Plant root associated biofilms: perspectives for natural product mining

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Introduction

For many years microbes in nature have been viewed as simple life forms growing as individual cells. This has enabled the characterization of the microorganisms. Most of our understanding of microbiology originates from experiments in liquid culture- free living bacteria. However, planktonic growth is not the natural situation for microorganisms and care needs to be taken then to interpret these results in their natural state. During the last decades an intensive research has been conducted in the area of biofilms: medical-industrial and plant associated biofilms. Usually biofilms are defined as complex microbial communities attached to the surface or interface enclosed in an extracellular matrix of microbial and host origin to produce a spatially organized three dimensional structure (9). It should also be noted that phenotypic variation in the biofilm forming bacteria is included (5, 36, 40, 41). Genotypically identical biofilm bacteria are inherently different from the planktonic bacteria. Individual cells within a population control their gene expression to ensure that regulation of cell differentiation will occur (41, 58). There are complete reviews in the literature covering biofilm biology and genetics (5, 21, 28, 35, 39, 40, 50, 57, 89, 97). Biofilm is a normal common existence in bacterial ecosystems. Within the biofilms bacteria have cooperative behavior and they may be susceptible to harsh environmental conditions. It is the preferred state of existence because bacterial community adds defenses and multiple mechanism of bacterial survival and enhances its fitness. Microorganisms also gain access to resources and niches that require critical mass and cannot effectively be utilized by isolated cells. Acquisition of new genetic traits, nutrient availability and metabolic cooperation have also been suggested as means for optimization of population survival in biofilms (2, 36, 40, 41, 49).

In several areas of medical and industrial biofilms, the microorganisms have relatively little to do with the surface quality. In the area of plant associated microorganisms it is generally accepted that plant roots live in firm teamwork with the surrounding microorganisms forming a unique self-regulating complex system (15, 71). Microorganisms are not only the most abundant organisms in natural systems, but are also key players in ecological processes.

Among other plant-associated bacteria, the aerobic endospore-forming bacteria, mainly those belonging to *Bacillus* and related genera, are ubiquitous in agricultural systems due to their multilayer cell wall structure, ability to form stress resistant endospores and to produce a wide variety of antibiotic substances. Exploiting these abilities, the bacteria can inhabit diverse niches in agro-ecosystems and outcompete other microorganisms on the plant root. Therefore, the colonization niches for the bacteria are more reproducibly stable and these bacteria are likely to be used in precision management of agro-ecosystems. For example, it was shown that an endospore forming species *Paenibacillus polymyxa* colonizes as biofilms the regions around root tips (78) (Fig 1). The bacterial biofilms can protect plants against pathogens as well as against abiotic stress conditions (24, 80, 81).

In this review we highlight themes regarding the nature and diversity of the bacterial biofilms and elucidate their potential as a rich source of novel biologically active compounds. The underground resources of plant rhizosphere could provide insights associated with global climate change. So far these resources have been neglected to large extent but hopefully with the help of new technologies we will be able to understand and employ the natural potential of biofilms for our agro-ecosystems.

Structure

Biofilms formation is a dynamic sequence of events that has been carefully studied in *Vibrio cholerae* in Kolter's laboratory (89, 90). Four general biofilm formation stages have been described. The first stage is initiated as an attachment stage. Here bacteria grow as planktonic cells and approach the surface so closely that motility is slowed as a result. The bacterium may form then a transient association with the surface and with other microbes that previously attached to the surface. The transient association refers to the search for a place to settle and is followed by a stable association. Stage two includes binding to the surface resulting in monolayer formation. After adhering to the surface the bacteria begin to multiply while emitting chemical signals that inter-communicate between bacterial cells and root. Once the signal intensity exceeds a certain level the genetic mechanisms underlying extracellular matrix production are activated. During this stage the cell motility is decreased and microcolonies are formed (58, 59, 64). The cell layers are progressively added by extracellular matrix production (4, 5, 51), and the biofilm three dimensional structure is

