Bacterial biofilm formation and its role against agricultural pathogens

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Bacterial biofilm is a microbial assemblage which forms by bacterial adhesion, growth and expansion process. In agriculture, PGPRs enhance plant growth in additional to quorum sensing in the mode of biofilm. Antibiotics produced by PGPRs acts as biocontrol agents. In a successful plant-microbe interaction, plant-root colonization by PGPR is attributed in addition to biofilm formation against microbial pathogens. *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Paenibacillus polymyxa* and *Bacillus subtilis* form biofilm and multicellular mode of growth likely predominates in nature as a shielding tool against hostile environmental conditions. They use quorum sensing mechanism to coordinate and regulate gene expression according to local population density against certain microbial pathogens. Moreover signalling mechanism coordinates and modulates plant-bacteria interaction with controlled release of antibiotic and toxins. In this chapter focus will be on the PGPRs use in biofilm formation and their contribution to battle against microbial pathogens involved in plant diseases.

Keywords: bacteria; biocontrol; biofilm formation; PGPR; quorum sensing; soil; roots

1. What is Biofilm

Biofilms are defined as the well-organized cooperating communities of surface-associated microorganisms enclosed in extracellular matrix produced by themselves. It is a trait of microorganisms (bacteria) that is why found everywhere either natural or artificial surface (both biotic and abiotic) [1, 2]. Naturally, bacteria live in aggregates and attach to solid surfaces along with intimate contact to other bacterial cells [3-5]. Bacterial cells produce variety of extracellular polymeric substances (EPS) along with various proteins, exopolysaccharides and DNA while attached on the surface. Configuration of biofilm is diversified, ranging from simple to complex, flat to tower, featureless to clustered film and making heterogeneous arrangements of bacterial cells. Biofilms have distinct physiological structures, cells within it vary from each other, up and down regulation of genes also vary from cell to cell. Biofilm cells are very responsive to various functions of their surroundings such as they modulate their metabolic functions, respond to nutrient products, waste product gradients, engage cell-cell communication and contact with adjacent cells.

2. Why biofilm is important

2.1 Functions of biofilm

Biofilm formation is predominant property in bacterial life style. Biofilms have great practical importance in medical, industrial and agricultural settings, exhibiting both beneficial and detrimental activities. In this chapter, we will highlight the importance of biofilm formation which describe its importance in biocontrol of the plant diseases and will focus on the situation that why microbes adopt this strategy of biofilm formation. Some key functions of biofilm are:

2.2 Protection from Environment

Bacteria residing in the biofilm are protected by the shelter and homeostasis as the EPS are produced having protein, nucleic acid, carbohydrate and other substances. It plays a key role in structure forming and functioning of biofilm communities in diverse environments. The EPS matrix acts as an anion exchanger which restricts the access of antimicrobial agents into the biofilm. Compounds surrounding the biofilm which may enter by diffusion are also restricted in this way. Nature of both agents; compounds and biofilm along with EPS matrix are the main determinants of this restriction. Toxins and different metals ions or cations are also reported to sequester in presence of EPS and protects from environmental stresses (UV radiation, pH shift, desiccation and osmotic shock).

2.3 Nutrient Availability

Water is a major medium for transport of nutrients and metabolites. Presence of water in biofilm establishes an aqueous phase and regulates nutrient availability, exchange of metabolites and removal of potentially toxic metabolites. Biofilm is the consortia of different bacteria where they work in syntrophic relationship e.g. three bacterial species are involved

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in the degradation of highly complex organic molecule into inorganic compounds like CH_4 and CO_2. The multispecies approach of biofilm leads to the effective nutrient ability by syntroposim and anaerobic degradation of compounds.

2.4 Acquisition of New Genetic Trait

Evolution is the process of environmental adaptations in organisms. Bacterial community also undergoes the process of evolution to adapt changing environmental circumstances. To cope up with the situation, they transfer their genetic material horizontally (conjunction) resulting in genetic diversity. Microbial communities acquire new genetic material and transcribe it to genes following to form an important member of biofilm.

