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Sticking together: building a biofilm the *Bacillus subtilis* way

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Preface

Biofilms are ubiquitous communities of tightly associated bacteria encased in an extracellular matrix. *Bacillus subtilis* has long-served as a robust model organism to examine the molecular mechanisms of biofilm formation and a number of studies have revealed that this process is subject to a number of integrated regulatory pathways. In this Review, we focus on the molecular mechanisms controlling biofilm assembly and briefly summarize the current state of knowledge regarding their disassembly. We also discuss recent progress that has expanded our understanding of biofilm formation on plant roots, which are a natural habitat for this soil bacterium.

Introduction

> "At the surface of the liquid… …The rods adhere together by their sides after the manner of the elements of columnar epithelium, but there is, I think, strong reason to believe that this adhesion is not direct, i.e that they are not in actual contact but glued together by a viscous intermediary substance."

The "viscous intermediary substance" described here by Burton-Sanderson in 1870 is a hallmark feature of biofilms and the image he portrays is in all likelihood a *Bacillus subtilis* biofilm. From the dawn of microbiology this Gram-positive bacterium has been the subject of thorough investigation. Its capacity to sporulate and form biofilms was already beautifully described in the classic work of Ferdinand Cohn in 1877[1].

Biofilms are communities of surface-associated microorganisms encased in a self-produced extracellular matrix. Biofilm formation is a nearly universal bacterial trait and biofilms are found on almost all natural and artificial surfaces[2,3]. They are widely studied because they represent a fascinating example of microbial development and also because they can be problematic in many man-made settings[4-6]. In clinical settings they form on virtually any indwelling device and in industrial settings they often clog pipes and tubing[7]. But there is also interest in exploiting the beneficial aspects of biofilms; they play a major role in wastewater treatment and are potential sources of energy in the form of microbial fuel cells[8-10]. While most natural biofilms are polymicrobial communities, much has been learned about the basic biology of biofilms through the study of single species biofilms using model bacteria. Because of the clinical relevance of biofilms, most of the model systems that were initially studied involved pathogenic bacteria that were predominantly Gram-negative. For example, *Pseudomonas aeruginosa* is arguably the most studied bacterium in the biofilm field[7,11].

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Over the past decade, *B. subtilis*, which is a non-pathogenic Gram-positive bacterium, has emerged as an alternative model organism for studying the molecular basis of biofilm formation. A general schematic depicting the different stages of *B. subtilis* biofilm formation is shown in Figure 1. Within the biofilm, genetically identical cells express different genes and produce subpopulations of functionally distinct co-existing cell types. The process begins with the expression of matrix genes in response to some external signal (such as surfactin). Initially, cells are short, motile rods but as the biofilm develops, they form long chains of non-motile cells that adhere to each other and the surface by secreting an extracellular matrix[12-14]. This substance is essential to the integrity of the biofilm as it holds the community together[15-17]. As the biofilm matures the cell clusters enlarge and the community is protected and organized by the extracellular matrix. In addition to matrix producers, motile cells and spores are also present and are spatially organized within the maturing biofilm (reviewed in[18,19]). The presence and localization of the different cell types is dynamic and there appears to be an ordered sequence of differentiation in which motile cells become matrix-producing cells, which go on to become spores[20]. Importantly, this is not terminal differentiation; as conditions change it is possible for cells to alter gene expression (in the case of motile or matrix-producing cells) or germinate (in the case of the spores). Phenotypic heterogeneity in *B. subtilis* is not limited to these three cell types. The topic of heterogeneity and the processes that regulate this heterogeneity have been extensively covered in several reviews[19,21-24]. In laboratory conditions, biofilms have a limited life span and they eventually disassemble in response to self-generated signals[25,26]. As biofilms disassemble, spores are released from the matrix giving them the potential to disperse and encounter environmental conditions that are propitious for germination.

Much is known about the molecular mechanisms that regulate entry into biofilm formation, the structural components that comprise the extracellular matrix and how the biofilm eventually disassembles. In this review we describe the major features of *B. subtilis* biofilms with a focus on the various signals and mechanisms that regulate expression of the matrix genes, which induce biofilm formation. We also discuss recent findings relating to the secreted molecules produced by cells within the biofilm that target the extracellular matrix to disassemble the community. This research has shed light on the potential to control biofilm growth of other pathogenic bacteria. Finally, we discuss the use of plant roots as a natural habitat for the study of *B. subtilis* biofilms.

Biofilm morphology and structural components

Study conditions and biofilm morphology

Several laboratory conditions have been used to study *B. subtilis* biofilm formation including colonies at the air-agar interface, floating biofilms that form at the air-liquid interface (also termed pellicles) and, in the case of certain domesticated strains, submerged, surface-adhered biofilms that form at the liquid-solid interface (Fig. 2). Colony biofilms are produced when cells are placed on a solid agar surface containing a medium that promotes the expression of genes required for extracellular matrix production. Subsequent growth of the cells leads to the appearance of complex wrinkled colonies within a few days[13] (supplementary information S1 (movie)). Wrinkles form as a consequence of localized cell death coupled with the stiffness provided by the extracellular matrix[27]. The *B. subtilis* matrix is primarily composed of exopolysaccharide (EPS) and proteins (Table 1)[15,16]. This matrix, in conjunction with the rough surface topography, provides the biofilm with a remarkably hydrophobic surface that is largely impermeable to aqueous liquids and organic solvents[28]. In liquid conditions cells will either float to the surface of the liquid where they produce extracellular matrix and form a pellicle at the air-liquid interface, or remain under the surface of the liquid where they adhere to the side of the container and form a submerged biofilm. The particular type of biofilm and its robustness varies depending on the strain of *B.*

subtilis and the experimental conditions used (Box 1). In addition to the artificial methods described above, *B. subtilis* also forms biofilms on natural surfaces such as plant roots where the bacteria provide the plant with many benefits (Box 2).

In all biofilms, a series of morphological changes occurs in cells during biofilm development (Fig. 1). Although the number of motile cells decreases as the biofilm develops, a small subpopulation of motile cells remains, even in mature biofilms[20]. The role of these motile cells in *B. subtilis* biofilms varies depending on growth conditions. Motility-defective mutants that do not have flagella are delayed in forming pellicle biofilms[14] and are defective in the formation of submerged surface-adhered biofilms[29], however, their colony morphology is akin to that of the wild type[20,30]. Similarly, although some cells in the biofilm eventually sporulate, sporulation *per se* is not a requirement for biofilm formation[13,31].

Exopolysaccharide and polymer components

The major exopolysaccharide component required for each biofilm type is synthesized by the products of the *epsA-epsO* operon [13,32,33]. Mutations in the *eps* genes result in defective biofilm formation, as do mutations in *pgcA* (formerly *yhxB* - encoding α-phosphoglucomutase) and *gtaB* (encoding UTP-glucose-1-phosphate uridylyltransferase), which are involved in the production of nucleotide sugars that likely feed into the *eps* pathway[34,35]. Indeed, mutants defective in the synthesis of uridine diphosphate-galactose (UDP-Gal), which is a precursor metabolite required for EPS biosynthesis[36], are defective in biofilm formation. UDP-Gal is a toxic intermediate product in galactose metabolism that is normally converted to the non-toxic UDP-glucose by the UDP-glucose 4-epimerase GalE. When the *galE* gene is mutated, growth on galactose is toxic because UDP-Gal accumulates. Interestingly, *galE* mutants grown in biofilm-inducing conditions or *galE* mutants that overexpress the *eps* genes can survive even in the presence of galactose because the UDP-Gal is shunted into the EPS pathway[36].

