August 2016

Molecular interactions of *Bacillus subtilis* biofilms with plant roots

Irene Marring[1] (s1664697), Oscar P Kuipers[2] (supervisor)

[1]Master Molecular Biology and Biotechnology, University of Groningen
[2]Molecular Genetics, University of Groningen

Plant surfaces and their internal tissues are inhabited by numerous microorganisms, such as bacteria, fungi and archaea. Biofilms on plant roots are highly spatiotemporally organized intricate networks of microorganisms, with the potential to provide benefits to both plant host and microorganism. Among the many biofilm-forming bacteria, *Bacillus subtilis*, often used as a model organism, is used to study biofilm formation on plant roots. The different components of biofilms are considered, as well as the interactions that take place between *Bacillus* and plants, and between *Bacillus* cells themselves.

Index

Introduction

Advances in microbiology have allowed us to gain more insight in the microorganisms living in soil. The development of non-culture-based methodology increased our understanding of the genetic and functional capacity of microbial communities (Arsène-Ploetze *et al.*, 2015). The structure of a microbial community is very dynamic and varies greatly with soil type (Bastida and Jehmlich, 2016).

Many soils harbor plants. And these plants provide yet another very unique habitat for microorganisms. All plant components can be colonized and inhabited by microorganisms: the surface of the aboveground parts can be colonized by epiphytic microorganisms, the internal tissues of the plant can be inhabited by endophytes, and the roots and the soil immediately surrounding the roots can be home to bacteria.

The plant microbiome, as these micro-organisms living on the surface and inner tissues of plants are collectively called, supplies an additional gene set to the plant (Rout, 2014). This symbiosis can be detrimental to plants, or beneficial for both plants and microorganisms. The microbiome can induce phenotypic changes of the plant, such as enhanced root growth, contributing to increased resistance to fungal infections (Chen *et al.*, 2012) and enhanced productivity (Wu *et al.*, 2004). The presence of microorganisms can also augment nutrient uptake, which is the case for the association of nitrogen-fixing bacteria with plant roots (Magalhães Cruz *et al*., 2001) and ameliorate plant immune function (Pineda *et al.*, 2010) and offer protection to a multitude of plant diseases. The exchange of root exudates, such as amino acids, offer a steady influx of nutrients to the microbial community (Moe, 2013).

This report will describe the process of *B. subtilis* biofilm formation. Also, it will shed a light on the main pathways and compounds that are involved in the different developmental stages of biofilms, as well as the different cell fates of *B.* subtilis. The interactions between *B. subtilis* and their plant host will be discussed, and the effects of biofilms, and their possible applications will be highlighted.

Plant roots

Plant growth starts when the initial root breaks through the wall of the seed. This primary roots, with a few exceptions, extends downwards, further into the ground, while the shoot starts to grow upwards, above the ground. Depending on the type of plant, there are several possible root systems: for example, the roots either develop one primary large root, after which lateral roots form (tap root) or a dense network (fibrous roots) (Nibau *et al.*, 2008). Roots function as an anchor for the plant, and via the roots, plants can get access to, and absorb water and nutrients from the soil.

In addition to acquiring products from the soil, roots exude various substances. Rhizodeposition of root exudates brings, amongst other things, carbon, electrons, ions and polysaccharides back into the rhizosphere (Rasmann and Turlings, 2016).

The rhizosphere

The term rhizosphere was first proposed by Hiltner in 1904, when he discovered bacteria that were closely associated with plant roots (Hartmann *et al.*, 2008). The rhizosphere is generally considered to be the soil immediately surrounding and affecting the roots of plants (***figure 1***). More specifically, it is defined as the soil attached to the roots up to a distance from 1 mm from the roots (Hirsch and Mauchline, 2012).

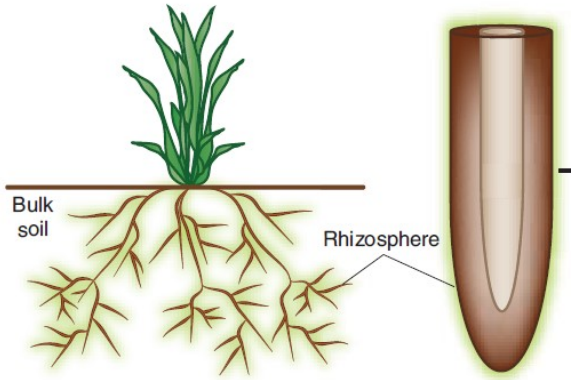

Figure 1: Schematic overview of plant roots, rhizo-sphere and bulk soil. *Depicted in green is the location of the rhizosphere (Taken from Hirsch and Mauchline, 2012).*

Bacteria in the rhizosphere

Rhizosphere microbiota is influenced by plants and vice versa. Interactions between plants and microorganisms take place in the rhizosphere, and therefore, the rhizosphere is affected to a higher extend by plant roots than is the bulk soil. Both soil characteristics and plant species influence the microbial community in the rhizosphere (Weinert *et al.*, 2011), but whether the soil or the plant species is the main driver, remains debatable (e.g. Costa *et al.*, 2006; Kielak *et al.*, 2008). The effects of plants on the rhizosphere microbiota is called the "rhizosphere effect" (Ĭnceoğlu *et al.*, 2013; Berg and Smalla, 2009).

The bacterial community in the bulk soil is phylogenetically more diverse than the

rhizospheric community. However, the rhizosphere, although less diverse, harbors more dominant bacterial species (Kielak *et al*., 2008). The existence of a microbiome belonging to specific plant species, is likely affected by the root exudates of these plant species (Moe, 2013; Doornbos *et al.*, 2012).

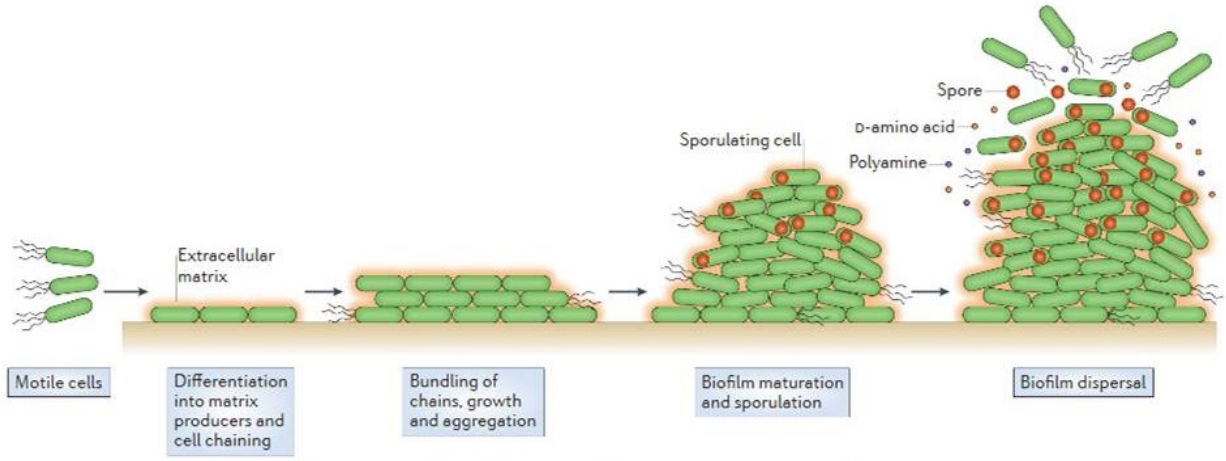

Figure 2: The different stages of biofilm formation. *Motile cells are recruited to the plant roots were they differentiate in various subpopulations. The biofilm grows and matures and eventually, the cells are dispersed. Taken from Vlamakis* et al.*, 2013.*

Among the bacterial species generally present in the rhizosphere are *Proteobacteria*, *Firmicutes*, *Actinobacteria*, *Bacteroidetes* and *Acidobacteria* (Weinert *et al.*, 2011).

Bacillus subtilis

Bacillus subtilis are gram positive bacteria that belong to the phylum of *Firmicutes.* During distinct developmental stages, *B. subtilis* can differentiate into different subpopulations: *B. subtilis* harbor the capacity to form endospores. Also, during a small window in its development, *B. subtilis* can become competent. This allows bacteria to internalize and incorporate foreign DNA (Yüksel *et al.*, 2016).