2.5 Penetration of Antimicrobial Agent

Antimicrobial agents and biofilm microbial community combat for dominancy where diffusion of molecules determine the success. Biofilm has the ability to develop a barrier of EPS against diffusion molecules of the antimicrobial agents.

3. Biofilm formation

3.1 Concept of biofilm formation

Every microbial community has distinctive chemical and physical properties, which shows challenges for the microbial colonists. In plant-microbe interactions, biofilms are formed on later stages and they modulate the plant growth along with microbial behaviour. As the soil environment is heterogeneous, microbial community residing there is also heterogeneous bringing about vigorously modifying colonized plant environment. Actively growing plant releases higher exudation into the soil by active root tissues. Biofilm present at the site is dramatically influenced by the nutrients present in root exudates.

3.2 Mechanism/steps/processes of biofilm formation

Bacterial biofilm formation involves a number of steps, including adhesion of bacteria to the surface, its growth and expansion of biofilm. Biofilm has the character of formation on every type of biotic and abiotic surface that is why it's equally important for soil particles in agricultural perspective, for medicinal implants and other food products. Fimbriae, pilli, flagella and EPS mediate bacterial attachment to the conditioning film. Bacterial biofilm can be of single species while in natural environment, many bacterial species are involved and moreover fungi, protozoa and algae may also contribute. Biofilms can be a single cell layer thick 6-8cm while in most of the cases, it is 100µm thick on average. Bacterial attachment to the surface and biofilm formation involves following steps:

3.2.1 Surface conditioning

Key function of surface conditioning includes organic substrates adsorption to the physical surface making it suitable for the microbes involved in biofilm formation. Adsorbed organic substances include a mixture of proteins, polysaccharides and glycoproteins. Major factors that control surface conditioning are surface tension, nature of fabricated material, roughness and wettability along with electrophoretic mobility. Property of adsorption carries change in physico-chemical structuring of the surface which smoothen microbial invasion. Rough surfaces provide more chances for the attachment of microbe to form biofilm.

3.2.2 Microbial cell adsorption to conditioned surface

Freely floating bacteria adhere to the surface after following the course of conditioning. In the preliminary stage, microbial adhesion to the conditioned surface is reversible. Freely floating microbes follow the principle of Brownian motion, attach to the surface and readily remove from the surface with mild rinsing. On electro-chemical basis, weak forces of attraction exist there, including Van der Waals, surface charge of microbes and some other electrostatic forces for interacting molecules.

3.2.3 Channelled and tangled network formation of EPS

On prolonged microbial adhesion to the conditioned surface, its attachment shifts from reversible to irreversible. If it is present on unwanted surface, its removal can be achieved by either mechanical or chemical treatment. The cementing substances like EPS in microbes make their adhesion irreversible. Microbes anchor the network of fibres on conditioned surface and forms spaghetti like strands of their connecting cells. As the diversified microbial consortia occupies, tangled polysaccharide fibres become sticky. EPS network provides nutrients from the medium, cohesive forces within biofilm and protection of microbes from environmental calamities. As described in the functions of biofilm, EPS enhances transfer of genetic material from one cell to another and acting as a short-term energy storage house.

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Chemically EPS contains neutral and acidic sugars e.g. fucose, galactose, glucose, gluconic acids, mannose, rhamnose, ribose and uronic etc.

3.2.4 Quorum sensing for durable biofilm

Quorum sensing is the communication mechanism among the microbial cells. Natural community of bacteria communicates by cell to cell signal and it can happen for genetically engineered and non-engineered bacteria in the form of bacterial products. They can diffuse from one cell to another. Acylhomoserine lactone (acyl-HSL) is a commonly produced quorum sensing molecule in biofilm which make it durable.