Of the 15 genes in the *epsA-O* operon, only a subset has been studied individually[13,37-40]. The best-studied gene product of this operon, EpsE, is a bifunctional protein that coordinates the production of EPS with the cessation of motility[40]. In addition to displaying glycosyltransferase activity required for EPS synthesis, EpsE also functions as a molecular clutch that inhibits flagella rotation by interacting with the flagella rotor protein, FliG[40,41]. Motility inhibition occurs independently of the glycosyltransferase activity of EpsE. This remarkable mechanism of regulation ensures that cells shut off motility when matrix production occurs for biofilm formation. Interestingly, in colony biofilms, it is EPS and not motility that is important for colony spreading: EPS is thought to generate osmotic pressure gradients that allow the colony to spread outwards and thus acquire nutrients[30]. This could explain the growth defect observed in colonies of mutants that are unable to produce EPS[42].

Another extracellular polymer, γ-poly-DL-glutamic acid (PGA), is produced in copious amounts by some *B. subtilis* strains, and can enhance submerged biofilm formation[43,44]. However, PGA is not required for wrinkled colony morphology or pellicle formation[12,14].

Protein components

In addition to several uncharacterized proteins that are present within the matrix, two structural protein components have been described for *B. subtilis* biofilms: TasA and BslA. TasA was the first described protein component of the extracellular matrix of colony and pellicle biofilms[12]. TasA assembles into long amyloid-like fibres that are attached to the cell wall by the protein TapA (TasA anchoring/assembly protein; formerly YqxM)[45,46]. TapA is found in the cell wall fraction of cells grown as pellicles or colonies and it plays a role not

only in anchoring TasA fibres to the cell, but also in assembly of the amyloid fibres[46]. In addition to its cell wall localization, TapA can be purified as a minor component of the amyloid fibres[46]. These proteins are encoded by the *tapA-sipW-tasA* operon, which also encodes SipW, the signal peptidase that processes both TapA and TasA[47-49]. SipW processes TapA and TasA by recognizing an N-terminal signal sequence and cleaving the proteins as they are secreted so that they can be released from the membrane and become cell-wall associated fibers.

While they are essential in colony and pellicle biofilms, TasA and TapA are not required for submerged biofilm formation[50]. However, mutation of *sipW* in certain domesticated strains results in defective attachment to glass or polyvinyl chloride surfaces[12,50]. This is because SipW is a bifunctional protein whose signal peptidase activity can process TasA and TapA, but whose C-terminal domain actually functions to activate *eps* gene expression. This activation is essential for attachment and occurs only when cells are growing in a submerged surface-adhered mode. Consistent with this, overexpression of the *eps* operon is sufficient to restore submerged biofilm formation in a *sipW* mutant[33].

In addition to TasA, another secreted protein, BslA (formerly YuaB) is important for surface hydrophobicity, complex colony morphology and pellicle formation[51-54]. The biofilm-defective phenotype of a *bslA* mutant can be extracellularly complemented by mixing this mutant with a mutant lacking *eps* and *tasA*[55]. This observation suggests that EPS and TasA are provided by the *bslA* mutant and BslA is provided by the *eps tasA* mutant[55]. BslA forms a hydrophobic layer on the surface of biofilms and is termed BslA for biofilm surface layer protein[51]. It has amphiphilic properties and when purified, BslA forms polymers in solution when the air – surface interface is increased by the addition of bubbles[51]. However, it is currently unclear exactly how BslA functions to confer hydrophobicity to the biofilm surface.

Regulatory pathways that control biofilm formation

Given all of the components that are necessary to assemble the matrix, how does this bacterium regulate their production and assembly? Indeed, *B. subtilis* has a complex regulatory network to coordinate expression of matrix genes in response to the shifting environmental conditions that it encounters in its natural environment. Figure 3 is a simplified schematic of this network and the four numbered sub-networks represent four pathways that regulate the expression of matrix genes (see Table 1).

The Spo0A pathway

Spo0A is a central transcriptional regulator that controls the expression of over one hundred genes, including those necessary for biofilm matrix gene expression and sporulation[56,57]. The activity of this protein is regulated by phosphorylation at a single aspartate residue and both phosphorylated and unphosphorylated forms of Spo0A are always found in the cell. The concentration of phosphorylated Spo0A (Spo0A~P) in a given cell determines the gene expression profile and changes in its concentration facilitate differential gene regulation[56]. For example, intermediate levels of Spo0A~P result in matrix gene expression and higher levels induce sporulation genes. In this way, when Spo0A is initially phosphorylated biofilm formation is induced as a result of matrix gene expression. As the biofilm matures, Spo0A~P accumulates in certain cells and activates sporulation.

The concentration of Spo0A~P is determined by the activity of at least four kinases (KinA, KinB, KinC and KinD) that either act directly on Spo0A or indirectly via a phosphorelay[58]. The phosphorelay consists of Spo0F, which is phosphorylated by either KinA, KinB, KinC or KinD and then passes its phosphoryl group to Spo0B, which goes on to phosphorylate

Spo0A. There is a fifth kinase, KinE, which can also feed into this pathway, but it does not appear to play a role in matrix gene expression[59]. There are many levels of regulation within the phosphorelay and this topic has been reviewed previously[60,61]. No single kinase is solely responsible for matrix gene expression, but rather the contribution of different kinases changes depending on the signals present in the growth conditions being analyzed[31,59,62]. Specific signalling molecules that trigger phosphorylation of Spo0A by these kinases are discussed in more detail later in this review.

Spo0A~P governs the regulatory pathway for matrix gene expression by influencing the activity of the master regulator SinR, a repressor of the *epsA-O* and the *tapA* operons. Derepression of the matrix genes is accomplished by the action of the SinR antirepressor SinI, which is under the control of Spo0A~P (see below). In addition to the matrix genes, SinR also represses the regulatory gene *slrR* (Fig. 3A, subnetwork I)[32,63,64]. However, when SinI is expressed, it blocks SinR-mediated repression through the formation of a SinI-SinR protein-protein complex that renders SinR incapable of DNA binding[65]. SinR is produced in all cells but is only inactivated by SinI in a fraction of cells, thus only a subpopulation of cells expresses the *tapA* and *eps* operons[20,66].

In addition to determining which cells express matrix genes, Spo0A~P levels also determine the duration of matrix gene expression. The promoter of *sinI* contains both a high-affinity activator and multiple low affinity operators for Spo0A~P[66]. When Spo0A~P levels are relatively low, the high affinity activator is bound and *sinI* is expressed. As the levels of Spo0A~P increase, the lower affinity operators are also occupied and further *sinI* expression is curtailed[66]. Meanwhile, sporulation genes become activated by the high levels of Spo0A~P[56]. A second and embedded mechanism exists to turn off matrix genes once sporulation commences. The function of SinI and SinR are remarkably sensitive to gene dose: a mere doubling of the *sinI* and *sinR* genes completely blocks matrix production[67]. While actively dividing cells do not maintain two chromosomes for very long, in early sporulation there is a prolonged presence of two copies of the chromosome in the mother cell that results in two copies of *sinI* and *sinR* which is sufficient to inhibit matrix gene expression. Together, Spo0A~P affinity for the *sinI* promoter and gene copy number of *sinI* and *sinR* ensures that matrix gene expression is transient and that sporulating cells do not expend energy producing the extracellular matrix.

Spo0A~P also represses a second matrix gene repressor, AbrB[68]. Like SinR, AbrB represses both the *tapA* and the *epsA-O* operons[32,50,63,64,69]. Furthermore, AbrB represses expression of the matrix protein BslA[54] and the regulatory proteins SlrR[64] and Abh[69]. The presence of two Spo0A-regulated repressors, SinR and AbrB, with highly overlapping targets is likely a means to fine-tune the regulation of biofilm formation and to ensure the coordinated expression of all of the matrix genes.

