudomonas has an extensive assortment of habitats with extreme changing conditions. One of the most important mechanisms is aggregation or attachment to develop surface-associated communities called biofilm (Davey and O'Toole, 2000). Initially, planktonic bacteria make weak and transient connections, referred to as reversible attachment, when they encounter surfaces. There are several factors such as flagella, type IV pili, cup fimbriae and sad genes that affect cell surface structures and properties in P. aeruginosa and P. syringae genomes, indicating that the nature of biofilm formation and irreversible attachment is distinct among various species of Pseudomonas.

Hinsa and Toole in 2006 suggest that LapD, an inner-membrane protein, modulates the secretion of the LapA protein, but does not have an obvious impression on LapA transcription or LapA levels in the cytoplasm. They reported that the identification of an ORF mapping adjacent to the lapAEBC locus, designated lapD, which is conditionally required for biofilm formation.

Later Nowell et al., (2009) anticipated that the LapD is a c-di-GMP effector protein that binds c-di-GMP via a degenerate c-di-GMP phosphodiesterase (EAL) domain. LapD gene is the c-di-GMP receptor in the signaling pathway by which inorganic phosphate (Pi) starvation controls biofilm formation. In contrast to c-di-GMP effectors identified to date, LapD is an inside-out signaling protein, communicating cytoplasmic c-di-GMP levels to the membrane localized attachment machinery via a periplasmic output domain.

Peter (2009) suggests that P. fluorescens requires the large adhesion LapA for stable accessory to surfaces, identification was extended that the inner-
membrane protein LapD as being required for biofilm formation and suggested that it played a role in the localization of LapA. The lapD mutant had decreased cellular levels of lapA compared with wild type. This suggests that this decrease is not caused by a difference in LapA transcription in the lapD mutant. Fuqua (2010) revealed that the remarkable Lap A proteins groups in P. putida protein has 8,682 amino acids, whereas the P. fluorescens protein has 4920 amino acids. The length variation is the larger size of the P. putida repeat sequence itself. These observations suggest that the Lap proteins and, by extension, similar large repetitive surface proteins, are rapidly evolving via these repeats. More comprehensive population level analyses will probably reveal interesting evolutionary trends for this remarkable group of proteins.

The LapD protein might affect the synthesis or function of the ABC transporter or the large adhesin LapA. It would be interesting to see if the increase in 2-OH-PCA induces the production of adhesions like lapA, lapD and Raps in P. chlororaphis (Madula et al., 2008). In P. fluorescens, secretion of lapA is regulated by LapD, a protein with degenerate GGDEF and EAL motifs and that responds to intracellular cyclic diguanylosine mono phosphate (c-di-GMP) levels to control adhesion. LapD, a gene located adjacent to the lapA gene, also plays a role in biofilm formation. A mutation in lapD results in a conditional biofilm, this biofilm phenotype is exacerbated when biofilm formation is assayed in a flow-cell system (George et al., 2008).

LapD and LapG play opposing roles in regulating attachment via LapA. The LapG and lapD genes occur in a putative operon adjacent to the genes encoding LapA and LapEBC, the ABC transporter required for LapA secretion. The overexpression of lapG eliminated biofilm formation, while overexpression of lapD increased biofilm to levels. Overproduction of LapD increased LapA in the cell and at the cell surface, but decreased the amount in the supernatant. Overproduction of LapG had the opposite effect reducing cellular and cell surface of LapA, while increasing the amount in the supernatant. Taken together, with the mutant and epistasis analyses, these data confirm that LapG and LapD exert opposing forces on the maintenance of LapA on the cell surface and suggest that they act in the same pathway (O’Toole et al., 2011).

**LapD Domains:**

The amino acid sequence of LapD contains 3 predicted domains: a HAMP domain, diguanylate cyclase (GGDEF) and EAL domains. GGDEF and EAL domain proteins regulate biofilm formation through c-di-GMP DGC and phosphodiesterase (PDE) activities, respectively (Newell et al., 2009). LapD binds with c-di-GMP through its cytoplasmic EAL domain and controls biofilm formation via a periplasmic output domain. LapD, a protein with predicted GGDEF and EAL domains that binds c-di-GMP. He proposed that LapD act as an effector protein linking this intracellular signaling molecule to the function of an extracellular adhesin, LapA, and does so through an inside-out signaling mechanism (Newell et al., 2009).

c-di-GMP is a novel global second messenger in bacteria; the metabolism of which is controlled by GGDEF and EAL domain. Roger et al. (2004) stated that cyclic nucleotides represent second messenger molecules in all kingdoms of life. In bacteria, mass sequencing of genomes detected the highly abundant protein domains GGDEF and EAL. He showed that the GGDEF and EAL domains are involved in the turnover of cyclic-di-GMP. The GGDEF domain stimulates c-di-GMP production and the EAL domain c-di-GMP degradation. GGDEF domains function as c-di-GMP cyclase and EAL domains as phosphodiesterase.