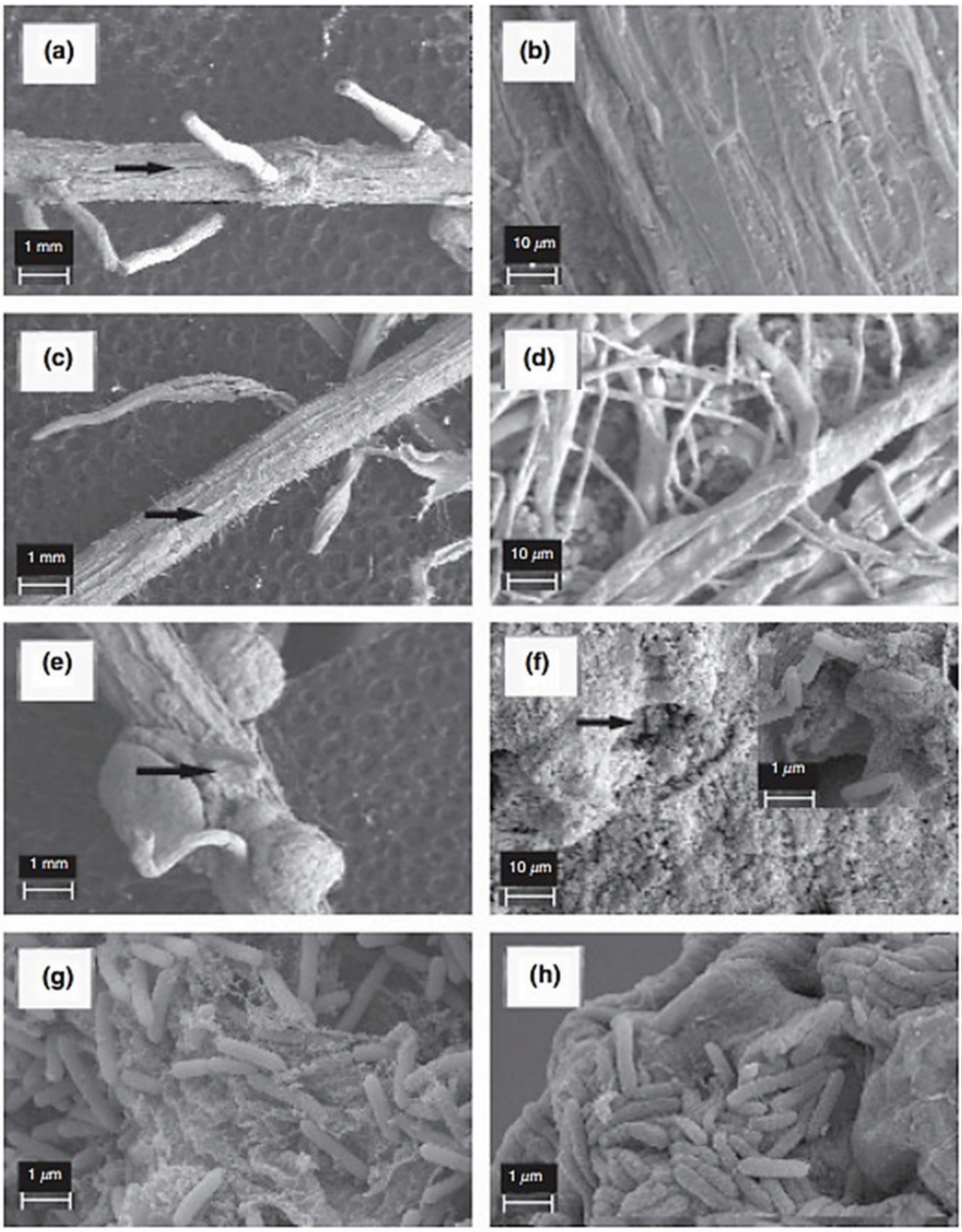

Fig. 1 Scanning electron micrographs of plant roots colonized by *Paenibacillus polymyxa* and *Aspergillus niger* (greenhouse experiment and gnotobiotic experiment). Peanut plant roots (a and b) in potting soil were infected with *Aspergillus niger* (c and d). *Paenibacillus polymyxa* B5 biofilm formation on peanut plant inhibits *Aspergillus niger* colonization (e and f). *Paenibacillus polymyxa* B5 (g) and B6 (h) biofilm formation on *Arabidopsis thaliana* root. Note that *Paenibacillus polymyxa* B5 isolate produces remarkably more extracellular matrix. Arrows indicate further magnification sites. Picture: courtesy of S. Timmusk.