The SlrR – SinR epigenetic switch

As mentioned above, SinR and AbrB inhibit expression of the regulatory protein SlrR[64,70,71]. SlrR is essential for the control of biofilm formation in two ways. First, SlrR binds to SinR (to form a SinR•SlrR complex), titrating SinR away and thus preventing it from repressing matrix gene promoters (*epsA-O, tapA-sipW-tasA* and *slrR* itself). Because SinR represses the *slrR* gene and the SlrR protein inhibits SinR, this results in a selfreinforcing double-negative feedback loop whereby the *slrR* gene remains de-repressed because SlrR prevents repression by SinR. Thus, once SlrR levels are high enough, they tend to stay high for many generations. In this state, the matrix genes are de-repressed because free SinR levels are low. Conversely, when SlrR levels are low, SinR is not inhibited and the matrix operons are switched off (Fig. 3B). Second, the complex of SinR•SlrR represses the promoters of the *hag* gene (which encodes flagellin and is required for motility), as well as

genes involved in cell separation (*lytABC* and *lytF*, which encode autolysins) (Fig. 3B) [72,73]. Thus, SlrR acts on SinR in two ways: it blocks it from repressing matrix gene and also re-purposes SinR in the form of the SinR•SlrR complex to become a repressor of autolysin and motility genes.

As mentioned earlier, cell chaining is essential at the onset of biofilm formation in *B. subtilis* (see Fig. 1). This cell chaining is achieved by a SinR•SlrR-mediated repression of genes encoding autolysins, which are required to separate chains of cells. SlrR is a member of the LexA family of autopeptidases and is proteolytically unstable and undergoes self-cleavage. In addition, cleavage of SlrR is dependent on the ClpCP protease. This instability results in the eventual degradation of SlrR and the derepression of genes encoding the autolysins, which allows the chains of cells to separate[72]. Inhibiting the separation of chains by using a non-cleavable mutant of SlrR[72] or by deleting three of the autolysins required for cell separation[14] does not alter pellicle formation. However, expression of the autolysin *lytC* prevents chaining and results in featureless colonies and pellicles containing cells that are delayed in sporulation[72].

As we have seen the SlrR-SinR switch can exist in two states: a state in which SlrR levels are low (corresponding to single, motile cells) and a state in which SlrR levels are high (corresponding to chains of matrix producing cells). How does the protein switch from the low state to the high state? This is accomplished by SinI, which is produced under the control of Spo0A~P in response to environmental signals that activate the histidine kinases that govern biofilm formation. SinI, in turn, is like SlrR, an antagonist of SinR that binds to and inhibits the SinR repressor. Thus, the production of SinI inhibits SinR, leading to the depression of the gene for SlrR. This results in the accumulation of SlrR to high levels and further inhibition of SinR by SlrR, driving the switch into an SlrR high state. Because the switch in self-reinforcing, it persists in the high state for many generations and can be said to be epigenetic.

The components of the epigenetic switch are subject to additional regulation from another pathway comprised of YwcC and SlrA (Fig. 3A, subnetwork II). SlrA is a paralogous antirepressor for SinR and functions similarly to SinI[70]. The *slrA* gene is repressed by YwcC, a TetR-type transcription repressor[70,71]. When YwcC receives an as-yet-unknown signal, *slrA* is derepressed and the matrix genes are induced by SlrA-mediated inactivation of SinR[70]. Unlike SinI, SlrA is produced in almost all cells, which transiently boosts matrix production in the entire population. In this sense, the YwcC-SlrA pathway might constitute a stress-response pathway to ensure that cells respond quickly to changing environmental conditions by forming a biofilm to protect the bacterial community[70].

Transcription of *slrR* is indirectly activated by the regulatory protein Abh (Fig. 3A, subnetwork III)[74]. The *abh* gene is itself repressed by AbrB[69] and its transcription is controlled by several extracytoplasmic function (ECF) sigma factors including σ^M, σ^X, and σ^W [14,74-78]. ECF sigma factors are activated by a variety of external stimuli including cell wall stresses and specific antibiotics[79], thereby providing a Spo0A-independent mechanism for responding to changes in external conditions.

Expression of *slrR* is positively regulated by several other proteins aside from Abh. For example, YmdB, a putative phosphoesterase, is necessary for *slrR* expression[80]. In addition, *slrR* gene expression also requires two small proteins RemA and RemB[81]. Genetic analyses show that RemA and RemB activate expression of the *eps* and *tapA* matrix operons via SlrR and in a manner independent of SlrR. The exact mechanism by which these small proteins function remains to be determined, however it appears that they act in parallel with SinR, AbrB and DegU (see below), the other known matrix gene regulatory proteins[81].

Other regulation

A fourth pathway that only regulates the expression of *bslA* and the *pgs* operon involves the DegS-DegU two-component system (Fig. 3A, subnetwork IV)[44,52,54,82]. In this system, DegS is a sensor histidine kinase that phosphorylates DegU, the response regulator. DegU is a global regulator in *B. subtilis* that is involved in the control of a variety of cellular processes such as competence, motility and secretion of degradative enzymes[83]. In addition, a *degU* mutant is defective in submerged biofilm formation, which requires the polymer PGA, the product of the enzymes encoded by the *pgs* operon [44]. Furthermore, colony biofilm formation is defective in a DegU mutant due to the loss of the surface hydrophobicity protein BslA[51,52,54,82].

Finally, in addition to the various transcription factors that are described above, the *epsA-O* operon is under the control of a cis-acting RNA element that is located between the second and third genes of the operon. This element is termed the "EAR" (*eps*-associated RNA element) and it is conserved among a subset of the Bacillales order. This element is thought to act as an anti-terminator and increases *eps* gene expression by interacting with RNA polymerase[84].

The foregoing list of regulators underscores the remarkably complex and multi-layered regulatory mechanisms controlling biofilm formation in *B. subtilis*. The particular regulators are specific for this organism and it is highly unlikely that we will find homologs with similar roles in other bacterial species. However, the take-home message is that such complex regulatory mechanisms are likely to have evolved to adequately respond to environmental changes by forming biofilms at the right time and under the right conditions.

Triggers of biofilm formation

As is clear from the previous section, much is known about the molecular mechanisms that regulate matrix gene expression. But what are the signals that trigger these pathways? Because of the numerous inputs to the system (the four histidine kinases, the ECF sigma factors, YwcC, and the DegS-DegU system) it is likely that many conditions exist that could trigger biofilm formation. As is described below, several signals and mechanisms that result in increased expression of extracellular matrix genes have been identified. The known mechanisms occur via two of the sensor kinases that phosphorylate Spo0A: KinC and KinD. Currently, the mechanisms by which the other kinases affect biofilm formation have not been elucidated. As we explore additional conditions that *B. subtilis* encounters in its natural environment, many more triggers for biofilm formation are likely to be identified.

KinC-mediated matrix gene expression

The first molecule identified as an inducer of matrix gene expression was surfactin, a molecule produced by constituent cells of the biofilm[62]. Surfactin has been studied for its potent surfactant and anti-microbial activities as well as its role in surface motility in *B. subtilis*[85-87]. Surprisingly, surfactin also acts as a signal that triggers phosphorylation of Spo0A via the sensor kinase KinC and positively regulates matrix gene expression[62]. Interestingly, surfactin is only produced by a sub-population of cells, and the cells that produce surfactin are not the same cells that respond to the molecule (i.e. those cells that express matrix genes or *sinI*, which are also only produced by a subset of biofilm cells)[88]. This concept represents a new way of thinking about self-generated quorum sensing signals in bacteria and has been referred to as paracrine signalling, whereby the signal is unidirectional and the signal producer does not respond to the signal it makes. This is in contrast to previously described quorum sensing systems where every cell in a population is thought to produce and respond to the signalling molecule (reviewed in [18,89]).