The rod-shaped *Bacillus* is capable of living under either aerobic or anaerobic conditions (Nakano and Zuber, 1998), which makes them ubiquitous. Also, *B. subtilis* is known to produce various antibiotics (Stein, 2005).

The main constituent of the *B. subtilis* cell wall is peptidoglycan, which is composed of glycan strands, and provides structural stability to the bacteria (Hayhurst *et al.*, 2008).

B. subtilis can live in a planktonic, single-cell state, but is also capable to form biofilms on various surfaces.

Bacillus subtilis biofilms

Biofilms are closely associated microbial aggregates, that can form on the interface of many surfaces, such as on liquid, on teeth, industrial surfaces, but also on plant roots (Lemon *et al.*, 2008). The constituent microorganisms of the biofilm are enclosed by a self-produced extracellular matrix (ECM), that enables adherence both from the bacterial cells to the surface and from bacterial cells to each other, and provides protection (Kobayashi and Iwano, 2012).

Biofilms can be constructed of multiple species or one single species. The remainder of this report will deal with single-species *B. subtilis* biofilms. The bacteria in these biofilms are, although consisting of one species, highly differentially specialized. The genetically identical cells are functionally very distinct (Chai *et al.*, 2008): a small proportion of the cells remains motile, a larger part of the cells is non-motile. The non-motile cells form chains and produce the extracellular matrix. Also, there are spore-forming cells and cannibalistic cells (Marlow *et al.,* 2014; Vlamakis *et al.*, 2013).

The formation of biofilms comprises multiple steps: assembly of bacteria, maturation of the biofilm and eventually the dispersal of biofilm bacteria (***figure 2***).

Biofilm assembly

Biofilm assembly starts with the recruitment of motile *B. subtilis* cells. In this stage, the transition from motile to sessile bacteria takes place, which is regulated by certain key genes. Matrix gene expression is upregulated, whereas genes involved in motility and autolysis are downregulated. The transition is initiated when phosphorylated Spo0A (Spo0A~P) reaches a certain threshold. Phosphorylation of Spo0A

leads to anti-repression of matrix genes via two parallel ways (***figure 3***):

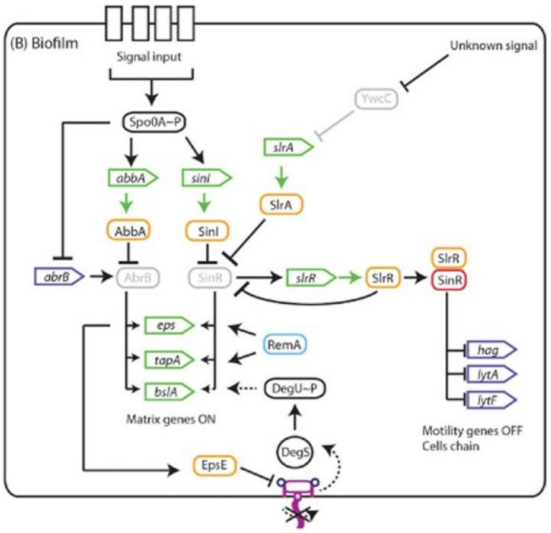

Figure 3: Regulatory pathways for matrix production and motility in B. subtilis. *In green: operons, in yellow: proteins, in red: transcription repressor, arrows: activation, T-bars: inhibition, dashed arrow: indirect activation, green arrow: translation. Taken from Cairns* et al.*, 2014.*

1: Direct or indirect repression of AbrB. The latter goes via the promotion of *abbA* expression, which produces the AbrB anti-repressor AbbA that binds to AbrB and thereby prevents it from binding to the DNA. Both the direct and indirect repression of AbrB enable the transcription of operons essential to biofilm matrix production, such as *eps* and *tapA* (Cairns *et al.*, 2014).

2: Direct inhibition of the *eps* operon and the *tapA-sipW-tasA* (also referred to as *tapA*) operon via SinR. Threshold Spo0A~P levels induce promotion of *sinI*, which produces SinI, that forms a complex with SinR. While in this complex, SinR is unable to bind to and repress matrix-gene operons, and as such, these promotors will be transcribed (Cairns *et al.*, 2014).

In the case of high levels of SinI, SinR is repressed, and as a consequence, the *slrR* gene is expressed, SlrR levels will rise, and the *eps* and *tapA* promotors will be transcribed. The self-reinforcing negative feedback loop that consists of SinR, SlrR and *slrR*, exists because *slrR* is repressed by SinR and SinR is repressed by SlrR, so the *slrR* gene remains de-repressed (***figure 4***). In a low SlrR state, the *slrR* gene is repressed, maintaining low SlrR levels, and matrix genes are repressed, but genes involved in motility and autolysin are expressed. In a high SlrR state, a SinR-SlrR complex is formed, that represses autolysin and motility genes and induces matrix-gene expression (Chai *et al.*, 2010).

The SinR-SlrR switch is set to high SlrR state in the case of biofilm formation, because *sinI* is induced by Spo0A~P. The state of the switch is self-reinforcing, and can therefore be considered an epigenetic switch (Vlamakis *et al.*, 2013).

Structural components of the ECM

During biofilm formation, the extracellular matrix is produced. The main constituents of the extracellular matrix (ECM) are exopoly-saccharides (EPS), proteins and nucleic acids.

EPSs are produced by genes that are controlled by the *epsA-O* (or simply called *eps*) operon (***figure 4***).

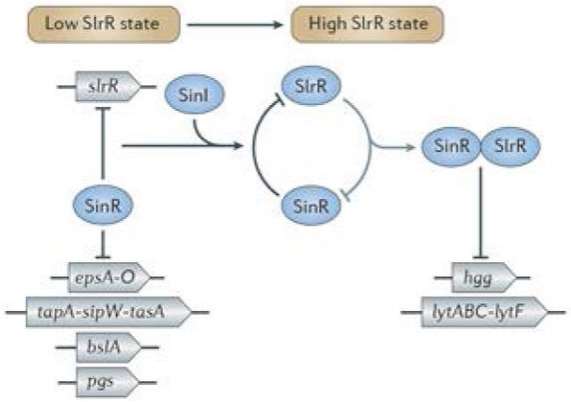

Figure 4: Mechanism of the epigenetic SinR-SlrR switch. *Taken from Vlamakis* et al.*, 2013.*

Four functionally distinct EPSs can be classified (Marvasi *et al.*, 2010):

1: Structural EPS: These neutral polysaccharides perform a structural role in the ECM. The structural EPSs are composed of glucose, fucose, galactose, glucuronic acid and O-acetyl groups (Morita *et al.*, 1979). The genes controlled by the *eps* promotor (***figure 3 and 4***) belong to this category. Also, levan type I and II are examples of structural exopolysaccharides (Dogsa *et al*., 2012).

2: Sorptive EPS: γ-polyglutamate (γ-PGA) is an example of a polymer from D- and L-glutamate of the second category. γ-PGA is involved in interactions between *B. subtilis* cells and the surface, and gives the biofilm a mucoid appearance (Chan *et al.*, 2014). However, γ-PGA is not essential to biofilm formation (Marvasi *et al.*, 2010; Morikawa *et al.*, 2006). DegU, DegQ and SwrA are involved in the regulation of γ-PGA production (Stanley and Lazazzera, 2005).

3: Surface-active EPS: These non-ribosomal lipopeptides can be divided in three groups: surfactin, iturin and fengycin. These lipopeptides are known to induce systemic resistance (Aleti *et al.*, 2016).

Not all *Bacillus subtilis* cells in a biofilm produce surfactin. A distinction can be made between a subpopulation of cells at the surface of the biofilm that produces surfactin (Dervaux *et al.*, 2014) and another subpopulation that expresses matrix genes in response to surfactin (López *et al.,* 2009a). Surfactin production is induced by a peptide pheromone called ComX, which is involved in quorum sensing (see the section ***Communication in biofilms*** below for a detailed description of quorum sensing). The differentially expressing subpopulations remain stable for certain periods of time, because the cells that produce surfactin, do not respond to surfactin themselves, and cells that express matrix genes, do not produce surfactin.