4. Agents involved in biofilm formation

Biofilm formation is not a simple process, it involves a number of factors that helps in initiation, expansion and stimulation of biofilm formation. Some of the key factors will be discussed here:

4.1 Involvement of certain proteins

The surface structure of bacteria like lipopolysaccharide concentration and proteins present on the surface membrane are important factors in the biofilm formation on roots. Recently a 900-kDa cell surface protein called LapA (Large adhesion protein A) is identified and reported to affect *Pseudomonas fluorescens* colonization [6]. Similarly, in *Pseudomonas putida* KT2440 LapA homologues is involved in the competitive root colonization and adhesion to seed [7]. It ultimately leads to work as biofertilizer and plant growth improves. LapA has domains which resemble adhesion is involved in the biofilm formation of specifically Gram-positive bacteria. Similarly, another domain Ca^{2+} -binding proteins and haemolysins are often involved in the interactions of host–cell [6]. In *Rhizobium leguminosarum*, a set of secreted agglutinins called biovartifolii is thought to bind Ca^{2+} known as Rap (Rhizobium adhering proteins) localized to poles of the cells and it is hypothesized that it plays a key role in rhizobial cells to plant tissues binding [8].

4.2 Transcription factor

In a recent study about the role of transcription factor for biofilm formation in Agrobacterium tumefaciens, SinR is found to be a regulator in biofilm formation [9]. SinR transcription factor is an oxygen-responsive regulator of FNR (fumerate and nitrate reductase) family of proteins. They control the transition in limited oxygen condition. Microbes like *Agrobacterium tumefaciens* having sinR disruption form thin heterogeneous biofilm while thick and less structured biofilm is observed with strong sinR expression. As the conditioned surface and associated layer of biofilm are oxygen limited, sinR functions for plant-microbial interaction [10, 11].

4.3 Exopolysaccharides

Production of exopolysaccharide by the microbes is a common and generally important phenomenon that interact with the root appendages to establish plant-microbe interaction [12, 13]. Many types of polysaccharides are involved in the modulation of physical and chemical attributes of *Pseudomonas aeruginosa* biofilm matrix [14]. For instance, cellulose is an important polysaccharide and required for the biofilm formation. Its optimum production in *Agrobacterium tumefaciens* regulates biofilm production and root adherence. On overproduction of cellulose, dense biofilm is produced [15].

5. Threats of pathogens to the agriculture

A disease of bacterial wilt in which vascular bundle of plant damage, caused by *Ralstonia solanacearum*; genetically diverse soil-borne pathogen [16] Due to this disease, agriculture as a whole and horticultural crops specifically at risk worldwide. Naturally occurring rhizobacteria such as *Bacillus* spp., *Streptomyces* spp. and *Pseudomonas* spp. are used for biological control of this disease. [17, 18]. Importantly *Bacillus subtilis* produces a number of biologically active compounds with a broad spectrum of activities towards phytopathogens [19, 20]. *Bacillus subtilius* form biofilm and combat with the pathogenic bacteria. Now *Bacillus subtilis* is commercially used for the biological control activity. Indeed, positive results about *Bacillus subtilis* strains have been reported to effectively suppress the Ralstonia wilt disease [21] Similarly *Paenibacillus polymyxa*, a PGPR bacterium, colonize plant root tips, by forming biofilm-like structures and moreover protecting the plants against infectious pathogens [22]. In an experiment on *Arabidopsis thaliana*, *Bacillus subtilis* strain (ATCC 6051) formed biofilm-like structures on the roots and protected *Arabidopsis* from infection of *Pseudomonas syringae* [23].