formed. Finally, the bacteria eventually return to the planktonic stage (89). Recently, a number of studies described the vast diversity in biofilm structure (34). Are there any principals of general nature? One feature that seems to apply to biofilms is that they all seem to create matrix. What is inside a matrix? An extracellular matrix can provide an almost infinite range of macromolecules. It was suggested that in the model bacterium *Bacillus subtlis* polysaccharides and a protein Tas A are the major components of its biofilm. Mutations that eliminate Tas A and extracellular polysaccharides (EPS) production have a severe effect on biofilm production (4, 34). The sugars in biofilms can be divided into simple sugars (monosaccarides, oligosaccharides, polysaccharides), and complex sugars: all of which can play various roles in host microbe interactions (39, 86). Water retention varies with the type of polysaccharides but EPS water retention capacity may exceed 70 g water per g polysaccharide (6, 74, 86, 99). Our experiments show that bacteria can engineer their own microenvironment in a form of porous EPS mixed soil particles. The environment immediately interacts with plant root providing buffered and predictable hydration and transport properties (Fig 4, Timmusk manuscript in preparation). The EPS producing *Paenibacillus* sp. strains significantly increased soil aggregation in comparison to the null mutants of the strains (Timmusk manuscript in preparation). The EPS may also contribute to mechanical stability of the biofilm and interact with other macromolecules and low molecular mass solutes, providing a multitude of microenvironments within the biofilm (86). Currently many of these effects can only be speculated. Due to their abundance in nature it is tempting to suggest polysaccharides as the vehicle for biofilm manipulation.The diverse structural variations of EPS produced by bacteria of different taxonomic lineages makes the task hardly realistic.

Signaling

Quorum sensing (QS) is a well-known relatively conserved general communication mechanism. Since the initial discovery of Davies et al (1998) the QS involvement in biofilm formation has been shown in variety of species. The cell to cell communication in this process is based on utilization signal molecules-the messengers that transform information across the space. QS is regulation of gene expression in response to cell population density. Gram

positive and gram negative bacteria use QS to regulate diverse physiological activities. It has been shown that such activity occurs both inside and between the species. In general gram negative bacteria use homoserine lactones and gram positive bacteria use small peptides. QS nature and potential applications are reviewed (7, 14, 16, 77). Kevin Foster and colleagues (51) recently published a study examining the evolution of QS within biofilms. They illustrated how in the process of gaining fitness some bacterial species activate EPS production, whereas other species repress EPS synthesis upon QS activation.

There is growing evidence that in addition to the well documented quorum sensing systems other molecules act as signal molecules (66). Initially it was shown by the Davies group that the subinhibitory concentration of various antibiotics may function as signals (94). Surprisingly, these small molecules have the activity to modulate global gene transcription. There are bacteria in plant rhizospheres that produce the antibiotics in concentrations that are capable of killing other microbial cells. However, most attempts to detect the high antibiotic concentrations produced under natural conditions have limited success. Hence, besides being weapons fighting against competitors they are also considered signaling molecules that regulate the homeostasis of microbial communities. Strangely enough it was shown that some antibiotics at low concentrations may even be beneficial to the bacteria in natural environments (13, 17, 23, 38, 47, 48, 69, 94, 95). If the antibiotics are handled as signaling compounds it gives also a totally new view to antibiotic resistance in the natural systems. In this case antibiotic resistance may serve as protection against new signals in environment in order to maintain the biofilm community (13, 94, 95). Beside antibiotics several other secondary metabolites are known to be involved in microbial signaling (66).

The environmental signals such as e.g. nutrient sources, local PH, temperature, and oxygen surface properties evoke changes in biofilms in order to be able to gain optimal nutrition and colonize the environment efficiently (12, 50). As mentioned above, biofilm formation has four steps surface attachment, micro colony formation, maturation and architecture formation. The initial steps attachment and microcolony formation are regulated by the signals that differ from bacteria to bacteria and reflect the natural habitat. The steps that follow are relatively more conserved and mainly reveal the physiology of cells inside the biofilm (72). It was shown in Kolter's laboratory that bacteria initiate biofilm formation through different

pathways depending on environmental conditions (58). Hence the bacterial strain can achieve biofilm phenotype under different conditions through different mechanisms (64). Studies on wild barley *Hordeum spontaneum* biofilms show that different types of biofilms are formed on the root tips from the 'Evolution Canyons' 'African' and 'European' slopes (Fig 4) (detailed below) (79). Since bacteria cannot escape stressful environmental conditions, their sensitive mechanisms must be evolved to allow the rapid perception of stress and homeostasis maintenance. This adds more dimensions to the complexity of biofilms and draws our attention to the necessity to study biofilms under contrasting environmental conditions e.g. stress and non-stress environments.