Induction of gene expression in response to surfactin does not occur by the canonical mechanism involving a sensor protein binding to a ligand. Instead of responding to the structure of surfactin, KinC is activated by the function of the molecule. Surfactin is a lipopeptide that inserts into the membrane and results in potassium leakage. This potassium leakage activates KinC by an unknown mechanism and the matrix genes are expressed[62]. Other compounds with different structures to surfactin that cause potassium leakage, such as the fungicide nystatin and the antibiotic valinomycin[62], also induce matrix gene expression via KinC. Importantly, molecules such as surfactin, nystatin and valinomycin are natural products produced by other organisms that reside in the soil, thus it is likely that *B. subtilis* encounters these molecules in nature. The fact that the antifungal agent nystatin, which functions by binding and displacing ergosterol in the membrane, affected signalling in *B. subtilis* led to the finding that *B. subtilis* harbors membrane microdomains analogous to lipid rafts in eukaryotes[90] (see Box 3). Chlorine dioxide, which is a potent biocide at high concentrations, induces matrix gene expression in a KinC dependent manner at sublethal concentrations[91]. Unlike surfactin, chlorine dioxide is thought to trigger KinC activation by collapsing the membrane potential of the cell. Thus it appears that KinC can be activated by at least two distinct membrane-disrupting mechanisms: potassium leakage and a decreased membrane potential. Increasing matrix production in the presence of membrane disruptors may be beneficial for *B. subtilis* survival; at least in the case of chlorine dioxide, wherein the presence of exopolysaccharide provides protection against the lethal effects of the molecule[91].

KinD-mediated matrix gene expression

Matrix itself appears to regulate matrix gene expression and mutants that are unable to produce EPS and TasA show prolonged expression of the promoters of the *eps* and *tasA* operons (as observed using transcriptional reporters) and delayed sporulation in biofilm conditions[20,42]. This is at least in part due to the activity of KinD. Like many other two component sensor kinases[92], KinD displays both kinase and phosphatase activity. KinD appears to function as a phosphatase to maintain low levels of Spo0A~P until matrix (or a component thereof) is sensed, at which point KinD functions as a kinase to promote sporulation[42]. Therefore, a checkpoint exists in which the differentiation into spores during biofilm conditions relies on the production of an extracellular matrix. KinD appears to be important in the response to extracellular matrix and it also activates matrix gene expression in response to compounds produced by soil microbes[93] and (as discussed in Box 2) in tomato root exudate[94].

Cannibalism to increase the matrix-producing sub-population

Matrix gene expression can also be increased by a non-signalling mechanism. In addition to activating the genes for extracellular matrix production, low levels of Spo0A~P also induce the expression of two “cannibalism” operons that produce secreted toxin peptides: SKF (for sporulation killing factor) and SDP (for sporulation delaying protein). Furthermore, these cells also express the resistance machinery for the toxins[95,96]. In the case of SKF, the exact mechanism of resistance is unclear, but requires an ABC transporter encoded within the operon that pumps the toxin out of the cell[96]. SDP resistance requires the membrane-associated immunity protein SdpI which is transcribed divergently relative to the gene encoding the toxin. SdpI is induced in the presence of the toxin but only in those cells that have high enough levels of Spo0A~P[97]. Thus the cannibal cells secrete toxins that kill siblings that are not expressing these genes. Because the cannibalism genes and matrix-producing genes are activated by low levels of Spo0A~P, the population of matrix-producing cells and cannibalism toxin-producing cells is highly overlapping[56,98]. Thus the same cells that have initiated matrix production also secrete toxins that effectively decrease

the population of non-matrix producers. This ultimately results in a population consisting of an amplified number of matrix-producing cells[98].

The cannibalism toxins do not only kill *B. subtilis* siblings. In fact, they preferentially kill different species when *B. subtilis* is grown in mixed cultures[99,100]. Consistent with this, cannibalism-like toxins secreted by close relatives of *B. subtilis* likely play a role in increasing the *B. subtilis* matrix-producing sub-population in mixed-species soil communities[93]. This was discovered in a screen for natural inducers of matrix gene expression in which a *B. subtilis* strain harboring a fluorescent reporter for matrix gene expression was co-cultured with soil organisms. Surprisingly, despite the diversity of bacteria in the soil samples, the vast majority of inducing organisms were other *Bacillus* species. In some instances, the inducing organisms produced a secreted molecule (cannibalism-like toxin) that preferentially killed non-matrix producing cells resulting in an increase in the matrix producing population[93]. The other inducing molecules required a functional KinD protein in the responding cells in order to be sensed.

In summary, diverse signalling molecules ranging from self-produced surfactin and cannibalism toxins to small molecules produced by other soil bacteria can trigger an increase in the number of matrix-producing cells in a population to stimulate biofilm formation. This can occur either via signalling, which results in differential gene expression, or by the selective killing of non-matrix producing *B. subtilis* cells.

Biofilm dispersal

Escaping the extracellular matrix

This review has focused on the process of building a biofilm. However, as the biofilm matures, it may be beneficial for the constituent cells to disperse owing to resource limitation and waste product accumulation[101,102]. One mechanism that *B. subtilis* has exploited to escape biofilms is the release of D-amino acids, a stationary phase phenomenon that naturally occurs in a number of organisms[26,103]. Cells in mature *B. subtilis* biofilms release a mixture of D-amino acids (D-tyrosine, D-leucine, D-tryptophan and D-methionine), which results in dissolution of the mature biofilm or inhibition of biofilm formation[26]. Furthermore, the accumulation of D-amino acids appears to be regulated by racemase enzymes (which catalyze the stereochemical conversion of L- to D-amino acids), as mutations in the encoding genes result in significantly delayed biofilm disassembly[26]. D-amino acids disrupt biofilm formation by becoming incorporated into peptidoglycan and thereby altering the association of certain proteins to the cell wall, including TapA, the protein that anchors TasA fibres to the cell wall. Thus, D-amino acids result in the release of the TasA amyloid fibres from the cell[26,46].

In addition to D-amino acids, the supernatant of aging *B. subtilis* biofilms also harbors the polyamine norspermidine, which efficiently disperses biofilms[25]. The inhibitory activity of norspermidine is synergistic with that of D-amino acids suggesting that it acts by a different mechanism. Indeed, norspermidine does not affect association of TasA to the cell wall; instead, it interacts directly and specifically with the exopolysaccharide component of the matrix[25]. This interaction results in a collapse of the EPS as visualized by microscopy and a change in cell size using light scattering[25]. This biofilm disrupting activity is also a feature of other polyamines such as norspermine, which harbor three methylene groups flanked by two amino groups. Similar molecules such as spermidine and spermine (which have an additional methylene group) do not exhibit inhibitory activity and it was proposed that the additional methelyne group alters the interaction with EPS[25]. Disrupting genes that interfere with norspermidine production result in more robust biofilms[25]. Interestingly, spermidine, which is also produced by *B. subtilis*, has the opposite effect; disrupting spermidine

biosynthesis results in less-robust biofilms and this phenotype can be rescued by the addition of exogenous spermidine[104]. Although spermidine and norspermidine are similar in a chemical sense, different genes are required for the biosynthesis of each and it is currently unknown how exactly spermidine functions to enhance biofilm formation or how the production of these two molecules is regulated. However, it is clear that *B. subtilis* is able to modulate biofilm formation using a number of secreted compounds.