6. Role of PGPRs in biofilm formation

Plant growth promoting rhizobacteria (PGPR) is extensively present in the rhizosphere. They enhance root growth and provides opportunities to the plant in getting maximum nutrients for their better growth. Along with growth promotion, PGPRs have a number of functions such as stress tolerance (heat, drought, salinity and disease), bioremediation of heavy metals, biodegradation of complex toxic organic compounds, biocontrol agents and biofilm formation. Mainly root-associated *Pseudomonas* sp. are used as biocontrol agents and have a major contribution in a highly dense biofilm formation that is why *Pseudomonas* is used as a model organism in biofilm studies [21]. *Pseudomonas putida* have the capability to respond rapidly in the presence of root exudates in rhizosphere and colonizes on root surface to form biofilm [22]. Along the epidermal fissures, plant-growth-promoting Pseudomonas discontinuously colonize to establish small biofilms and large biofilm on the root surface [23]. Besides that, some pathogenic *Pseudomonas* sp. also form biofilms on root surface [24, 25]. *Pseudomonas* sp. form very diverse, multicellular clusters and too extensive biofilm networks. In maize, wheat and cereals, *Azospirillum brasilense* and other *Azospirillum* sp. as plant growth promoters are present, which form biofilm on the root surface [26]. By nature *Azospirillum brasilense* is a free living nitrogen fixer that promotes the plant growth and stimulates root proliferation rather than providing the ready-made nitrogen to the plant. Bacteria form dense biofilm on the root zone by colonization [27]. Various *Agrobacterium* sp. and genera of symbiotic rhizobia induce nodulation in the roots along with effective colonization in roots. In case of Rhizobia, it

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stimulates infection of the root hairs, curling and nodulation on an appropriate host plant [28, 29]. On observing the rhziobial cells under microscope within curled root hairs, very small biofilm-type aggregates are present, which provide inocula for root invasion where they penetrate to the root interior and forms threads as filament like structure [30]. Similarly *Agrobacterium tumefaciens* form structurally dense and complex biofilms on the surface of root extensively on the epidermis and root hairs [31, 32]. Moreover Gram positive bacteria effectively colonize the rhizoplane [33]. Besides that microbes involved in the biocontrol, for instance *Bacillus cereus* also establish dense surface associated population which leads to the formation of biofilm [34]. Several functions known to influence biocontrol activity are also likely to play a role in biofilm formation [35].

7. Remediation methods against pathogens

The Myxobacteria are often come across in soil habitats [36, 37]. Their highly structured biofilms and surface-gliding social behaviour, along with their ability to kill competing microbes, have attracted significant interest in cell-cell interacting molecules [37]. Previously it is documented that metabolites, including enzymes, antibiotics and volatiles produced by soil-plant-associated bacteria, are crucial factors in the suppression of plant pathogens [38-40]. The bacteria identified to date for the potential of biocontrol activity represent diverse genera, including *Agrobacterium, Burkholderia*, *Collimonas*, *Pseudomonas*, *Stenotrophomonas*, *Arthrobacter*, *Bacillus*, *Streptomyces*, *Azotobacter*, *Serratia* and *Pantoea*. Antibiotics produced bacteria for biocontrol action exhibit broad-spectrum activity. For instance, pyrrolnitrin produced by *Pseudomonas* and *Burkholderia* species has activity against basidiomycetes, ascomycetes, deuteromycetes, plant pathogens *Botrytis cinerea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahlia*.

Among the biocontrol bacteria, *Pseudomonas* and *Bacillus* are the most widely studied genera. The bacilli are the most successful commercially available biocontrol agents which can form the biofilm [41]. After the initial discovery of the insecticidal activities of the Cry proteins produced by *Bacillus thuringiensis*, a number of other species, including *Bacillus subtilis*, *Bacillus pumilis*, *Bacillus amyloliquefaciens* and *Bacillus licheniformis*, are now exploited for plant growth promotion and biological control of plant diseases. Analysis of the genome sequence of the biocontrol strain *Bacillus amyloliquefaciens* FZB42 revealed that approximately 8.5% is dedicated to secondary metabolism [42]. Similarly, in *Bacillus subtilis* approximately 4% of the genome is dedicated to secondary metabolite production [43].