"Evolution Canyon"

Insights into microbial biofilms biological and evolutionary significance necessitates the study of coevolution with the host plant, ideally under contrasting environmental stresses. The 'Evolution Canyon' (EC) model (Fig 2) is a natural laboratory focusing on the study of the evolution of biodiversity and adaptation at a microsite. The project is navigated by the Institute of Evolution at the Haifa University in Israel. The model present sharp interslope ecological contrasts caused by interslope microclimate divergence (61). Both the geology and macro-climate are similar for both slopes. Since the canyon runs east-west, the canyon slopes display opposite orientations. The south- facing "African" slope, AS or SFS, receives 200-800% more solar radiation than the north- facing "European" slope, ES or NFS. Consequently, the savannoid AS is warmer and drier and more drought- stressed than the cooler and more humid, ES. The opposite slopes are separated at bottom by 100 m and at top by 400 m, averaging 200m (54).

The EC model reveals evolution in action across life at a microscale involving biodiversity divergence, adaptation and incipient sympatric ecological speciation (54-57). The model highlights diverse taxa species richness, genomics, proteomics and phenomics phenomena by exploring genetic polymorphisms at protein and DNA levels. Four EC's are currently being investigated in Israel in the Carmel, Galilee, Negev, and Golan Mountains (EC I-IV), respectively. We identified 2,500 species in ECI (Carmel) from bacteria to mammals in an

area of 7,000 m^2. Local biodiversity patterns parallel global patterns (54). Higher terrestrial species richness was found on the AS. Aquatic species richness prevails on the ES. In 9 out of 14 (64%) model organisms across life, we identified a significantly higher genetic polymorphism on the more drought-stressful AS (55). Likewise, in some model taxa, we found largely higher levels of mutation rates, gene conversion, recombination, DNA repair, genome size, small sequence repeats (SSRs), single nucleotide polymorphism (SNPs), retrotransposons, transposons and candidate gene diversity on the more stressful AS. Remarkably, interslope incipient sympatric ecological speciation was found across life from bacteria to mammals. The EC model could potentially highlight many mysteries of evolutionary biology, including the genetic basis of adaptation and speciation, especially now with the rapid high-throughput techniques of whole genome analysis (29, 52-55).

Among other model organisms wild progenitors of cereals emmer wheat (*Triticum dicoccoides*) and wild barley (*Hordeum spontaneum*) have been studied at the 'EC' for more than 30 years. The work has produced more than 200 publications (see the full list at http://enevo.haifa.ac.il and at http://evolution.haifa.ac.il) and the book, 'Evolution of Wild Emmer and Wheat Improvement' (56). This book contains interdisciplinary studies on the ecological, genetic, genomic, agronomic, and evolutionary aspects of wild emmer, conducted at the Institute of Evolution from 1980 to 2002. Wild emmer and wild barley are the progenitors of most cultivated wheat and barley and thus are important sources of wheat and barley improvement. It is known that plants have co-evolved together with biofilm-forming rhizobacteria over millennia. It is not clear, however, whether the modern cropping systems have retained all the beneficial components that are present in the naturally coevolved systems. *Paenibacillus polymyxa* as a representative of the wild progenitors rhizobacteria has been thoroughly studied. This bacterium is capable of imparting resistance to pathogens and improve drought tolerance (81). A model system to study and compare the bacterial biofilm formation in soil was developed (78). To investigate bacterial interactions in natural systems real-time PCR for the biofilm forming bacterial rapid detection was also developed (82). *P. polymyxa* antagonism studies in interaction with agricultural plants against different pathogens e.g. *Aspergillus niger*, *Pythium and Phytophthora spp.* highlighted the importance of biofilms in biocontrol initiation (24) (Fig 3).