Phosphorylation of Spo0A (mentioned in the section ***Biofilm assembly*** as the start of the regulatory pathways) can in fact be initiated by surfactin (Kearns *et al.*, 2005; Lopez *et al.*, 2009a), as well as by a number of other mechanisms (Vlamakis *et al.*, 2013), which will be covered in the section ***Determining cell fate within* Bacillus subtilis *biofilms***. In addition, surfactin is involved in spore-formation (Branda *et al.*, 2001), via the formation of aerial structures that are necessary to form spores.

4: Active EPS: The enzymes belonging to this category are transported from the cytoplasm to the exterior of the bacterial cells, via multiple possible pathways (Cheng *et al.*, 2016 and 2015; Simone *et al.*, 2013). The description of these pathways is beyond the scope of this paper. Among the exported proteins are antibiotics.

The TasA protein is, in contrast to the aforementioned EPSs, which are controlled by the *epsA-O* operon, under control by the *tapA-sipW-tasA* operon (Romero *et al.*, 2014), see ***figure 3*** and ***4***. TasA is capable of forming amyloid fibers. The fibers are a structural part of the ECM and are also necessary for biofilm formation (Romero *et al.*, 2010). Another product of the *tapA-sipW-tasA* operon, TapA, plays a role in the localization of the amyloid fibers to the ECM (Ostrowski *et al.,* 2011; Branda *et al.,* 2006).

The cooperation between the exopolysaccharides and amyloid fibers results in the formation of chains of *Bacillus subtilis* cells (Kobayashi and Iwano, 2012). Furthermore, the extracellular matrix provides physical strength and protection to *B. subtilis* in biofilms, provided by the structural EPSs and amyloid fibers, as well as the hydrophobic outer surface, that is capable of repelling water and even ethanol (Epstein *et al.*, 2011). The hydrophobic properties of the biofilm are in part due to the structure of the surface.

BslA, an amphiphilic protein (i.e. harboring both hydrophilic and hydrophobic properties) is associated with the ECM. BslA is involved in the surface structure of the biofilm. Regulation of *bslA* goes directly via AbrA or indirectly via Rok and DegU (Kovács *et al.*, 2012). Transcription of the *bslA* gene commences after the chains of bacterial cells have formed under influence of *eps* and *tasA* (Kobayashi and Iwano, 2012). The wrinkled morphology of the matrix, induced by BslA, gives the biofilm a rough surface, that aids in liquid repellence.

Determining cell fate within* Bacillus subtilis *biofilms

Phosphorylation of Spo0A, as the main regulator of the expression of genes involved in matrix production, motility or sporulation, is affected via various pathways. Phosphorylation of Spo0A can be regulated by surfactin, as was discussed in the section ***Structural components of the ECM***. This goes via the transmembrane histidine kinase KinC (Devi *et al.*, 2015). In addition to KinC, other kinases also play a role in the activation of Spo0A via a phosphor-relay (Yan *et al.*, 2016; McLoon *et al.*, 2011). KinB, KinC and KinD are transmembrane kinases. Together with the cytoplasmic kinases KinA and KinE, they act on the phosphorylation of Spo0F. Spo0F~P, on its turn, transfer a phosphoryl group to Spo0B, and Spo0B~P transfers its phosphoryl group to Spo0A (***figure 5***).

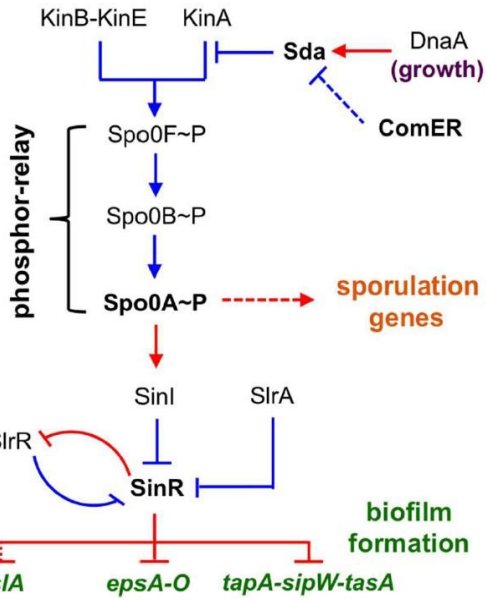

Figure 5: Phosphor-relay pathway for Spo0A phosphorylation. *Taken from Yan* et al.*, 2016.*

Upon maturation of the biofilm, an increasing number of *B. subtilis* cells starts forming spores - dormant cells that enable bacteria to survive less favorable conditions - (Bassler and Losick, 2006). Sporulation, matrix production and cannibalism are all regulated by Spo0A~P.

In situations of higher levels of Spo0A~P, which is often the case when cells are starved due to an increasingly limited availability of nutrients, the spore-production machinery is upregulated (Vlamakis *et al.*, 2008). In fact, the onset of sporulation depends not only on the concentration of phosphorylated Spo0A, but also on the rate of phosphorylation (Vishnoi *et al.*, 2013).

Before sporulation becomes the committed step and therefore irreversible, an intermediate step of cannibalism is deployed (Bassler and Losick, 2006). A subpopulation of the cells is involved in both matrix production and cannibalism. In relatively low levels of Spo0A~P, the toxins Skf and Sdp are produced, via the direct expression of *skfA-H*, or indirectly, via the repression of AbrB, such that *sdpABC* is expressed. This specific subpopulation is resistant to its own toxins, but the spore-producing subpopulation, as well as other bacterial species, will die (López *et al.*, 2009b). Cannibalism allows *B. subtilis* to kill its siblings, in order to release nutrients. The presence of nutrients, either exogenously or acquired via cannibalism, ceases sporulation.

Another cell fate directed by nutrient limitation is competence. When *B. subtilis* cells are committed to sporulation, a daughter cell (the endospore) is produced and the mother cell is lysed. Within the sporulation route, an alternative path can be taken that leads to the uptake of DNA from the lysed cells via a process called competence (Schultz *et al.*, 2009).

The regulation of the decision between sporulation or competence, depends on an intricate connection of many parameters. Within a certain range of Spo0A~P concentrations, there is a possibility for *B. subtilis* to become competent. If the Spo0A~P concentration is below the lower threshold, competence is inhibited by the repression of ComK by AbrB-Rok (Schultz *et al.*, 2009). Between the first and second threshold, ComK is no longer inhibited, because AbrB is repressed by Spo0A~P. It depends on the immediate surroundings of *B. subtilis* whether the cells actually become competent: ComS prevents proteolysis of ComK. As a consequence, higher levels of ComS increase the likelyhood for the cells to become competent (Yüksel *et al.*, 2016). When Spo0A~P levels exceed the second threshold, the concentration of ComK increases. These high levels of ComK inhibits ComS and eventually, sporulation is inevitable (Schultz *et al.* 2009).

Biofilm spreading and dispersal

In the process of maturation, biofilms expand and cover an increasing surface. Propagation of the biofilm does not involve motility genes, but instead there are several ways that possibly contribute to the expansion of biofilms across a surface:

The first method is based on the reduction of surface tension caused by the production of surfactin. The production of surfactin by a specific subpopulation of *B. subtilis* cells creates a surfactin-gradient due to the higher concentration of surfactin at the center of the growing colony. As a consequence, the biofilm expands outwards, via a motion called sliding (Ghelardi *et al.*, 2012; Angelini *et al.*, 2009).

The second sliding method for biofilm propagation involves osmotic pressure. The production of exopolysaccharides causes an osmotic gradient which results in expansion of the cells over the surface (Seminara *et al.*, 2012).

The protein BslA, which is also a structural component of biofilms (see ***Structural components of the ECM***), provides another mechanism for sliding motility (Grau *et al.*, 2015). BslA forms a water-repellent layer that enables surface motility.

The above mentioned mechanisms involved in sliding of *B. subtilis* are controlled by KinB and KinC. In the initial stage of sliding, the concentration of intracellular potassium is high. KinB is activated by potassium, whereas KinC is inhibited. At later stages, the potassium levels diminish, and KinC is activated. Via a phosphor-relay (described in ***Determining cell fate within* Bacillus subtilis *biofilms***) Spo0A is phosphorylated, which in turn controls the formation of the components responsible for sliding (Grau *et al.*, 2015).