Controlling biofilms in other species with small molecules

Efforts to decipher the molecular mechanisms regulating biofilm formation in *B. subtilis* have led to the discovery of a number of compounds that could function as general biofilm inhibitors. For example, D-amino acids are also able to disrupt biofilm formation in the pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*[26]. Similar to what was observed in *B. subtilis*, where D-amino acids disrupt TapA association with the cell wall, D-amino acids also prevent surface localization of proteins in *S. aureus*[105]. The molecular basis for the inhibitory effect of D-amino acids on biofilm formation in Gram-negative bacteria is currently unknown. However, a recent study that analyzed the effect of D-amino acids on *P. aeruginosa* strain PAO1 flow cell biofilms showed a lethal effect on cells as well as an increase in extracellular matrix production and no biofilm inhibition. This suggests that there is condition or strain specificity in the response, at least in *P. aeruginosa* [106].

The polyamines norspermine and norspermidine are also potent inhibitors of biofilm formation in *S. aureus* and *Escherichia coli* [25]. Studies have yet to be performed showing exactly what the targets of the polyamines are in these systems, however it is likely that the EPS component of the matrix is altered in the presence of the polyamines.

In a similar vein, inhibitors of key lipid synthesis enzymes (such as the squalene synthase inhibitor zaragozic acid (see box 3) and cholesterol-lowering statins) are effective against biofilm formation in *S. aureus*. At the same time, these molecules also disrupt the production of virulence factors such as proteases and the carotenoid staphyloxanthin[90]. This might contribute to the ability of cholesterol biosynthesis inhibitors to block *S. aureus* virulence[107].

Importantly, zaragozic acid, D-amino acids and polyamines do not inhibit growth of the target Gram-positive organisms. This is an appealing feature of these compounds as their use should reduce the selection pressure for the emergence of resistant mutants, which is associated with traditionally used antibiotics. Since many nosocomial infections are associated with biofilm formation, these molecules might represent a promising alternative to antibiotics[101,108]. Alternatively, using these compounds to disrupt biofilms prior to treatment with other antimicrobials could provide a more effective means of eliminating harmful bacteria.

Concluding remarks

B. subtilis, a bacterium for which a multitude of genetic and cell-biology tools have been developed, has proven to be an ideal model organism for characterizing the molecular mechanisms underpinning biofilm formation and disassembly. *B. subtilis* has evolved a number of regulatory pathways that trigger and control biofilm formation. Among these, activation of Spo0A is central for the induction of matrix gene expression in response to a wide variety of extracellular signals. Matrix producers also use cannibalism to amplify the population of matrixproducing cells and this appears to be a more general feature observed when *B. subtilis* is in contact with close relatives that produce similar cannibalism-like molecules. This leads to enhanced biofilm formation in the presence of potential competition. It is very likely that as *B. subtilis* is studied in more complex environments,

such as on plant roots or in the presence of other bacteria, even more mechanisms to induce matrix gene expression will be discovered. Understanding how cells are able to form biofilms at the appropriate time in the presence of diverse inputs is the next step in enhancing our understanding of biofilm formation in this organism. Another major challenge will be to decipher how the many regulatory pathways, often with overlapping outputs, converge to control matrix gene expression. Perhaps in different conditions only subsets of matrix genes are important (such as the dispensability of TasA in submerged biofilms) and this is why such complexity in regulation has evolved.

Unlike the abundance of information that has been amassed on the regulation of biofilm assembly, studies on biofilm disassembly in *B. subtilis* are still in their infancy. There are many questions that remain to be addressed. How is the production of small molecules such as norspermidine and D-amino acids regulated to ensure that biofilms do not disassemble at inappropriate times? Is their production regulated by even more external stimuli? What are the exact mechanisms by which these molecules exert their inhibitory effects?

Many features, such as the requirement for extracellular matrix components comprising exopolysaccharides and proteins, are general for the formation of all bacterial biofilms. This has prompted the successful use of small molecules that disrupt *B. subtilis* biofilms for the targeting of biofilms produced by pathogenic organisms including *S. aureus*. Much remains to be done as far as understanding how the biofilm disassembly factors identified in *B. subtilis* are able to inhibit biofilm formation in other diverse organisms. Despite the many outstanding mechanistic questions, it is exciting to imagine how combinatorial therapy, such as the coupling of these molecules with improved antibiotics, has the potential for more successful eradication of detrimental biofilms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Definitions

Biofilms	Surface-associated communities of microbial cells that are encased in a self-produced extracellular matrix composed of exopolysaccharide, protein and occasionally DNA.
Bimodality	In statistics, bimodality represents a continuous probability distribution with two different modes, appearing often as two distinct peaks in the probability density function.
Population heterogeneity	A population of genetically identical cells that demonstrate heterogeneous phenotypes.
Epigenetic switch	A regulatory switch that can impose heritable changes in gene expression or phenotype caused by mechanisms other than genetic changes.

Chaining	Occurs when daughter cells divide but are remain connected by polar cell wall peptidoglycans.
Biocontrol	A method to protect against disease using biological control agents such as beneficial bacteria.
Rhizosphere	A narrow region of soil that surrounds the root of a plant and is directly influenced by plant root secretions and associated soil microorganisms.
TetR-type transcriptional repressor	Two-domain proteins with a DNA-binding domain and a small ligand-binding domain. Normally, repression is relieved upon binding of a small molecule to the ligand-binding domain.
Cannibalism	In the context of *B. subtilis*, this term is used to indicate that in a genetically identical population some cells secrete toxins that kill siblings that are not expressing the genes encoding resistance to these toxins.

Biographies

Hera Vlamakis received her B.S. from the University of IL at Urbana-Champaign and her Ph.D. from the University of CA at Berkeley. She currently holds an instructor position in Roberto Kolter's lab at Harvard Medical School and she is interested in how individual bacteria coordinate their behaviors in complex communities.

Yunrong Chai received his Ph.D in microbiology from Cornell University, studying bacterial quorum sensing mechanisms. He then carried out postdoctoral training with Dr. Richard Losick at Harvard University, working on biofilm formation in *Bacillus subtilis*. Yunrong Chai is now an assistant professor in the Biology Department at Northeastern University.

Pascale B. Beauregard received her Ph.D. under the supervision of Luis Rokeach (Université de Montréal, Quebec, Canada) where she worked on a prion-like phenomenon in yeast. She is now a post-doctoral fellow in Roberto Kolter's laboratory (Harvard Medical School, Massachusetts), where she studies *Bacillus subtilis* biofilm formation on plant roots.

Richard Losick received his A.B. in Chemistry at Princeton University and his Ph.D. from MIT. He then moved to Harvard University where he is a Professor. He studies spore and biofilm formation by *Bacillus* subtilis and the role of RNA polymerase sigma factors in the control of gene transcription.

Professor Roberto Kolter studied at Carnegie-Mellon University (B.S.), UCSD (Ph.D.), and Stanford (post-doc). He has been a faculty member at Harvard Medical School since 1983. There he has worked on antibiotic synthesis, bacterial starvation physiology, experimental evolution, bacterial biofilms, and chemical communication in the microbial world.