Biofilm formation is a complex phenomenon and is affected by physicochemical environment. For example, nutrient resources, attachment efficiency, cyclic stage of the bacteria are factors that affect crosstalk between bacteria and plant roots (3). Using scanning electron microscopy (SEM) it was shown that wild barley seedlings from AS and ES have different types of biofilms formed around their root tips (Fig 4). Both AS and ES biofilms are formed mainly by rod-shaped bacilli. Significantly more EPS containing biofilm is formed on the stressful AS (Fig 4, Timmusk manuscript in preparation). The EPS role in protection against desiccation was shown by Tamaru et al (75). Their results confirm that EPS directly contributes to desiccation resistance enhancement. Bacteria from the biofilm forming regions of both slopes were isolated and screened for their metabolic properties (79). The drought-stressful AS slope contains significantly higher population of 1-aminocyclopropane-1-carboxylate deaminase (ACCd) producing, phosphorus solubilizing, osmotic stress tolerant bacteria (79). The features are likely to have provided a selective advantage for the plant-bacterial biofilm complex survival, and the bacteria may have helped the plant to tolerate various stresses using one or more of those mechanisms. These results suggest that bacterial biofilms on the plant root behave much like a multicellular organism. They excrete the 'matrix' to provide a buffer against the environment and hold themselves in place. Whatever is produced inside the biofilm has a suitable environment and higher probability to get through to the target. This indicates that the rhizosphere bacteria, together with the plant roots at the AS wild barley rhizosphere, might function as communities with elevated complexity and plasticity which, in aggregate, have afforded the plant the adaptability to the harsh conditions encountered. The bacteria that coevolved with their hosts, over millennia, are likely to control, to a large extent, plant adaptation to the environment and have a huge potential for application in our agricultural systems enhancing plant stress tolerance.

New perspectives

Biofilm research is currently one of the most topical research issues of molecular microbial ecology. First, it is expected that an improved understanding of the bacterial behavior will lead to develop agents that control the biology of biofilms. Secondly, biofilms are a rich source for novel natural products. Natural products are chemical compounds that usually

exhibit biological activity and are presumed to have an ecological function. The compounds underwent an evolutionary process during which they were optimized for specific purposes. One of the most promising resources for new drugs, signaling compounds and plant growth promoting substances are biofilm secondary metabolites (SM) (87). There are millions of these compounds produced in the microbial world and several of them successfully applied. The biosynthetic pathways of secondary metabolites are rather complex (68).

The two most common classes of SMs are the nonribosomal peptides (NRP) and the polyketides (PK) (33, 46, 93, 98). PK synthetases (PKS) and NRP synthetases (NRPS) are both multienzyme multimodular biocatalysts containing numerous enzymatic domains organized into functional units (62, 63, 91, 92). The vast structural diversity is due to a wide range of available substrates compared to 20 amino acids available for ribosomal synthesis. There are over 300 different amino, hydroxy or carboxy acid substrates that have been identified in nonribosomal peptide compounds (32). Additionally NRP compounds also include fatty acid chains, macrocyclic and heterocyclic rings. NRP usually contains between 2 to 20 amino acids. However, exceptionally the longest NRP known so far contains 48 AA (25). The evolution of nonribosomal expression systems has allowed evolving the peptide based compounds with relatively low ATP cost. It is suggested to be sixfold lower in cost than the consumption for ribosomal synthesis where ATP is required for aminoacyl-tRNA sysnthesis proofreading, elongation and translation (30, 31). Both PKS and NRPS contain conserved domains. These domains are used in the overall assembly process. Three types of domains adenylation (A) thiolation (T) and condensation (C) domains are essential for the compound synthesis. A domain activates the corresponding AA as aminoacy-adenylates are subsequently transferred to 4-phospho-pantheinyl cofactors attached to downstream T-domains. During the stepwise elongation formation of the peptide bond between two adjacent aminoacyl intermediates bound to T domain is carried out by the intervening C domain. In some cases there is a additional Epimerisation (E) domain which catalyses the racemization of activator L amino acid to D amino acid.

How does one identify the compounds and correspondents in complex mixtures of microbes? The conserved domains have been valuable in predicting the metabolites into the structurally difficult to characterize PKS and NRPS groups. Usually the cosmid libraries from the

microbial isolates are constructed, the libraries are screened with radioactive, degenerate DNA probes or PCR primers, which target conserved regions of PKS or NRPS gene clusters. Then chromosome walking is used from identified genes to retrieve the sequence of the entire gene. Gene knockouts coupled with comparative metabolic profiling of wild type and mutant strains are then used tool to identify the actual products (96).Yet it is also known that there is an heterologous expression of the single biosynthetic genes. This can be found out by Northern blotting, DNA microarray analysis or RT-PCR. The pleiotropic SM regulator manipulation at the cellular level is a good strategy to find and activate the silent cryptic pathways.