The physical dispersal of biofilms follows a different path. Biofilm dispersal can be beneficial in instances were the biofilm has grown so much that the distance between certain cells and the interface is simply too large to acquire nutrients. Also, metabolic waste products and toxins can accumulate, creating an unpleasant environment (Petrova and Sauer, 2016; Karatan and Watnick, 2009). In addition to signals coming from the environment, biofilm dispersal can also be initiated by internal signals. For example, *Bacillus subtilis* cells in aged biofilms start producing a mixture of D-amino acids (D-tyrosine, D-leucine, D-tryptophan and D-

methionine, Lam *et al.*, 2009). The amino acids are then incorporated in the peptidoglycan of the cell wall. TapA is responsible for the localization of TasA to the cell wall (Romero *et al.*, 2011). In the presence of D-amino acids, the specific locations for the attachment of TasA to the cell wall disappear, and the amyloid TasA fibres that were originally attached to the cell wall, are released (Romero *et al.*, 2011; Kolodkin-Gal *et al.*, 2010). The presence of D-amino acids, via the release of the TasA amyloid fibers, results in the detachment of the cells from the surface (Yu *et al.*, 2016).

Components involved in motility

Flagella in planktonic *Bacillus subtilis* cells are under control of the SinR-SlrR switch (see ***Biofilm assembly***). When biofilms are being formed, the SinR-SlrR switch is in a high SlrR state and genes involved in motility are repressed. In later stages of biofilm development, it becomes beneficial again to regain motility. Upon maturation of the biofilm, the SinR-SlrR switch slowly goes to a low SlrR state (Chai *et al.*, 2010). Consequently, genes involved in motility (such as *hag*) are being expressed. *B. subtilis*, with its new regained motility, is now capable of colonizing new substrates, via a process called swarming.

Communication in biofilms

Quorum sensing is a means of communication, for example between bacteria or between plant and bacteria. Quorum sensing enables bacteria to act in a multicellular way to control behavior. Short signaling peptides produced by the *B. subtilis* community accumulate extracellularly and are sensed by the bacteria after exceeding a certain threshold. Only then, communal action takes place, such as biofilm formation, or sporulation (Bassler and Losick, 2006).

Communication between gram-positive bacteria, such as *B. subtilis*, occurs in two different ways. Autoinducing peptides (AIPs) are produced by ribosomes (Sturme *et al.*, 2002), and while they are exported from the cell, they can undergo posttranslational modifications. Autoinducing peptides are involved in signaling, however, certain AIP have an additional antimicrobial function.

The briefly mentioned peptide pheromone ComX is a quorum sensing peptide involved in biofilm formation (see ***Structural components of the ECM***). In response to ComX, a subpopulation of *B. subtilis* produces surfactin (López *et al.*, 2009a). ComX belongs to the isoprenylated tryptophan peptide family (Okada, 2011), which is one of the three categories of autoinducing peptides (AIP).

The second category of AIPs are the oligopeptide lantibiotics. Subtilin is an example of a lantibiotic with know antimicrobial activity against gram-positive bacteria (Spieß *et al.*, 2015).

Thiolactone peptides are the third category of AIPs.

The AIPs of all three categories are sensed by a two-component signal transduction system (***figure 6***).

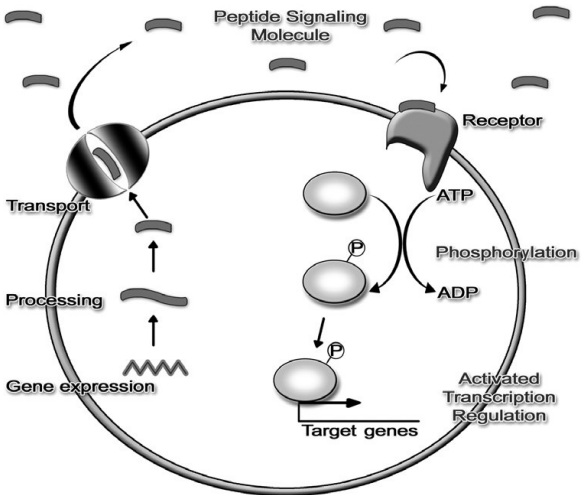

Figure 6: Schematic representation of quorum sensing via autoinducing peptides (AIP). *The peptides are transported outside the cell and accumulate extracellularly. After exceeding a threshold, the peptides are sensed by a receptor. Next, a cascade of reactions take place resulting in target gene expression. Taken from Turovskiy* et al.*, 2007.*

The diagram in ***figure 6*** shows the process of signaling via autoinducing peptides. The left side of the figure displays the synthesis of the peptides. Some precursor peptides undergo posttranslational modifications before they are transported out of the cell. When the extracellular signaling peptides reach a threshold, the signal is recognized by a histidine sensor kinase protein receptor (right side of the figure). The histidine sensor kinase protein is part of a two-component signal transduction system. A phosphoryl group, resulting from autophosphorylation, is then transported to a response regulator protein, which is also part of the two-component signal transduction system, and target genes are being expressed (Nazzaro *et al.*, 2013).

***Interactions between plants and* Bacillus subtilis**

The recruitment of *B. subtilis* by plants is presumably based on the attraction of *B. subtilis* to nutrients and carbon (Bais *et al.*, 2006). In

addition, polysaccharides exuded by plants trigger biofilm formation via the regulation of Spo0A~P (Beauregard *et al.*, 2013). Once the roots are colonized, a broad range of signals can be exchanged between *B. subtilis* and plants.

Communication between plants and *B. subtilis* can be bidirectional. Plants are capable of releasing compounds which evoke a response in *B. subtilis*. The opposite can also be the case: the signals can also be emitted by *B. subtilis* and received by the plant.

Quorum sensing is described above as a means of interbacterial communication, but it can also play a role in the communication between plants and bacteria. Root exudates are an example of quorum sensing signals transmitted by plants. These chemicals produced by plants are either actively (via ABC-transporters) or passively (via leakage or detachment of root cells) exuded from the roots (Hassan and Methesius, 2012). In addition to inorganic products and ions, root exudates can be subdivided in high and low molecular weight organic components. The latter consists, among other things, of amino acids and sugars, which are the main products exuded by roots (Moe, 2013). Other low molecular weight root exudates are organic acids and secondary metabolites (Badri *et al.*, 2009). The high molecular weight exudation products are proteins, polysaccharides and mucilage (Moe, 2003; Badri *et al.*, 2009).

The quantity and composition of exudates from plant roots is influenced by the environment. Physiological parameters, such as nutrient availability and drought, affect root deposition (Badri *et al.*, 2009).

The plant root cells that are responsible for the passive transport of metabolites are called border cells (Driouich *et al.*, 2013). Root border cells are released from the root cap and exist as single cells or aggregates. The border cells produce polysaccharides, proteins and extracellular DNA in a mucilage slime layer (Driouich *et al.*, 2013). The border cell products can directly protect the plant from pathogens.

Plant roots are also capable of actively transporting compounds that have direct antimicrobial properties. The main transporters involved in this active transport are the ATP-Binding Cassette (ABC) and Multidrug and Toxic Compound Extrusion (MATE) (Baetz and Martinoia, 2014).

Phenolics produced by plants have direct antifungal and antibacterial characteristics. Moreover, some phenolics, such as amino acids, can attract beneficial bacteria and indirectly influence protection against pathogens (Baetz and Martinoia, 2014).

Plant-produced flavonoids, which are also phenolic secondary metabolites, consist of p-coumaroyl-CoA and malonyl-CoA. Flavonoids harbor a great variety of compounds, involved in both direct and indirect protection against pathogens. Flavonoids are known to mimic bacterial quorum sensing signals, and as such, they affect the ability of bacteria to aid in protection against pathogens (Hassan and Methesius, 2012). Flavonoids are actively transported out of the cell via the ABC transporter.

Plants also produce volatiles which belong to the terpenoids. Like flavonoids, volatiles also have direct and indirect effects on the protection of plants against pathogens (Baetz and Martinoia, 2014).

Bacillus subtilis can aid plants in their protection against pathogens. Among the many threats that plants potentially face are predation by (in)vertebrates and infection by viruses, bacteria and fungi. Protective measures against the latter three, via antagonistic activity of *B. subtilis* against pathogens or via an induced immune reaction, are described here. Plant protection by *B. subtilis* is possible via a number of ways (Doornbos *et al.*, 2012):

1: Antibiotics: *B. subtilis* is known to produce a multitude of antibiotic compounds, approximately 5% of its genes are involved in the synthesis of antibiotics (Stein, 2005). The antibiotics can be divided in several categories and subcategories based on their structure, synthesis or function.