8. Molecular mechanism of biofilm formation and biocontrol

Two matrix gene operons: *tapA-sipW-tasA* and *epsA-O*, are involved in the formation of biofilm. They are responsible for the synthesis of two major matrix components: amyloid-like fibres and an exopolysaccharide [44, 45]. The two matrix operons are directly controlled by a repressor SinR [46]. De-repression is triggered by SinI, an anti-repressor whose gene is activated by phosphorylated Spo0A (Spo0A~P) [47, 48]. Spo0A~P is a master regulatory protein and of central importance for biofilm formation. Phosphorylation of Spo0A is controlled by a network of kinases and phosphatases in response to various environmental signals [49, 50]. Spo0A~P also represses the gene for AbrB, a repressor that contributes to repression of the matrix operons (Fig. 2) [51]. Under as yet poorly defined conditions, matrix gene expression is alternately turned on by a Spo0A~P-independent pathway consisting of TetR, and YwcC-type repressor, and SlrA, a paralogue of SinI whose gene is repressed by YwcC. SlrA contributes to biofilm formation by antagonizing SinR and thereby de-repressing matrix genes.

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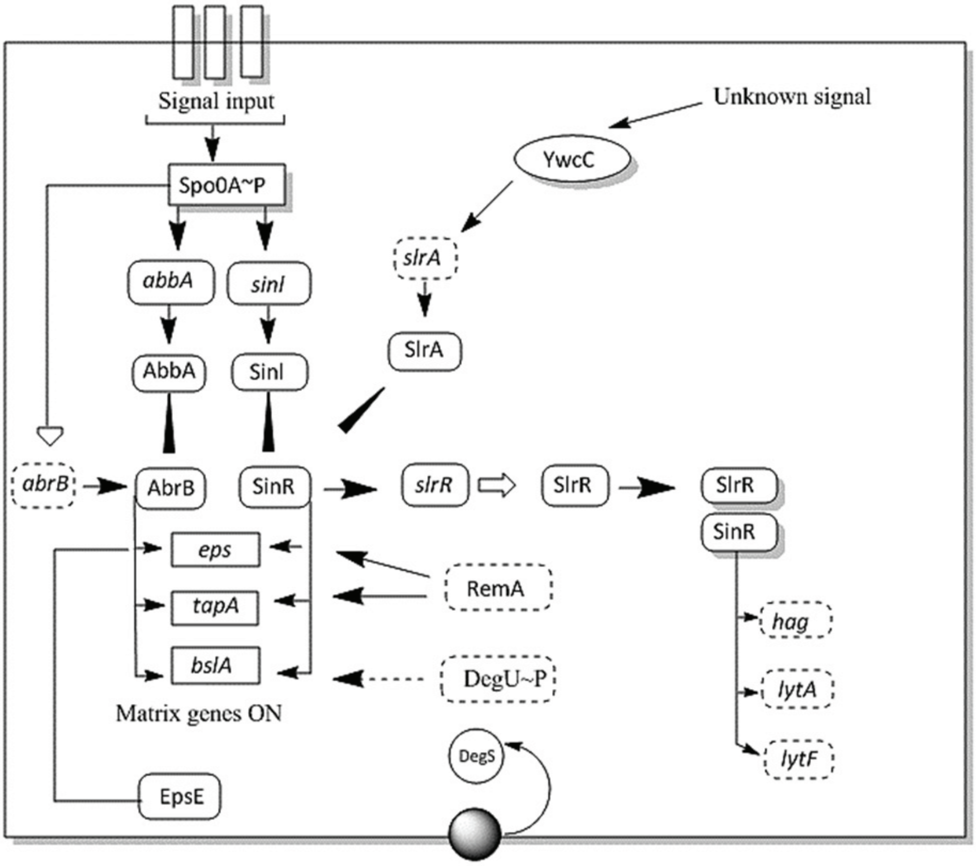

Fig. 2 Regulatory network governing biofilm formation. Schematic diagram of the complex regulatory pathways that control gene transcription during growth as a biofilm. It includes boxes indicating proteins, open reading frames (ORFs), arrows indicate activation, and triangles show repression. Moreover indirect activation and repression, active gene transcription, translation, absence of gene transcription, transcriptional repressor and protein–protein interaction is shown. Here protein that is able to bind to DNA to activate transcription, flagellum with the curved arrow indicating rotation and the cross indicating inhibition of flagella rotation. Vertical rectangles labelled with "signal input" indicate sensor kinases for the Spo0A pathway.