Taking into account that 99% of the microorganisms from most environments on earth cannot be grown under laboratory conditions DNA based technologies should also be applied in the process of compound isolation and identification. Microbe and community genome sequences have revealed many genes and gene clusters encoding compounds similar to the ones known to be involved in the biosynthesis of biologically active compounds (8) (Fig 5). Often the gene clusters represent biosynthesis of novel natural products. Significant advances have been made in the past 20 years through the application of metagenomics also referred to as environmental and community genomics. Metagenomics is the genomic analysis of microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms (26). Comprehensive reviews have been written on the area (18, 19, 22, 37, 65, 67, 68, 76, 83, 88) It became apparent that metagenomic approach could allow the isolation of genes encoding novel compounds from any environment (11, 35, 42). It was proposed that if the gene clusters could be expressed in heterologous hosts it would provide a direct route to the production of bioactive compounds. Hence it was hoped that characterization of the communication networks and the natural roles of secondary metabolites was an available task. Even though several of the initial efforts encountered shortage of suitable techniques and tools for the natural product discovery it was a necessary platform to reach the current stage. Nowadays, protocols have been developed to capture unexplored microbial diversity to overcome the existing barriers in estimation of diversity. New screening methods have been designed to select specific functional genes within metagenomic libraries to detect novel biocatalysts as well as other bioactive molecules (68). To study the complete gene or operon clusters, various vectors including cosmid, fosmid or

bacterial artificial chromosomes are being developed (76). Bioinformatics tools and databases have added enormously to the study of microbial diversity (67).

If the compound is identified and isolated then atomic force microscopy (AFM) can be used as a tool to study its production and performance under complex microbial associations. The earlier works mainly focused on gaining morphological and topographic information of the biofilm surface (73). The components of biofilm forming bacterial metabolism can be visualized in real time assays. One way to do it is immobilization of molecules at AFM probes. The AFM cantilever tips can then measure breakaway forces between biomolecules. With the specific antibodies on the cantilevers researchers have measured antibody- antigen interactions and at the same time imagined their target antigens (27). The molecular recognition force (27) is applicable to study the biomolecule localization and function on the surface of biofilms. Single molecule studies have elucidated the important parameters of microbial protein folding and rupture. For example, the AFM imaging and force measurements studies have been performed on surface polysaccharides of *Lactobacillus* sp. Lecithin modified tips were used to study individual polysaccharides molecules on the surface of biofilms (20). In order to understand their function in biofilms polysaccharides were characterized with single molecule force spectroscopy (70). Glucans were characterized on the *Streptococcus mutans* biofilms and their possible role in substrate day biofilms was studied (10). The study was conducted with various mutants which ability to synthesize glucans was affected. The technique also provides the possibility for microbial surface molecular recognition using specific binding such as antibody antigen interaction. Employing AFM it is possible to study properties of attachment to the surfaces under natural conditions. The studies of pathogens were performed and structural details of Hif-typ pili at the early stage of biofilm were described (1). Force measurements of chemically fixed planktonic cells and native biofilm cells showed major difference in physical properties such as elasticity and adhesion (84, 85). It has been also shown that biofilm formation is strongly dependent on the characteristics of substrate material (60). AFM was used to image ate *Bdellovibrio bacteriovorus* attack on *E. coli* biofilms. The morphological changes in nanoscale of *E.coli* cells were monitored while attacked by the predator (57). AFM studies are even more efficient when combined with other methods. As such AFM can’t produce information about the chemical composition of the biofilm under the surface. Hence it can be used in

combination of florescent and confocal microscopy (43, 44). Raman spectroscopy would also facilitate to identify the materials. It uses a nondestructive laser to identify the components peaks of the Raman spectra (45).

In sum, we are just beginning to understand the complexity and potential of biofilms. Yet it is already clear that much is to be gained from studying this area. Intelligent biofilm engineering will be crucial in meeting the needs of handling the biofilms in agro-ecological systems. The contrasting environmental study locations where plants have coevolved with microbial representatives under stress over long period of time such as the contrasting opposite slopes of "Evolution Canyon" (AS and ES) are especially good source for microbial representatives in order to study the biofilm structure, properties as well as production and composition biologically active compounds.