Box 1

Domestication of lab strains

As cells are passaged in liquid culture in the laboratory, mutations can arise that decrease the ability of these cells to form biofilms. For example, Branda *et al*. analyzed a common laboratory strain (PY79) and a "less domesticated" strain (NCIB 3610), which is a close relative of PY79[109]. NCIB 3610, forms robust wrinkled colonies (a hallmark of biofilm formation) and floating pellicle biofilms in a defined medium, but adheres poorly to glass surfaces (See Fig. 2 and movie of colony growing)[12,13]. At the same time, Hamon and Lazzazzera developed a submerged surface-adhered biofilm assay with the commonly used lab strain JH642[31]. This strain formed submerged surface-adhered biofilms on polyvinylchloride (PVC) and glass surfaces, but this strain and its close relatives (strains 168 and PY79) do not produce robust wrinkled colonies[13,110]. All of these strains are extremely similar at the nucleotide level, thus the specific genotypic differences that conferred these phenotypes were investigated. Using gross morphology of complex colony wrinkling (which is easily observed by visual screening of colonies on agar plates) as a read-out for matrix production, five specific genes were identified as being central to this process. Point mutations in four genes *sfp*, *epsC*, *degQ*, and *swrA* and the lack of *rapP*, a regulatory gene found on the plasmid of strain NCIB 3610 were responsible for the diminished matrix gene expression in the domesticated strain 168 relative to NCIB 3610[110]. The *sfp* gene encodes a phosphopantetheinyl transferase that is required for surfactin production and the point mutation in strain 168 impairs surfactin production. The *epsC* gene is in the *epsA – O* operon and the point mutation results in a decrease in EPS production. Both surfactin and EPS are required for biofilm formation. DegQ is a small protein that stimulates phosphotransfer from DegS to DegU and has previously been shown to be involved in biofilm formation[44,52]. SwrA is a regulatory protein that is important in swarming motility and PGA synthesis[44,52]. The mechanism by which RapP regulates biofilm formation has yet to be determined, although this protein is similar at the amino acid level to a family of regulatory proteins that antagonize response regulators, including Spo0F, a member of the Spo0A phosphorelay[111]. It is possible that the effect of RapP is mediated by interactions with a regulatory protein. More specific details regarding the genome differences of different *B. subtilis* strains have been discussed in several papers[109,112].

Box 2

Plant roots as a natural habitat for B. subtilis biofilm formation

In order to proliferate in the soil, *B. subtilis* requires a nutrient source such as decaying organic material or plant roots[113]. The rhizosphere, or region of soil directly surrounding plant roots, is rich in plant secretions that can provide bacteria with nutrients[114-116]. Bacteria in the rhizosphere can benefit the plant, and *Bacillus* species - including *B. subtilis* - are sold commercially as biological control agents for agriculture[115,117,118]. *Bacillus* species can promote growth and protect plants from infections by pathogenic bacteria, fungi and even nematodes. This protection is due to the secretion of antimicrobial compounds by *B. subtilis*, coupled with induced systemic resistance in response to *B. subtilis* that enhances the capacity of the plant to resist various pathogens[119-123].

B. subtilis is readily isolated from the rhizosphere of plants and the majority of root-associated strains are capable of forming robust biofilms in laboratory conditions[124,125]. In addition, several other *Bacillus* species form biofilms on plant roots[126-128]. As shown in the figure, biofilm formation on plant roots parallels *in vitro* biofilm formation in that the matrix exopolysaccharide is required ([94,125] and P. Beauregard unpublished results). Similarly, the master regulator Spo0A and the antirepressor SinI are also required for root colonization ([125] and P. Beauregard unpublished results). In many wild isolates, the presence of these genes, and thus the capacity to form a biofilm on the root, is also required for the strain to exert its maximal biocontrol effect[125].

*B. subtilis c*olonization of *Arabidopsis thaliana* roots also requires the production of surfactin, a lipopeptide antimicrobial that is also important for biofilm formation *in vitro*[126]. The production of surfactin and other lipopeptides by *Bacillus* cells is one of main mechanisms for plant biocontrol since these molecules can induce systemic resistance as well as strongly inhibit the growth of common plant pathogens such as *Pseudomonas syringae*[125,126,129].

To recruit *B. subtilis*, plants secrete small molecules. For example, when *A. thaliana* is infected with *P. syringae*, malic acid is secreted and this enhances *B. subtilis* biofilm formation on the root[127]. Furthermore, root exudates from *P. syringae*-infected plants or purified malic acid induce matrix gene expression in *B. subtilis*[127]. This phenomenon is not specific to *A. thaliana*, malic acid is also found in tomato root exudates and, at high concentrations, can stimulate matrix gene expression and biofilm formation *in vitro*[94]. Tomato root exudates stimulate matrix gene expression in a manner that is dependent on the Spo0A kinase KinD. Mutants specifically lacking the extracellular CACHE domain of KinD are less efficient colonizers of tomato roots[94].

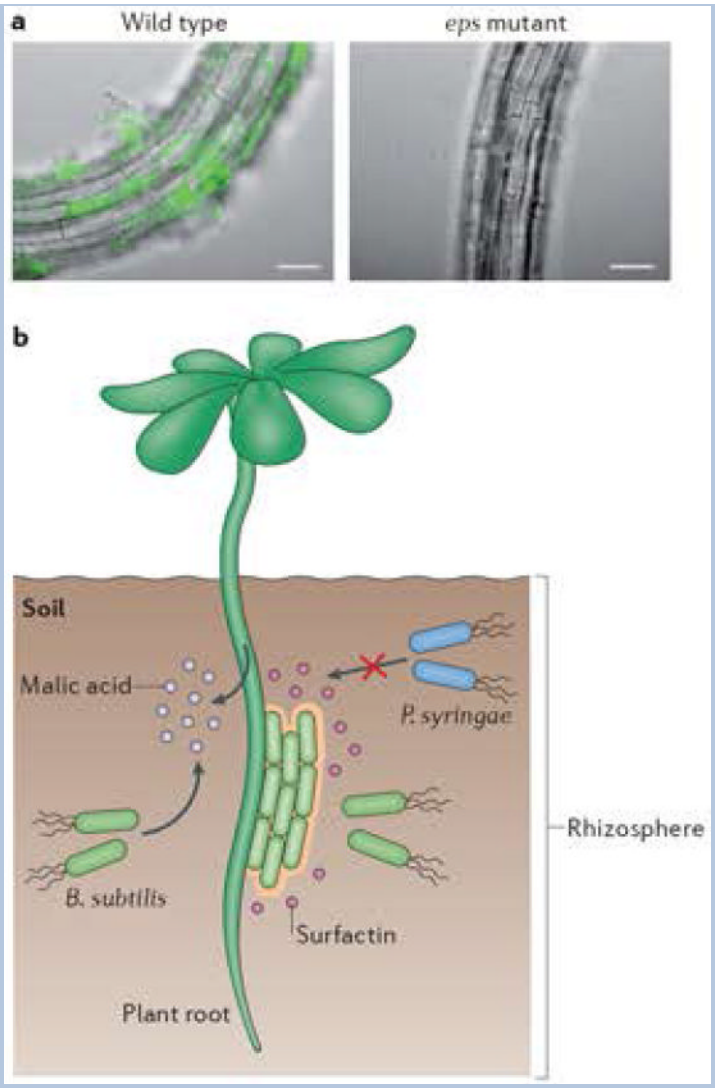

Figure.

A) Wild-type *B. subtlis* cells or an *eps* mutant constitutively expressing YFP were inoculated with 6 day-old seedlings of *A. thaliana*. Colonization of the root was observed after 24h. Overlays of fluorescence (false-coloured green) and transmitted light images (gray) are shown. Bars are 50 μm. Images in A) by P. Beauregard (unpublished). B) Schematic illustration of *B. subtilis* colonizing a plant root. *B. subtilis* secretes the lipopetide surfactin, which is required for *B. subtilis* (green rods) biofilm formation on the root. A second trigger for *B. subtilis* matrix gene expression is malic acid, which is constitutively secreted in the rhizosphere by tomato plants but secreted by *A. thaliana* only when the plant is infected with the pathogen *P. syringae*. *B. subtilis* exerts beneficial effects on the plant by promoting its growth and helping to fight of pathogens (such as *P. syringae*), directly via the secretion of surfactin and other antimicrobials and indirectly by eliciting induced systemic resistance in the plant.