9. Genetics involved in biofilm formation and bio-control

The extensive interest in *Pseudomonas* sp. is due, in part, to the production of a variety of bioactive compounds. Over the past 30 years, specific emphasis has been given to the role of siderophores, hydrogen cyanide, 2,4-DAPG, pyrrolnitrin, pyoluteorin, phenazines, 2,5-dialkylresorcinol, quinolones, gluconic acid, rhamnolipids, and various LP antibiotics in biological control of plant pathogens [52-54]. The first genome sequence of the biocontrol agent *Pseudomonas fluorescens* strain Pf-5 appeared in 2005 [55] and since then numerous other *Pseudomonas* sp. and strains have been sequenced. Genome sizes range from 4.6 Mb for *Pseudomonas stutzeri* strain A1501 to 7.1 Mb for *Pseudomonas fluorescens* strain Pf-5 [56]. By mining these genome sequences, a number of novel gene clusters and metabolites have been discovered, including the LPs orfamide [57], viscosin [58], syringafactin [59] and entolysin [60] as well as the rhizoxins [61] and the insecticidal Fit toxins [62].

The genes responsible for the production of EPS are part of the *epsA-O* gene operon (*eps*) [63, 64]. The molecular structure of the EPS is yet to be elucidated, however, it is known that *eps*-defective mutants developed flat colonies and extremely fragile pellicles. These mutants are still able to grow in cell chains and contain remaining extracellular material due to the presence of additional matrix components of a protein nature. Two secreted proteins provide structural integrity to the matrix. These are *TasA* and *TapA*, which are encoded by the three-gene operon *tapA-sipW-tasA* (*tapA* operon). *TasA* is a functional amyloid protein [65] which is secreted into the extracellular space with the help of *SipW*, where it self-assembles into fibers that are anchored to the cell wall by *TapA* [66].

Bacillus subtilis has an average of 4–5% of its genome devoted to antibiotic synthesis and has the potential to produce more than two dozen of structurally diverse antimicrobial compounds [67]. In some studies, cyclic lipopeptides such as surfactin, iturin and fengycin, as one class of antibiotics, have been reported to be involved in the biocontrol of plant diseases [68, 69]. Strong antagonistic activity in vitro is considered critical in selecting for *B. subtilis* strain as biocontrol agents against *R. solanacearum*.

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10. Case study of biofilm

Bacillus subtilis is traditionally known as a soil-living organism and preferentially remain in association with plant roots [70]. It has been recently shown that *B. subtilis* colonizes plant roots, as well as plant leaves in a biofilm-dependent manner and the presence of the biofilms increases protection of the plants form a variety of pathogenic insults [71, 72] The regulatory and structural components of the biofilms revealed in laboratories have proven their importance for the plant-associated biofilm development. In a recent study, another information was revealed that robust biofilm forming ability on plant-roots relate with disease development and protection. Moreover, a blessing is also observed that plants also favour the formation of biofilm and favours microbial colonization by releasing special type of root exudates [73].