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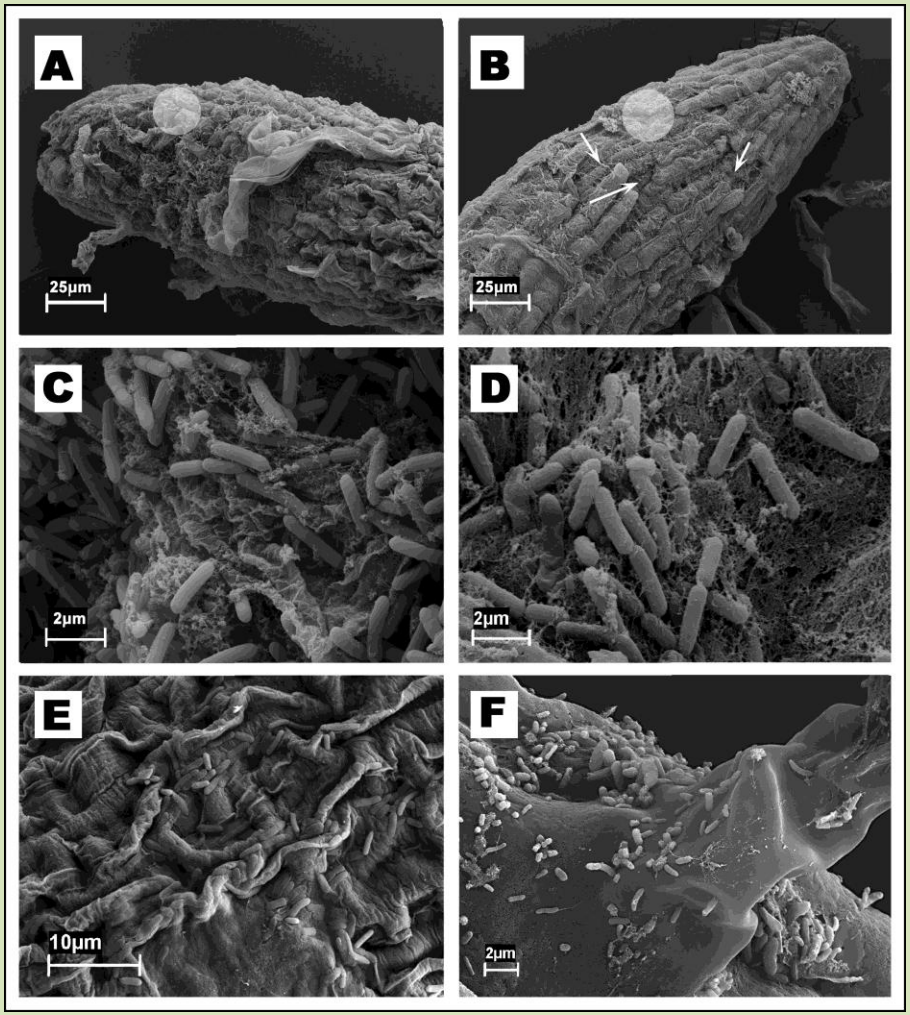

***Figure 1*. Scanning electron microscopy micrographs of plant roots colonized by *Paenibacillus polymyxa*.** *P. polymyxa* B1 colonization and biofilm formation on *plant* roots in the gnotobiotic system (A, C, E), and in soil assays after one week of colonization (B, D, F). Roots were prepared and analyzed as described in Timmusk et al 2005. Images were taken from the root tips (A, B, C and D) and from tip-distal regions (E and F). Note the biofilm formation on root tips (A, B, C, D). Much fewer bacteria colonize the regions behind root tip (E, F). In the non-sterile system only *P. polymyxa* was present at the biofilm-covered regions (D), whereas *P. polymyxa* cells mixed with indigenous bacteria were found on the distant regions of the plant root (F).