Lantibiotics and sactibiotics belong to the bacteriocin category. Bacteriocins are produced by ribosomes and are characterized by their thioether bonds. Bacteriocins are posttranslationally modified and their structure dictates their function (Stein, 2015). The antimictobial activity of these antibiotics is mainly directed against gram-positive bacteria. Examples of lantibiotics are: subtilin, ericin, mersacidin, sublancin. Examples of sactibiotics are: thuricin, subtilosin and propionicin (Jung *et al*., 2014).

Non-ribosomally synthesized antibiotics harbor, for example, lipopeptides and polyketides (Hofemeister *et al.*, 2004). The surface-active EPSs surfactin, iterin and fengycin, which were previously described (***Structural components of the ECM***), are non-ribosomal amphiphilic lipopeptides. The lipopeptides from these three families have a cyclic structure in common,

however, they differ in their the amino acid composition.

The cyclic structure is composed of peptides, which are slightly different between the different cyclic lipopeptides. Attached to the ring is a fatty acyl chain (***figure 8***; Falardeau *et al.*, 2013).

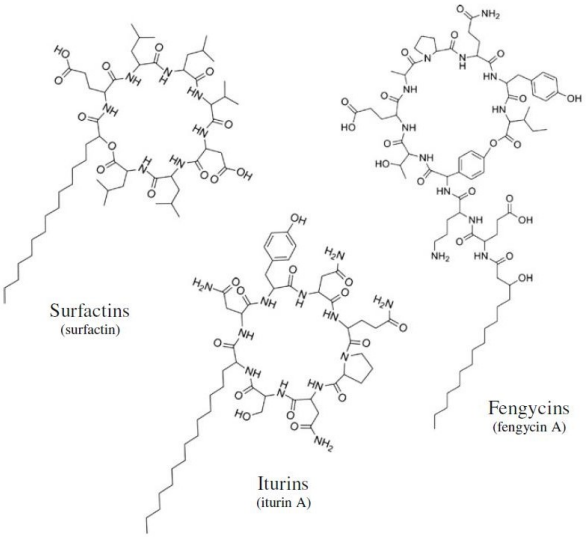

Figure 8: The general structures of the cyclic lipopeptides from the surfactin, iturin and fengycin families. *Taken from Falardeau* et al.*, 2013.*

In addition to the role in the extracellular matrix, the cyclic lipopeptide surfactin has antibacterial and antiviral properties. Fengycin and iturin are most active against fungi. The cyclic lipopeptides disrupt the membrane of pathogens by creating pores (Falardeau *et al.*, 2013).

The pathways of antibiotic regulation is shown in ***figure 9***. The ribosomal- and non-ribosomal antibiotics are interconnected via an dense network.

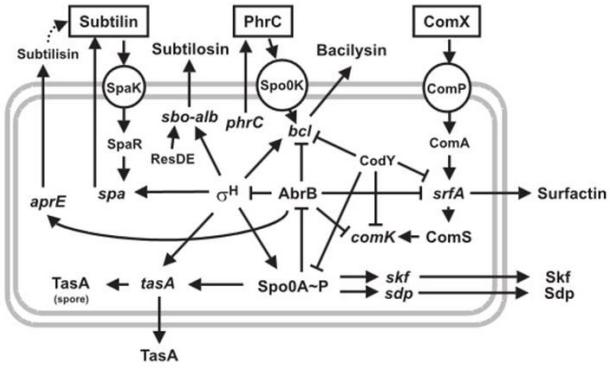

Figure 9: Regulatory pathways of antibiotic production. *In boxes: pheromones, in circles: transmembrane histidine kinases, arrows: activation, T-bars: inhibition. Taken from Stein, 2005.*

2: Lytic enzymes: Spore-forming bacteria produce lytic enzymes in order to lyse the mother cell after sporulation cell division, and degrade the spore cortex and resume a vegetative state (Moir and Cooper, 2015). Also, as described in ***Determining cell fate within* Bacillus subtilis *biofilms***, in the process cannibalism, non-matrix-producing cells are lysed (Bassler and Losick, 2006). These autolytic enzymes are under control of the *lyt*-operon or *skfA-H* and *sdpABC*, respectively, which are regulated by Spo0A~P (***figure 3***). In addition, *B. subtilis* produces other lytic enzymes that can hydrolyze cell walls of (pathogenic) bacteria and fungi. The fungal cell wall consists mainly of chitin ((1,4)-2-acetamide-2-deoxy-β-D-glucan) and β-1,3-glucan (Solanski *et al.*, 2012). Gram-positive bacteria have cell walls with a thick peptidoglycan layer (*N*-acetylmuramoyl and *N*-acetylglucosaminyl linked via a glycosidic bond), whereas the peptidoglycan portion in gram-negative bacteria is considerably smaller. However, gram negative bacteria possess an additional layer of lipopolysaccharides (Popham, 2013; Scheurwater *et al.*, 2008). The individual cell wall constituents can be degraded by different enzymes. Hydrolysis of the cell walls results in lysis of the pathogen. Peptidoglycan of bacterial cell walls can be hydrolyzed by lysozyme. Alternatively, the glycosidic bond between *N*-actetylmuramoyl and *N*-acetylglucosaminyl can be broken by transglycosylase (Scheurwater *et al.*, 2008).

The main fungal cell wall component chitin can be hydrolyzed by chitinase and beta-hexosaminidase, which are produced by the genes *chiA* and *nagZ*, respectively (Senol *et al.*, 2014). *B. subtilis* possesses both of these genes. The enzyme glucanase is capable of hydrolyzing glucan, which is also a main constituent of the fungal cell wall.

3: Competion for iron: Iron is a limiting nutrient in soil. Via siderophores produced by *B. subtilis*, the poorly soluble iron is bound and internalized by bacteria possessing this machinery. Iron is therefore not available to (pathogenic) microorganisms that do not harbor the iron-chelating capacity (Yu *et al.*, 2011). As a consequence, the growth rate of microorganisms that are unable to bind iron is reduced as a result of iron depletion.

4: Induced systemic resistance (ISR): ISR is an indirect reaction in which an immune response from the plant against pathogens is induced by bacteria (Ongena *et al.*, 2007).

The lipopeptides surfactin and fengycin have direct antimicrobial properties, and via ISR, they can also have indirect protective effects on plants. Surfactin and fengycin, but not iturin, are

capable of triggering the immune response of plants, when produced by bacteria living in association with plants. Bacterial strains that produce fengycin and surfactin induce lipid hydroperoxydase and lipoxygenase in plants (Ongena *et al.*, 2007). These enzymes are involved in the lipid peroxidase pathway which can lead to many distinct antifungal, antibacterial and antiviral products (Feussner and Wasternack, 2002). Surfactin is known to induce systemic resistance against both bacteria and viruses, whereas fengycin provides induction of systemic resistance against fungi (Falardeau *et al.*, 2013).

Volatiles can be produced by plants, as was described above. Interestingly, bacteria can also produce volatiles. The volatile compounds produced by rhizobacteria are generally lipophilic and can act over relatively long distances (Kanchiswamy *et al.*, 2015). Microbial volatile organic compounds (MVOCs) can be directed towards other bacteria or fungi. Amongst other things, *B. subtilis* produces volatiles that induce cannibalism in other microorganisms (Kai *et al.*, 2016).

MVOCs can also be directed towards plants (***figure 10***). MVOCs are associated with induced systemic resistance and plant growth promotion.

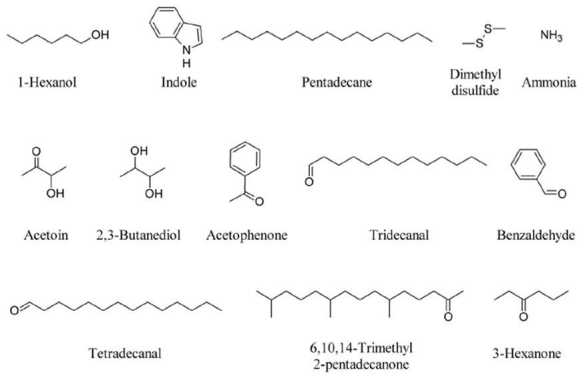

Figure 10: The main microbial volatile organic compounds involved in plant responses. *Taken from Kanchiswamy* et al.*, 2015.*

Three possibly redundant pathways are used by various *B. subtilis* strains to trigger induced systemic resistance (Pieterse *et al.*, 2009; Ryu *et al.*, 2004). Ethylene (ET), salicilic acid (SA) and jasmonic acid (JA) are phytohormones that regulate these pathways (***figure 11***).