Box 3

Lipid rafts coordinate signalling molecules in bacterial membranes

Membrane microdomains, which are analogous to the cholesterol-rich lipid rafts found in eukaryotic cells, also exist in bacteria. *B. subtilis* membranes do not contain sterols, however, lipids that are synthesized by the same precursor (isoprenyl pyrophosphate) are present. Different lipid components of the membrane can be separated by their ability to withstand detergent treatment and proteins found in detergent-resistant microdomains can be purified from the membrane of *B. subtilis* using techniques similar to those used for the purification of detergent-resistant lipids in eukaryotes[88,124]. KinC localizes in these microdomains along with a variety of other proteins involved in signalling[90]. In addition, FloT (formerly YuaG) and YqfA, two homologs of the eukaryotic lipid raft protein Flotillin-1, are associated with the detergent-resistant microdomains and localize in a punctate pattern throughout the membrane[90,130,131]. The figure shows a FloT-YFP protein fusion localized in puncta throughout the *B. subtilis* membrane. A mutant in the gene *yisP*, which encodes a squalene synthase that is required for the production of the detergent-resistant lipids is defective in biofilm formation. Furthermore, known inhibitors of squalene synthase, such as zaragozic acid, disrupt these domains and inhibit biofilm formation. The series of images show dissipation of FloT-YFP puncta over time after treatment with zaragozic acid. By eight hours, all of the FloT-YFP puncta have dispersed[90]. As is discussed at the end of this review, this knowledge led to studies in other bacteria where the disruption of membrane microdomains using inhibitory molecules of lipid biosynthesis also inhibited biofilm formation and blocked the production of several virulence factors.

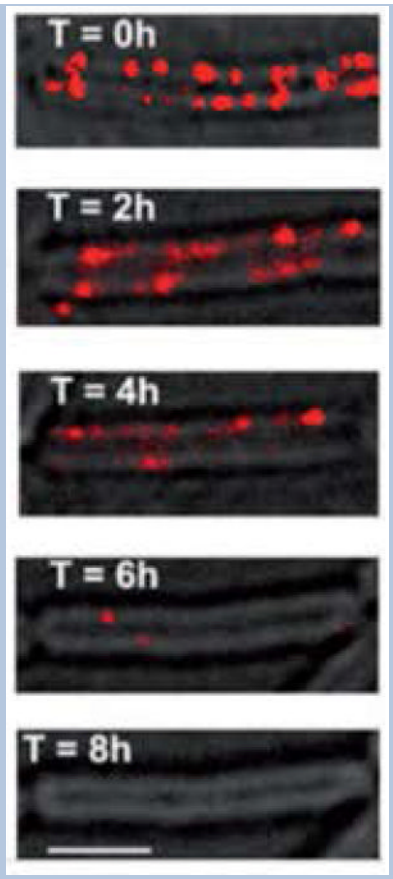

Figure.
FloT-YFP localizes in a punctate pattern in the cell membrane and is disrupted by inhibitors of squalene synthase. Fluorescence image (false-coloured red) is overlayed on the transmitted light image. An untreated cell is shown (top image) and a series of images taken over a period of 8 h after treatment with zaragozic acid. Scale bar is 2 μm. Images from [90].

Online summary

- *B. subtilis* is a model non-pathogenic bacterium that has been used to study biofilms. These ubiquitous communities of tightly associated bacteria are encased in a selfproduced extracellular matrix that holds cells together.
- Within a biofilm, genetically identical *B. subtilis* cells differentiate and cells expressing different sets of genes serve distinct functions.
- *B. subtilis* cells are able to sense and respond to diverse extracellular cues using a complex regulatory network to ultimately express matrix genes and form a biofilm. These cues range from self-produced signals, such as surfactin, to natural products produced by other organisms found in the soil.
- Several self-produced molecules are secreted late during the life cycle of a biofilm to trigger disassembly of the community. These molecules appear to have properties that are active against biofilms formed by other bacteria.
- *B. subtilis* naturally colonizes plant roots and is commonly used as a biocontrol agent. Biofilm formation is important for plant root colonization and protection.

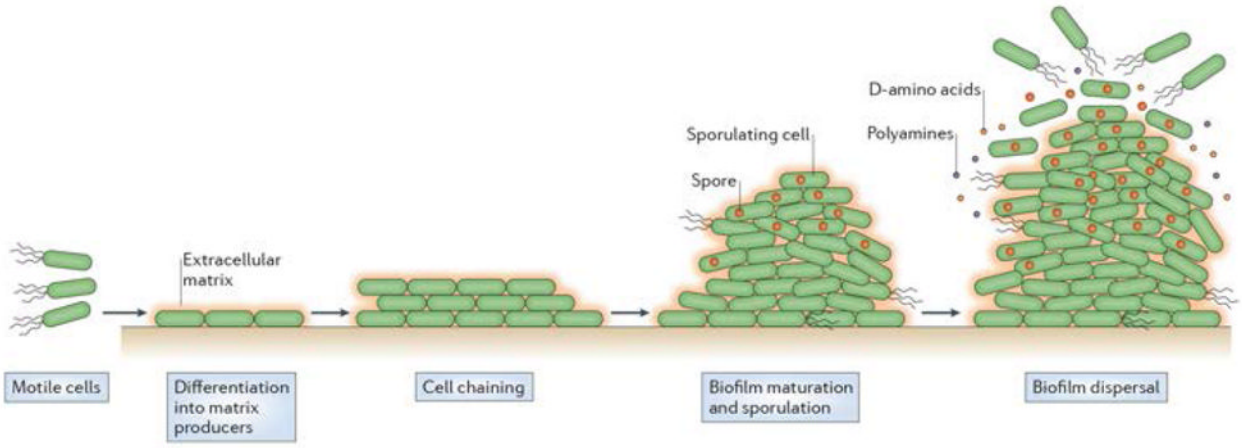

Figure 1.

The life-cycle of a *Bacillus subtilis* biofilm. This process occurs in stages which comprise the development, maturation and disassembly of the community. Motile cells with flagella differentiate into non-motile matrix-producing cells that become organized in chains and are surrounded by extracellular matrix (orange). In mature biofilms matrix-producing cells sporulate (dark brown spores). In aged biofilms, some cells secrete small molecules such as D-amino acids and polyamines that break down the extracellular matrix and the cells disperse in the environment.

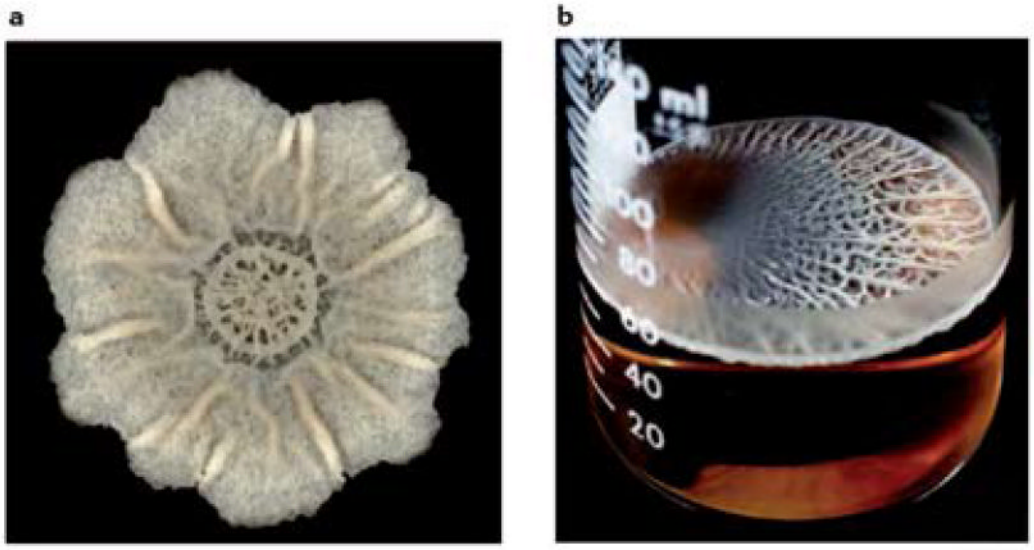

Figure 2.