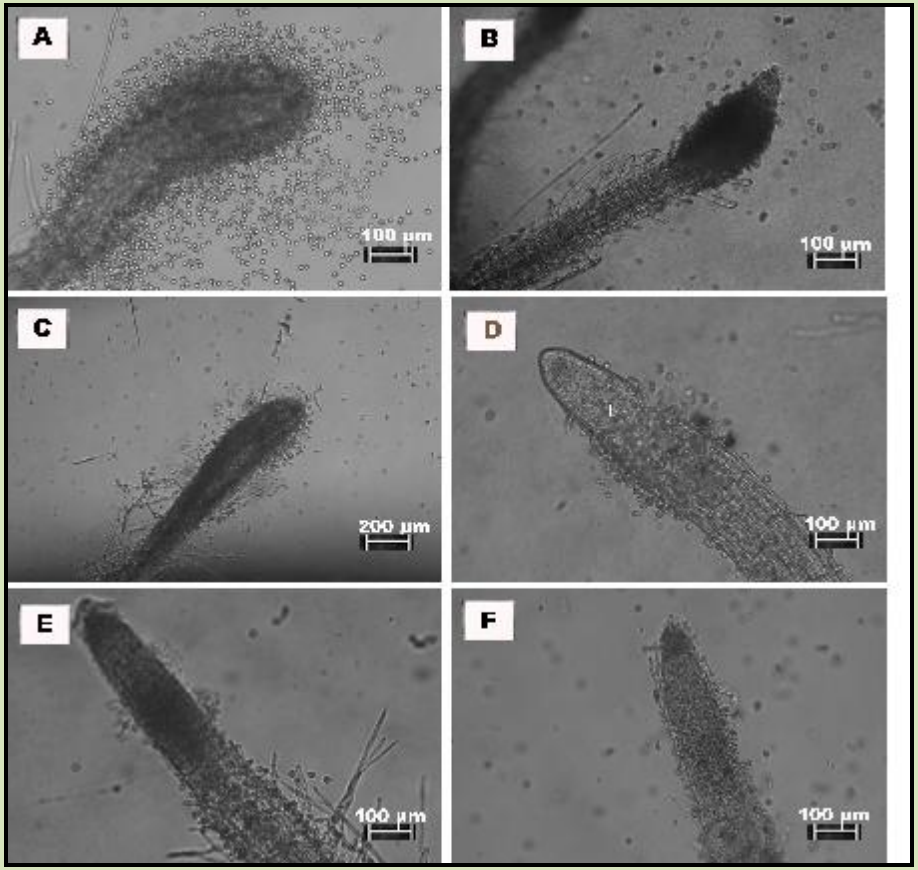

Figure 2. **Inhibitory effect of *Paenibacillus polymyxa* biofilm formation to *Pythium aphanidermatum* and *Phytophthora palmivora* root colonization**

Arabidopsis thaliana seedlings were grown and inoculated with the *P. polymyxa* and pathogens as described in Timmusk et al 2009. The pattern of *P. aphanidermatum* (A) *and P. palmivora* (B) zoospore colonization on plant root is affected by *P. polymyxa* pre-inoculation (C to F). *P. polymyxa* relatively poor biofilm forming strain caused somewhat reduced *P. aphanidermatum* (C) *and P. palmivora* (D) zoospore colonization. Efficient biofilm forming *P. polymyxa* strains pretreated sample showed significantly less *P. aphanidermatum* (typical example on E) *and P. palmivora* (F) zoospore colonization.

Figure 3. **Cross section of the 'Evolution Canyon' indicating the collection sites on 'African Slope' (AS) 1 and 2 and 'European Slope' (ES) 5 and 7**

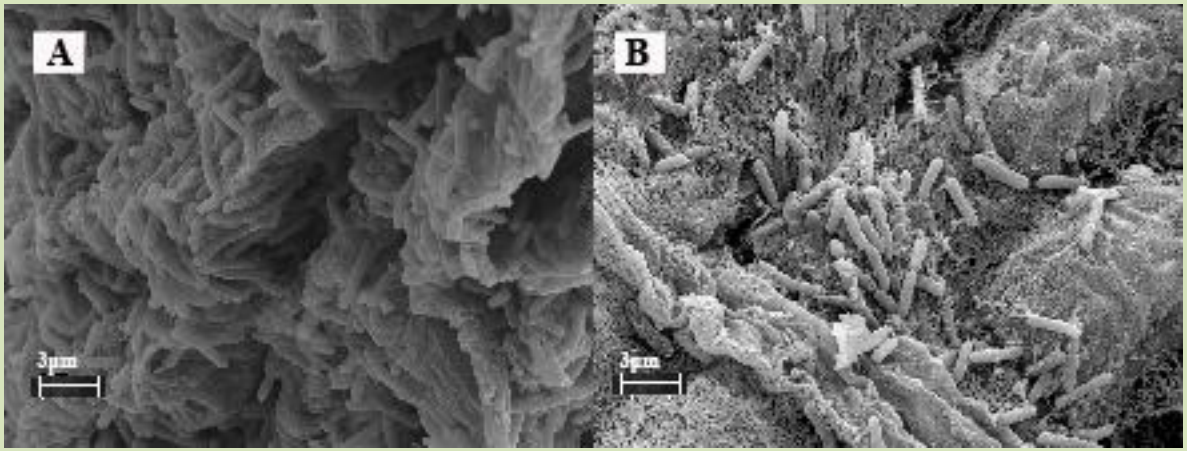

***Figure 4*. Scanning electron microscopy micrographs of wild barley *Hordeum spontaneum* roots colonized by biofilm forming bacteria**

Typical pattern of bacterial biofilm formation on wild barley root tips at AS (A) and ES (B).
Wild barley plants were sampled, prepared and analyzed as described in Timmusk et al 2009,
Note that wild barley root tips at AS (A) are well colonized with mainly rod-shaped biofilm forming bacilli. Significantly less biofilm is formed on ES wild barley root tips (B).