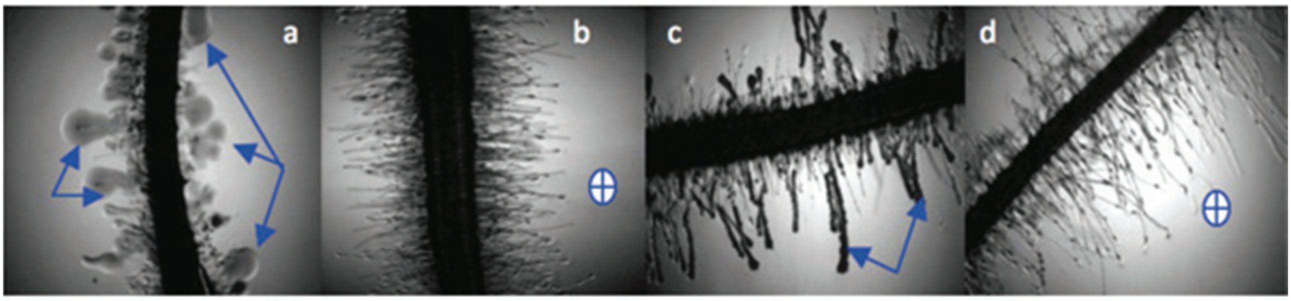

Fig. 3 Root colonization by strain S499 of *Bacillus subtilis*; it is microscopy visualization of root of (a) treated salad; (b) untreated salad; (c) treated tomato and (d) untreated tomato. The arrows in (a) and (c) indicate absence of biofilm formation by the strain on the root system. Picture: courtesy of V. Nihorimbere

11. Recent advances in biofilm formation and its role against agricultural pathogens

Recently biofilm formation in the rhizosphere has been a key interest for the scientists and researchers. They are keen to know about the battle going on in the rhizosphere among microbes in the presence of plant root exudates. PGPRs are reported to be the plant growth promoting bacteria, but it's not all about the PGPRs. They work beyond this and provide numerous benefits to the plants. They participate in the release of antagonistic metabolites involved in the inhibition of plant pathogens directly [74, 75]. It is known as the process of antibiosis i.e. microbial growth inhibition by diffusible antibiotics and volatile organic compounds (VOCs), toxins and biosurfactants. Besides that parasitism may involve in production of extracellular cell wall-degrading enzymes known as chitinases and β-1,3-glucanase [76]. The degradation of pathogenicity factors of the pathogen such as toxins by the organisms which are beneficial for plants have also been reported as a protective mechanism [77]. To demonstrate the role of antibiotics in biocontrol, mutants impaired in biosynthesis or over-producing mutants have been used together with, in some cases, the use of reporter genes or probes to show efficient production of the compound in the rhizosphere. As an example, *Bacillus subtilis* strains produce a variety of powerful antifungal metabolites, e.g., kanosamine, lipopeptides and zwittermicin-A, from the fengycin, iturin and surfactin families [78-80] showed that overproduction of extracellular protease in the mutant strains of *Stenotrophomonas maltophilia* W81 results in improved biocontrol of *Pythium ultimum*. Excretion of glucanases and chitinases by species of *Streptomyces* and *Trichoderma* has also been shown to play an important role in mycoparasitism [81].

Another way of dealing with the opponents is doing a healthy competition. Soil microbes, especially PGPRs compete in rhizosphere for the food, water, air and space. Competition for resources generally in soil between soil-inhabiting organisms are of key interest for the plant security and health. For biocontrol purpose, it occurs when the antagonist directly competes with pathogens for these resources. On the root, microorganisms compete with each other for suitable sites at the root surfaces so they can easily colonize and develop a symbiotic relationship. Competition for nutrients, especially for food source carbon, is assumed to be responsible for the well-known phenomenon of fungistasis characterizing the inhibition of fungal spore germination in the soil [82].

12. Concluding remarks

Positivity and negativity go side by side. Similarly beneficial and pathogenic bacteria remain present in the rhizosphere to combat. It is the battle field for microbes to win their goal of either plant growth promotion or affect the health of plant by inducing plant diseases. To fulfil the future demand of a growing population for food, healthy and high yielding plants are the requirement. Use of microbes in the form of biofertilizer for plant growth promotion and biocontrol activity, is a hope for the food security issue. To combat with pathogenic microbes, concept of biofilm formation by the PGPRs has great potential in it. They can communicate with each other by quorum sensing and can confuse the pathogenic bacteria. *Bacillus subtilius* case study showed remarkable results in biocontrol. Here is a dire need to explore other microorganisms with the capability of biofilm formation to participate in biocontrol activities and ensure the healthy life of plants and the nation. Moreover mechanism behind biofilm formation and its contribution in biocontrol should be addressed optimistically.

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