A) Top-down view of a colony grown at room temperature on biofilm-inducing medium (MSgg) for 7 days. Scale bar is 2 mm. A time-lapse movie of the growth of this colony can be viewed in the supplemental material (H. Vlamakis, unpublished movie). B) Top-down view of a pellicle grown at room temperature for 5 days (image from [13]).

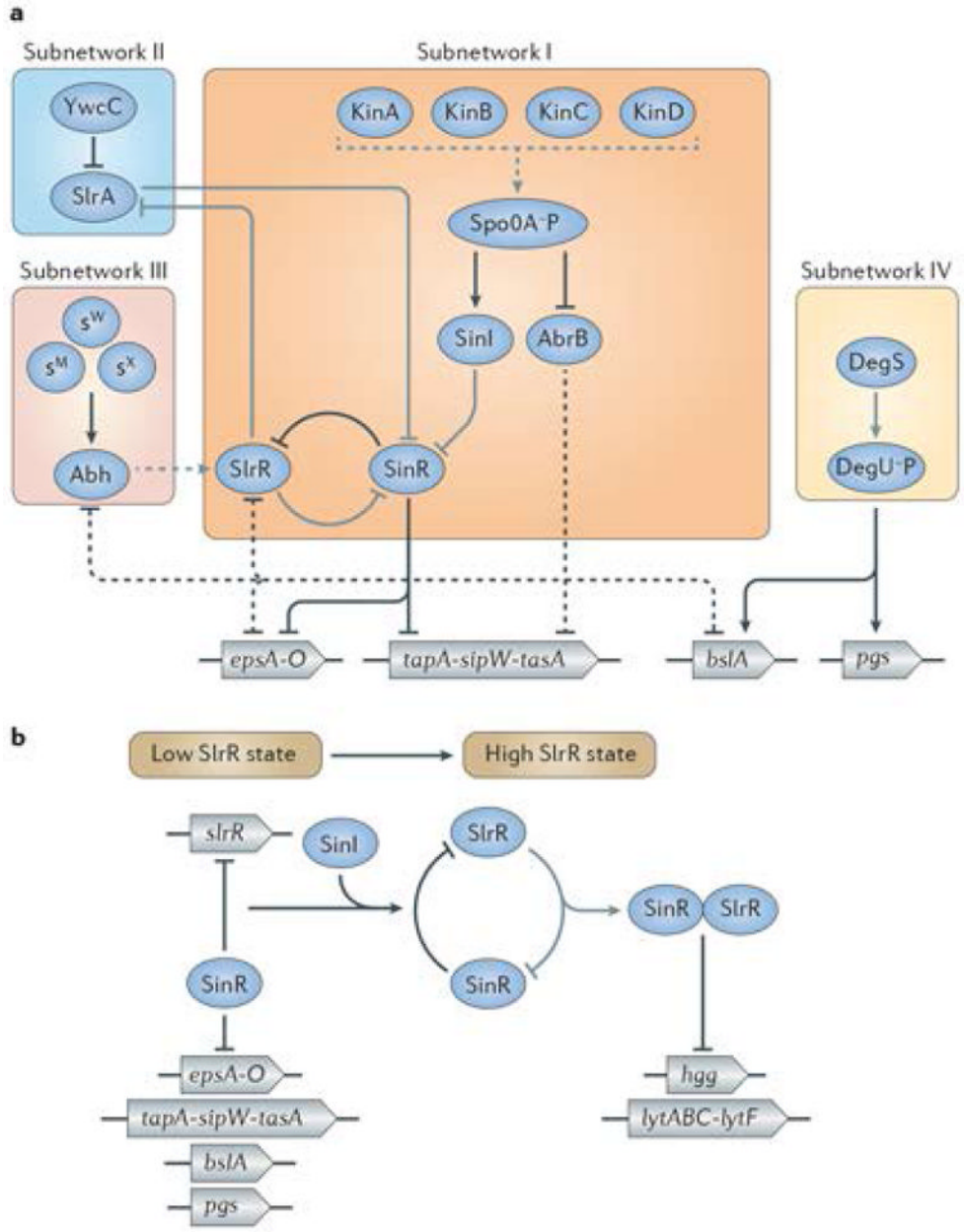

Figure 3.

Simplified schematic of the regulatory network that controls biofilm formation in *B. subtilis*. A) Several sub-networks (I-IV) are integrated to activate (arrows) or repress (T-bars) matrix gene expression depending on the environmental condition. Details are discussed in the text. The genes encoding components of the extracellular matrix are coloured in blue and encode: EPS (*epsA*-O), TasA (*tapA-sipW*-tasA), BslA (*bslA*), and PGA (*pgs*). Red and pink lines indicate gene regulation whereas yellow lines indicate protein-protein interactions. Dashed lines indicate indirect activity. B) The double negative feedback loop (involving the *slrR* gene, the SlrR protein and the SinR protein) exists in SlrR low (left side of figure) and SlrR high (right side of figure) states. The SinR–SlrR switch regulates matrix genes (*epsA*-O and *tapA-sipW-tasA*), autolysin (*lytABC* and *lytF*) and motility genes (*hag*), as well as the gene (*slrR*) for SlrR itself. In the SlrR low state (left) the SinR protein represses the *slrR* gene,

keeping the levels of the SlrR protein low. In the SlrR high state (right), SlrR binds to SinR, trapping it in the heteromeric SinR•SlrR complex. This titrates SinR, resulting in derepression of matrix genes and *slrR*, setting up a self-reinforcing switch leading to high SlrR levels. At the same time, SlrR repurposes SinR in that the SinR•SlrR complex represses autolysin and motility genes (right). Image adapted from[72]. The double negative loop is epigenetic in that both the SlrR low and SlrR high states self-reinforcing and are stable for many generations. During biofilm formation, SinI produced under the control of Spo0A~P (see text) drives the switch into the high state by binding to and inhibiting SinR.

Table 1

Genes involved in extracellular matrix production.

Gene	Role in matrix production	Mutant phenotype
epsA – O operon	Enzymes that produce exopolysaccharide (EPS).	Flat colonies (Δ operon, *epsE* [41], *epsG* [13], *epsH* [13]; other genes not tested) Thin pellicles (Δ operon, *epsE* [41], *epsG* [13], *epsH* [13]; other genes not tested) Defective submerged biofilm (*epsG* [33]; other genes not tested)
tapA (formerly *yqxM*)	TasA accessory/anchoring protein. Minor component of TasA fibers.	Flat colonies [12] Thin pellicles [12] Wild type submerged biofilm [50]
sipW	Bifunctional signal peptidase required for TapA and TasA processing and secretion and for *eps* gene expression.	Flat colonies [12] Thin pellicles [12] Defective submerged biofilm [50]
tasA	Major protein component of TasA fibers.	Flat colonies [12] Thin pellicles [12] Wild type submerged biofilm [50]
pgsB (formerly *ywsC*)	First gene in operon that encodes enzymes that produce γ-poly-DL-glutamic acid (PGA).	Wild type colonies [12] Wild type pellicles [12] Over expression enhances submerged biofilms [44]
bslA (formerly *yuaB*)	Surface hydrophobic layer protein, BslA.	Flat colonies [55] Thin pellicles [52,55] Untested in submerged assay