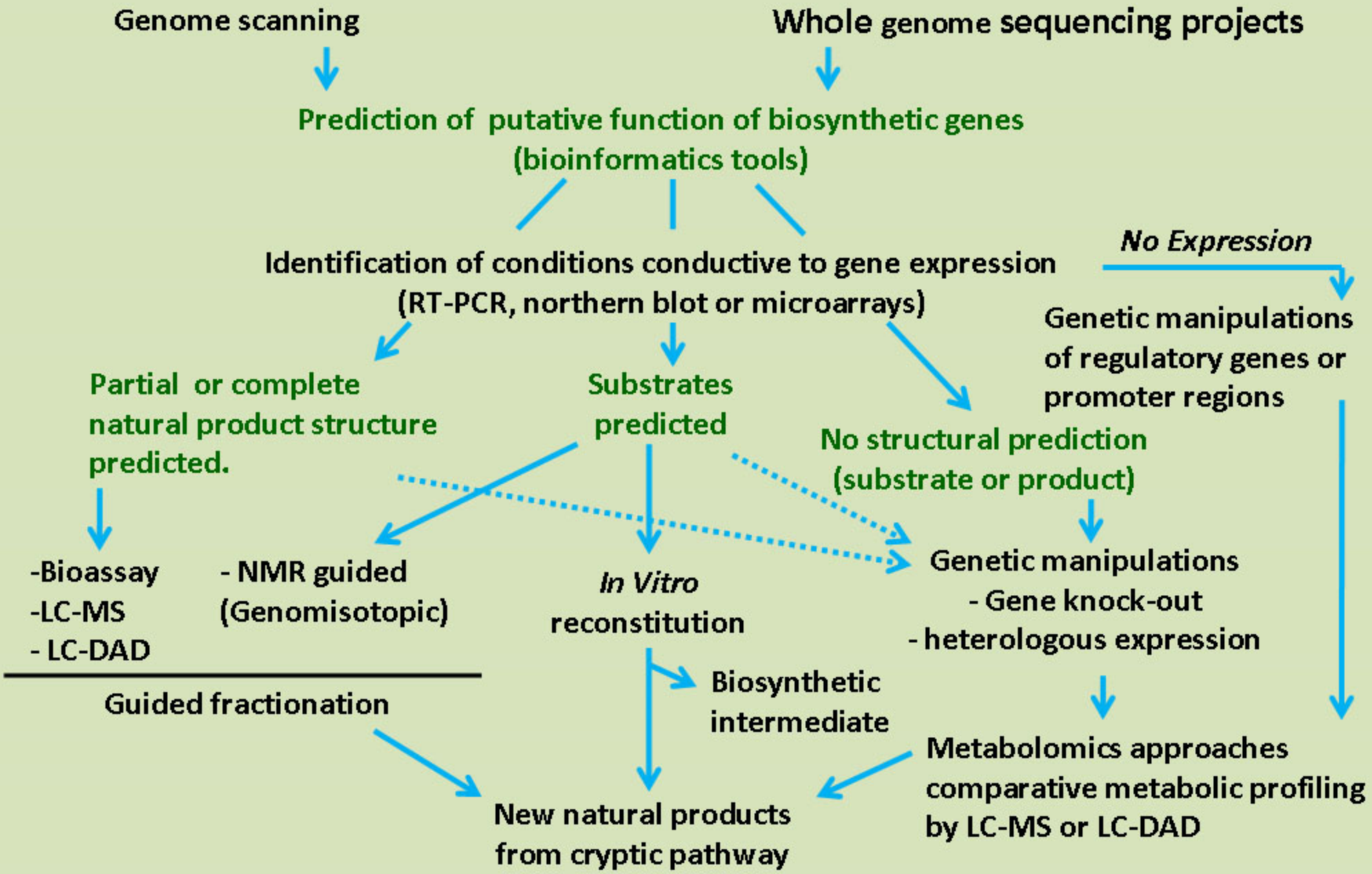

Figure 5. **Strategies for discovery of novel natural products by genome mining (modified from Corre and Challis, 2009)**