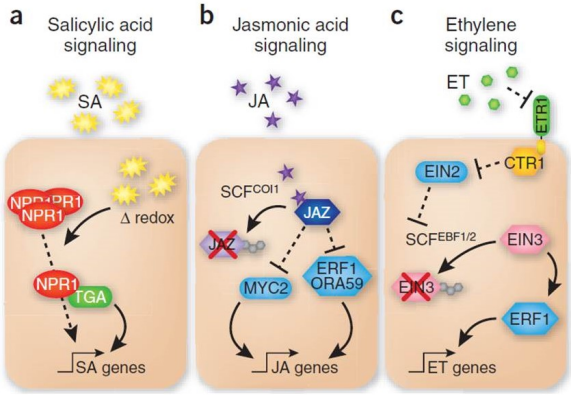

Figure 11: Signaling pathways to induce systemic resistance in plants. *A: The SA pathway is based on redox potentials. B: The JA pathway is based on JA-complex formation to induce gene expression. C: The ET pathway relieves the intrinsic repression of genes in presence of ET. Taken from Pieterse* et al.*, 2009.*

Upon detection of pathogens, the signaling pathways can be activated by the volatile phytohormones produced by *B. subtilis*, leading to ISR.

The SA signaling pathway (***figure 11A***) is induced by increasing levels of salicylic acid. The changing SA concentration causes a difference in the redox potential. NPR1, which is usually present in the cell as a non-active polymer, is reduced to active monomeric subunits. NPR1 forms a complex with TGA, and facilitates gene transcription in plant cells.

In the JA signaling pathway (***figure 11B***), jasmonic acid enables JAZ complex formation. Next, the complex is broken down and this results in gene transcription.

Ethylene signaling (***figure 11C***) is based on the detection of ET by membrane receptors. In a normal state, CTR1 and its subsequent reactions are repressed. However, CTR1 is activated in the presence of ET. Which, via multiple steps, leads to gene expression.

The protection of plants against pathogenic microorganisms is of course beneficial to the plant host. But *B. subtilis* also increases the likelihood of its own survival by diminishing or eliminating potential competitors for resources.

Plant growth promotion by Bacillus subtilis

B. subtilis, together with many other rhizobacteria, portrays plant growth promoting (PGP) properties. PGP bacteria increase the size and biomass of plants (Majeed *et al.*, 2015). PGP can be acquired via multiple methods:

1: Production of plant growth-promoting compounds. Indole acetic acid (IAA, also known as auxin), cytokinins and gibberellins are examples of bacterial products that are produced by plants themselves, but also

by PGP bacteria (Santner *et al.*, 2008). The compounds mentioned, as well as many others, are known to enhance plant growth (Majeed *et al.*, 2015).

2: Nitrogen fixation: Nitrogen is often a limiting nutrient in the soil. Fixation of atmospheric nitrogen is necessary to make it available as a nutrient. Due to the limited availability, there is harsh competition for N_2 (Liu *et al.*, 2016), between plants, between bacteria, but also between plants and bacteria. However, *B. subtilis* is capable of nitrogen fixation (Satapute *et al.*, 2012), and excess N_2 will be available for plant growth.

3: Processing of organic and inorganic nutrients: In addition to nitrogen, other compounds are also essential for plant growth. The form in which some compounds are present in soil, leaves plants unable to use them.

Solubilization of inorganic phosphate, as well as the mineralization of organic nutrients by *B. subtilis* makes it available for uptake by plants (Jeon *et al.*, 2003).

4: The four methods of plant protection offered by *B. subtilis* mentioned in the section ***Interactions between plants and*** **Bacillus subtilis** are in fact also considered plant growth promoting (Majeed *et al.*, 2015).

Biofilm effects on plant functioning

During the different stages of biofilm development, different subpopulations of *Bacillus subtilis* are the key players. External and internal signals trigger differentiation of *B. subtilis* and biofilms formation. And the inhabitants of the biofilm itself, also send signals, directed at its neighboring *B. subtilis* cells, other microorganisms and their plant host.

Bacillus subtilis biofilms on plant roots provide the plant with protection against biotic and abiotic stressors. By producing antimicrobial compounds, *B. subtilis* can directly interfere with pathogenic microorganisms. Indirect assistance in protection of the plant host against pathogens, is also possible via a multitude of reactions.

B. subtilis does not only contribute to the survival of plants, but also to the maintenance and growth of their plant host.

Applications

Due to the protective properties of *Bacillus subtilis* biofilms, the potential of *B. subtilis* in biocontrol is worth investigating. Usage of chemical pesticides in the protection of crops against microbial infections can have detrimental effects, such as resistance of the pathogens or accumulation of the pesticides on crops, in soil or in water (R4P Network, 2016). Natural antimicrobial methods, possibly in the form of inoculation with *B. subtilis*, is therefore preferred.

Fertilizers are often used to increase crop yield, but they, too, can accumulate in the environment (Savci, 2012).

Crop yield can potentially increase as a result of the plant growth-promoting properties of *B. subtilis*, as well as increased nutrient availability.

Plant seeds can be inoculated with microorganisms such as *B. subtilis* via a number of methods: Seeds can be primed with the beneficial microorganism. This leads to the presence of microorganisms on the inside of the seeds. The outside of the seeds can also be coated with microorganisms (O'Callaghan, 2016).

Bacillus subtilis is an ideal microorganism to use for biocontrol. In addition to its antimicrobial and plant growth-promoting properties, it is also an endospore-forming bacterium. Endospores allow *B. subtilis* to endure the treatments necessary for the production of inoculated seeds (O'Callaghan, 2016).

References

Aleti G, Lehner S. Bacher M, Compant S, Nikolic B, Plesko M, Schuhmacher R, Sessitsch A, Brader G (2016) Surfactin variants mediate species-specific biofilm formation and root colonization in *Bacillus. Environmental Microbiology*

Angelini TE, Roper M, Kolter R, Weitz DA, Brenner MP (2009) *Bacillus subtilis* biofilms spreads by surfing on waves of surfactant. *PNAS* 106 (43): 18109-18113

Arsène-Ploetze F, Bertin PN, Carapito C (2015) Proteomic tools to decipher microbial community structure and functioning. *Environ. Sci. Pollut. Res*. 22: 13599-13612

Badri DV, Vivanco JM (2009) Regulation and function of root exudates. *Plant, Cell and Environment* 32: 666-681

Baetz U, Martinoia E (2014) Root exudates: the hidden part of plant defense. *Trends in Plant Science* 19 (2):90-98

Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM (2006) The role of root exudates in rhizosphere interactions with plants and other organisms. *Annu. Rev. Plant Biol.* 57: 233-266

Bassler BL, Losick R (2006) Bacterially speaking. *Cell* 125: 237-246

Bastida F, Jehmlich N (2016) It's all about functionality: How can metaproteomics help us to discuss the attributes of ecological relevance in soil? *Journal of Proteomics*

Beauregard PB, Chai Y, Vlamakis H, Losick R, Kolter R (2013) *Bacillus subtilis* biofilm induction by plant polysaccharides. *PNAS*: 1621-1630

Branda SS, González-Pastor JE, Ben-Yehuda S, Kolter R (2001) Fruiting body formation by *Bacillus subtilis*. *PNAS* 98 (20): 11621-11626

Branda SS, Chu F, Kearns DB, Losick R, Kolter R (2006) A major protein component of the *Bacillus subtilis* biofilm matrix. *Molecular Microbiology* 59 (4): 1229-1238

Chai Y, Kolter R, Losick R (2010) Reversal of an epigenetic switch governing cell chaining in *Bacillus subtilis* by protein instability. *Mol Microbiol* 78 (1): 218-229

Chan JM, Guttenplan SB, Kearns DB (2014) Defects in the flagellar motor increase synthesis of Poly-γ-Glutamate in *Bacillus subtilis. J. Bacteriol* 196 (4): 740-753