The second messenger cyclic dimeric GMP (c-di-GMP) regulates surface attachment and biofilm formation by many bacteria. *Pseudomonas fluorescens* Pf0-1, c-di-GMP impacts the secretion and localization of the adhesin LapA, which is absolutely required for stable surface attachment and biofilm formation by this bacterium. LapD serves as the c-di-GMP receptor connecting environmental modulation of intracellular c-di-GMP levels by inorganic phosphate to regulation of LapA localization and thus surface commitment by *P. fluorescens*. The structure/function analysis presented by Newell et al. (2009). He suggested that LapD controls LapA localization by a unique inside-out signaling mechanism binding c-di-GMP in the cytoplasm and transmitting this signal through the inner membrane to the periplasm via a HAMP domain.

The HAMP domain plays an essential role in many signaling proteins. HAMP is named by Aravindand Ponting (1999) for their occurrence in histidine kinases, adenyl cyclases, methyl-accepting chemotaxisproteins, and phosphatases. HAMP domains connect extracellular sensory with intracellular signaling domains in over 7500 proteins, including histidine kinases, adenyl cyclases, chemotaxis receptors, and phosphatases. These domains typically relay signals across the cytoplasmic membrane, altering their conformation in response to activation of an extracellular input domain and propagating that conformational change to a cytoplasmic output domain. (Hulk et al., 2006)

Gjermansen et al. (2003) stated that local starvation-induced biofilm dissolution appears to be an integrated part of *P. putida* biofilm development that causes characteristic structural rearrangements. *P. putida* biofilms was shown to occur in response to carbon starvation. Genetic analysis suggested that the adjacent *P. putida* genes PP0164 and PP0165 play a role in *P. putida* biofilm formation and dissolution. When c-di-GMP levels are low, the LapD protein is kept in an “off” state that allows LapG, a periplasmic protease, to interact with LapA and cleave the N-terminal domain of this adhesion, releasing LapA from the cell surface and promoting biofilm detachment. Under abundant phosphate conditions, LapD binds c-di-GMP in the cytoplasm and binds to and sequesters LapG in the periplasm, promoting cell adhesion via maintenance of LapA on the cell surface.

Stable surface adhesion of cells is one of the early pivotal steps in bacterial biofilm formation, a prevalent adaptation strategy in response to changing conditions.
environments. In *Pseudomonas fluorescens*, this process is regulated by the Lap system and the second messenger cyclic-di-GMP. High cytoplasmic levels of cyclic-di-GMP activate the transmembrane receptor LapD that in turn recruits the periplasmic protease LapG, preventing it from cleaving a cell surface-bound adhesin, thereby promoting cell adhesion. (Cooley et al., 2015).

**PCR amplification of lapD gene**

The approximate size of the *lapD* gene is of about 1.9kb with 648bp, it is sequenced in *Pseudomonas fluorescens* WCS365 (Hinsa et al., 2006). By using the PCR amplification of *lapD* gene it is possible to find the *pseudomonas* strain is forming biofilm or not. The primers for amplification are F: 5'-GTTCCTGGTGCTGCGCTT-3' and R: 5'-AATCGCTTGTCACTTTCC-3'. The reaction was performed with initial heating at 94°C for 7 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The reaction tubes were cooled and a small aliquot of PCR product was run on agarose gel electrophoresis along with the DNA molecular weight marker to analyze the expected amplicon size was 875 bp.

**Conclusion**

Biofilm formation of the bacteria in early days were considered as the pathogens and now a days the exploit of the biofilm formation in the bacteria towards the plant interaction plays a major role in plant growth promotion and also protects against the pathogens. The interaction of prokaryotes with plants can range from mutualism and commensalism (*Pseudomonas putida*, *Pseudomonas fluorescens* and related *Pseudomonas*f's found on leaves and roots).

Various models of biofilm formation on plants have been proposed from studies of single species biofilms *in vitro*, however little is known about the relative importance of these models of biofilm development outside the laboratory. The biofilm forming plant growth promoting bacteria may be commercialized and used as a biofertilizer in different fields. The outcomes confirmed that the LapA is used for the adhesion of the bacteria towards the root and helps in the biofilm formation. The lapD is an important gene for the formation of the adhesion for stable biofilm by *Pseudomonas* sp., This shall be easily confirmed by the amplification of the specific gene.

*LapD* is an important factor biofilms formation in *Pseudomonas* sp.. If the biofilms formation is present in the non pathogenic *Pseudomonas* sp., like *P. putida* and *P. fluorescens* and also if the strains were plant associated it is further constructive for plant growth.

**Reference**