Chen J, Zhao L, Fu G, Zhou W, Sun Y, Zheng P, Sun J, Zhang D (2016) A novel strategy for protein production using non-classical secretion pathway in *Bacillus subtilis*. *Microb Cell Fact* 15 (69)

Chen J, Fu G, Gai Y, Zheng P, Zhang D, Wen J (2015) Combinatorial Sec pathway analysis for improved heterologous protein secretion in *Bacillus subtilis*: identification of bottlenecks by systematic gene overexpression. *Microb Cell Fact* 14 (92)

Costa R, Götz M, Mrotzek N, Lottmann J, Berg G, Smalla K (2006) Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiol Ecol* 56: 236-249

Dervaux J, Magniez JC, Libchaber A (2014) On growth and form of *Bacillus subtilis* biofilms. *Interface Focus* 4

Devi SN, Vishnoi M, Kiehler B, Haggett L, Fujita M (2015) *In vivo* characterization of the transmembrane histidine kinase KinC in *Bacillus subtilis. Microbiology* 161: 1092-1104

Dogsa I, Brloznik M, Stopar D, Mandic-Mulec I (2012) Exopolymer diversity and the role of Levan in *Bacillus subtilis* biofilms. *PLOS One* 8 (4)

Doornbos RF, Van Loon LC, Bakker PAHM (2012) Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. *Agron Sustain. Dev.* 32: 227-243

Driouich A, Follet-Gueye ML, Vicré-Gibouin M, Hawes M (2013) Root border cells and secretions as critical elements in plant host defense. *Curent Opinion in Plant Biology* 16: 489-495

Epstein AK, Pokroy B, Seminara A, Aizenberg J (2011) Bacterial biofilm shows persistent resistance to liquid wetting and gas penetration. *PNAS* 108 (3): 995-1000

Falardeau J, Wise C, Novitsky L, Avis TJ (2013) Ecological and mechanistic insights into the direct and indirect antimicrobial properties of *Bacillus subtilis* lipopeptides on plant pathogens. *J Chem Ecol* 39: 869-878

Feussner I, Wasternack C (2002) The lipogenase pathway. *Annual Review of Plant Biology* 53: 275-297

Garbeva P, van Elsas JD, van Veen JA (2008) Rhizosphere microbial community and its response to plant species and soil history. *Plant Soil* 302: 19-32

Ghelardi E, Salvetti S, Ceragioli M, Gueye SA, Celandroni F, Senesi S (2012) Contribution of surfactin and SwrA to flagellin expression, swimming, and surface motility in *Bacillus subtilis. Applied and Environmental Microbiology* 78 (18): 6540-6544

Grau RR, de Oña P, Kunert M, Leñini C, Gallegos-Monterrosa R, Mhatre E, Vileta D, Donato V, Hölscher T, Boland W, Kuipers OP, Kovács AT (2015) A duo of potassium-responsive histidine kinases govern the multicellular destiny of *Bacillus subtilis. mBio* 6 (4)

Hartmann A, Rothballer M, Schmid M (2008) Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. *Plant and Soil* 312 (1): 7-14

Hassan S, Mathesius U (2012) The role of flavonoids in root-rhizosphere signalling: opportunities and challenges for improving plant- microbe interactions. *Journal of Experimental Botany* 63 (9): 3429-3444

Hayhurst EJ, Kailas L, Hobbs JK, Foster SJ (2008) Cell wall peptidoglycan architecture in *Bacillus subtilis. PNAS* 105 (38): 14603-14608

Hirsch PR, Mauchline TH (2012) Who's who in the plant root microbiome? *Nature Biotechnology* 30 (10): 961-962

Hofemeister J, Conrad B, Adler B, Hofemeister B, Feesche J, Kucheryava N, Steinborn G, Franke P, Grammel N, Zwintscher A, Leenders F, Hitzeroth G, Vater J (2004) Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide-like antibiotics, iron uptake and biofilm formation by *Bacillus subtilis* A1/3. *Mol. Gen. Genomics* 272: 363-378

Ĭnceoğlu O, Van Overbeek LS, Salles JF, Van Elsas JD (2013) Normal operating range of bacterial communities in soil used for potato cropping. *Applied and Environmental Microbiology* 79 (4): 1160-1170

Jaillais Y, Chory J (2010) Unraveling the paradoxes of plant hormone signaling integration. *Nature Structural & Molecular Biology* 17 (6): 642-645

Jeon JS, Lee SS, Kim HY, Ahn TS, Song HG (2003) Plant growth promotion in soil by some inoculated microorganisms. *The Journal of Microbiology* 41 (4): 271-276

Jung WJ, Mabood F, Souleimanov A, Whyte LG, Niederberger TD, Smith DL (2014) Antibacterial activity of antagonistic bacterium *Bacillus subtilis* DjM-51 against phytopathogenic *Clavibacter michiganense* subs. m*ichiganense* ATCC 7429 *in vitro. Microbial Pathogenesis* 77: 13-16

Kai M, Effmert U, Piechulla B (2016) Bacterial-plant-interactions: Approaches to unravel the biological function of bacterial volatiles in the rhizosphere. *Frontiers in Microbiology* 7 (108)

Kanchiswamy CN, Malnoy M, Maffei ME (2015) Chemical diversity of microbial volatiles and their potential for plant growth and productivity. *Frontiers in Plant Science* 6 (151)

Karatan E, Watnick P (2009) Signals, regulatory networks, and materials that build and break bacterial biofilms. *Microbiology and Molecular Biology Reviews* 73 (2): 310-347

Kearns DB, Chu F, Branda SS, Kolter R, Losick R (2005) A master regulator for biofilm formation by *Bacillus subtilis. Molecular Microbiology* 55 (3): 739-749

Kobayashi K, Iwano M (2012) BslA (YuaB) forms a hydrophobic layer on the surface of *Bacillus subtilis* biofilms. *Molecular Microbiology* 85 (1): 51-66

Kolodkin-Gal I, Romero D, Cao S, Clardy J, Kolter R, Losick R (2010) D-amino acids trigger biofilm disassembly. *Science* 328 (5978): 627-629

Kovács AT, Van Gestel J, Kuipers OP (2012) The protective layer of biofilms: A repellent function for a new class of amphiphilic proteins. *Molecular Microbiology* 85 (1): 8-11

Lam H, Oh DC, Cava F, Takacs CN, Clardy J, De Pedro MA, Waldor MK (2009) D-amino acids govern stationary phase cell wall re-modeling in Bacteria. *Science* 325 (5947): 1552-1555

Lemon KP, Earl AM, Vlamakis HC, Aguilar C, Kolter R (2008) Biofilm development with an emphasis on *Bacillus subtilis*. *Curr Top Microbiol Immunol* 322: 1-16

Liu Q, Qiao N, Xu X, Xin X, Han JY, Tian Y, Ouyang H, Kuzyakov Y (2016) Nitrogen acquisition by plants and microorganisms in a temperate grassland. *Scientific Reports* 6 (22642)

López D, Vlamakis H, Losick R, Kolter R (2009a) Paracrine signaling in a bacterium. *Genes and Development* 23: 1631-1638

López D, Vlamakis H, Losick R, Kolter R (2009b) Cannibalism enhances biofilm development in *Bacillus subtilis. Mol. Microbiol.* 74 (3): 609-618

Magalhães Cruz L, Maltempi de Souza E, Weber OB, Baldani JI, Döbereiner J, De Oliveira Pedrosa F (2001) 16S Ribosomal DNA characterization of nitrogen-fixing bacteria isolated from banana (Musa spp.) and pineapple (Ananas comosus (L.) Merril). *Applied and Environmental Microbiology* 67 (5): 2375-2379

Majeed A, Abbasi MK, Hameed S, Imran A, Rahim N (2015) Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. *Frontiers in Microbiology* 6 (198)

Marlow VL, Cianfanelli FR, Porter M, Cairns LS, Dale JK, Stanley-Wall NR (2014) The prevalence and origin of exoprotease-producing cells in the *Bacillus subtilis* biofilm. *Microbiology* 160: 56-66

Marvasi M, Visscher PT, Casillas Martinez L (2010) Exopolymeric substances (EPS) from *Bacillus subtilis*: polymers and genes encoding their synthesis. *FEMS Microbiol Lett* 313: 1-9

McLoon A, Kolodkin-Gal I, Rubinstein SM, Kolter R, Losick R (2011) Spatial regulation of histidine kinases governing biofilm formation in *Bacillus subtilis. Journal of Bacteriology* 193 (3): 679-685

Moe LA (2013) Amino acids in the rhizosphere: From plants to microbes. *American Journal of Botany* 100 (9): 1692-1705

Morita N, Takagi M, Murao S (1979) A new gel-forming polysaccharide produced by *Bacillus subtilis* FT-3. Its structure and its physical and chemical characteristics. *Bull Univ Osaka Pref Ser. B* 31: 27-41

Nakano MM, Zuber P (1998) Anaerobic growth of a "strict aerobe" (*Bacillus subtilis*). *Annu Rev Microbiol* 52: 165-190

Nazzaro F, Fratianni F, Coppola R (2013) Quorum sensing and phytochemicals. *Int. J. Mol. Sci.* 14: 12607-12619

Nibau C, Gibbs DJ, Coates JC (2008) Branching out in new directions: the control of root architecture by lateral root formation. *New Phytologist* 179: 595-614

O'Callaghan M (2016) Microbial inoculation of seeds for improved crop performance: issues and opportunities. *Appl. Microbiol. Biotechnol.* 100: 5729-5746

Okada M (2011) Post-translational isoprenylation of tryprophan. *Biosci. Biotechnol. Biochem.* 75 (8): 1413-1417

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environmental Microbiology* 9 (4): 1084-1090

Osera C, Amati G, Calvio C, Galizzi A (2009) SwrAA activates poly-γ-glutamate synthesis in addition to swarming in *Bacillus subtilis. Microbiology* 155: 2282-2287

Ostrowski A, Mehert A, Prescott A, Kiley TB, Stanley-Wall NR (2011) YuaB functions synergistically with the exopolysaccharide and TasA amyloid fibers to allow biofilm formation by *Bacillus subtilis. Journal of Bacteriology* 193 (18): 4821-4831

Petrova OE, Sauer K (2016) Escaping the biofilm in more than one way: desorption, detachment or dispersion. *Current Opinion in Microbiology* 30: 67-78

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* 5 (5): 308-316

Pineda A, Zheng SJ, Van Loon JJA, Pieterse CMJ, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. *Trends in Plant Science* 15: 507-514

Popham DL (2013) Visualizing the production and arrangement of peptidoglycan in gram-positive cells. *Molecular Microbiology* 88 (4): 645-649

R4P Network (2016) Trends and challenges in pesticide resistance detection. *Trends in Plant Science* 1445

Rasmann S, Turlings TCJ (2016) Root signals that mediate mutualistic interactions in the rhizosphere. *Current Opinion in Plant Biology* 32: 62-68

Romero D, Vlamakis H, Losick R, Kolter R (2014) Functional analysis of the accessory protein TapA in *Bacillus subtilis* amyloid fiber assembly. *Journal of Bacteriology* 196 (8): 1501-1013

Romero D, Vlamakis H, Losick R, Kolter R (2011) An accessory protein required for anchoring and assembly of amyloid fibers in *B. subtilis* biofilms. *Mol Microbiol* 80 (5): 1155-1168

Romero D, Aguilar C, Losick R, Kolter R (2010) Amyloid fibers provide structural integrity to *Bacillus subtilis* biofilms. *PNAS* 107 (5): 2230-2234

Rout ME (2014) The plant microbiome. *Advances in Botanical Research* 69

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloeppner JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in Arabidopsis. *Plant Physiology* 134 (3): 1017-1026

Santner A, Calderon-Villalobos LIA, Estelle M (2009) Plant hormones are versatile chemical regulators of plant growth. *Nature Chemical Biology* 5 (5): 301-307

Satapute PP, Olekar HS, Shetti AA, Kulkarni AG, Hiremath GB, Patagundi BI, Shivsharan CT, Kaliwal BB (2012) Isolation and characterization of nitrogen fixing *Bacillus subtilis* strain AS-4 from agricultural soil. *IJRSR* 3 (9): 762-765

Savci S (2012) An agricultural pollutant: Chemical fertilizer. *International Journal of Environmental Science and Development* 3 (1): 77-80

Scheurwater E, Reid CW, Clarke AJ (2008) Lytic transglycosylases: Bacterial space-making autolysins. *Molecules in Focus 40 (4): 586-591*

Schultz D, Wolynes PG, Jacob EB, Onuchic JN (2009) Deciding fate in adverse times: Sporulation and competence in *Bacillus subtilis. PNAS* 106 (50): 21027-21034

Seminara A, Angelini TE, Wilking JN, Vlamakis H, Ebrahim S, Kolter R, Weitz DA, Brenner MP (2012) Osmotic spreading of *Bacillus subtilis* biofilms driven by an extracellular matrix. *PNAS* 109 (4): 1116-1121

Senol M, Nadaroglu H, Dikbas N, Kotan R (2014) Purification of chitinase enzymes from *Bacillus subtilis* bacteria TV-125, investigation of kinetic properties and antifungal activity against *Fusarium culmorum. Annals of Clinical Microbiology and Antimicrobials* 13 (35)

Solanski MK, Robert AS, Singh RK, Kumar S, Pandey AK, Srivastava AK, Arora DK (2012) Characterization of mycolytic enzymes of *Bacillus* strains and their bio-protection role against *Rhizoctonia solani* in tomato. *Curr. Microbiol.* 65: 330-336

Spieß T, Korn SM, Kötter P, Entian KD (2015) Autoinduction specificities of the lantibiotics Subtilin and Nisin. *Applied and Environmental Microbiology* 81 (22): 7914-7923

Stanley NR, Lazzazera BA (2005) Defining the genetic differences between wild and domestic strains of *Bacillus subtilis* that affect poly-γ-DL-glutamic acid production and biofilm formation. *Molecular Microbiology* 57 (4): 1143-1158

Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular Microbiology* 56 (4); 845-857

Sturme MHJ, Kleerebezem M, Nakayama J, Akkermans ADL, Vaughan EE, De Vos WM (2002) Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie van Leeuwenhoek* 81: 233-243

Vishnoi M, Narula J, Devi SN, Dao HA, Igoshin OA, Fujita M (2013) Triggering sporulation in *Bacillus subtilis* with artificial two-component systems reveals the importance of proper Spo0A activation dynamics. *Molecular Microbiology* 90 (1): 181-194

Vlamakis H, Chai Y, Beauregard P, Losick R, Kolter R (2013) Sticking together: building a biofilm the *Bacillus subtilis* way. *Nat Rev Microbiol* 11 (3): 157-168

Vlamakis H, Aguilar C, Losick R, Kolter R (2008) Control of cell fate by the formation of an architecturally complex bacterial community. *Genes and Development* 22: 945-953

Weinert N, Piceno Y, Ding GC, Meincke R, Heuer H, Berg G, Schloter M, Andersen G, Smalla K (2011) PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: Many common and few cultivar-dependent taxa. *Fems Microbiol Ecol* 75: 497-506

Wu R, Grissom JE, McKeand SE, O'Malley DM (2004) Phenotypic plasticity of fine root growth increases plant productivity in pine seedlings. *BMC Ecology* 4: 14

Yan F, Yu Y, Wang L, Luo Y, Guo JH, Chai Y (2016) The *comER* gene plays an important role in biofilm formation and sporulation in both *Bacillus subtilis* and *Bacillus cereus. Frontiers in Microbiology* 7 (1025)

Yu C, Li X, Xhang N, Wen D, Liu C, Li Q (2016) Inhibition of biofilm formation by D-tyrosine: Effect of bacterial type and D-tyrosine concentration. *Water Research* 92: 173-179

Yu X, Ai C, Xin L, Zhou G (2011) The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on Fusarium wilt and promotes the growth of pepper. *European Journal of Soil Biology* 47 (2): 138-145

Yüksel M, Power JJ, Ribbe J, Volkmann T, Maier B (2016) Fitness trade-offs in competence differentiation of *Bacillus subtilis. Frontiers in Microbiology* 7 (888)

Zhou H, Luo C, Fang X, Xiang Y, Wang X, Zhang R, Chen Z (2016) Loss of GltB inhibits biofilm formation and biocontrol efficiency of *Bacillus subtilis* Bs916 by altering the production of γ-polyglutamate and three lipopeptides. *PLOS One* 11 (5)