Sticking together: Building a biofilm the Bacillus subtilis way

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

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Abstract | 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 regulated by several integrated pathways. In this Review, we focus on the molecular mechanisms that control *B. subtilis* biofilm assembly, and then briefly summarize the current state of knowledge regarding biofilm disassembly. We also discuss recent progress that has expanded our understanding of *B. subtilis* biofilm formation on plant roots, which are a natural habitat for this soil bacterium.

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, that is, that they are not in actual contact but glued together by a viscous intermediary substance. Burton-Sanderson[1]

The "viscous intermediary substance" described here by Burton-Sanderson in 1870 is a hallmark feature of biofilms, and the image he describes is in all likelihood a *Bacillus subtilis* biofilm. From the dawn of microbiology, this Gram-positive bacterium has been the subject of thorough investigation; indeed, its capacity to sporulate and form biofilms was beautifully described in the classic work of Ferdinand Cohn in 1877 (REF. 2).

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[3,4]. 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[5–7]. In clinical settings, they form on virtually any indwelling device, and in industrial settings, they often clog pipes and tubing[8]. But there is also interest in exploiting the beneficial aspects of biofilms; they have a major role in wastewater treatment and are potential sources of energy in the form of microbial fuel cells[9–11]. Although 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 Gram-negative pathogenic bacteria. For example, *Pseudomonas aeruginosa* is arguably the most studied bacterium in the biofilm field[8,12].

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 FIG. 1. Within the biofilm, genetically identical cells express different genes and produce subpopulations of functionally distinct, coexisting cell types. The process begins with the expression of matrix genes in response to an external signal, such as the lipopeptide 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 to the surface by secreting an extracellular matrix[13–15]. This substance is essential to the integrity of the biofilm, as it holds the community together[16–18]. 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 present and are spatially organized within the maturing biofilm (reviewed in REFS 19,20). The presence and localization of the different cell types is dynamic, and there seems to be an ordered sequence of differentiation such that motile cells become matrix-producing cells, which go on to become spores[21]. Importantly, this process of differentiation is not terminal; as environmental conditions change, it is possible for cells to alter their gene expression (in the case of motile or matrix-producing cells) or to germinate (in the

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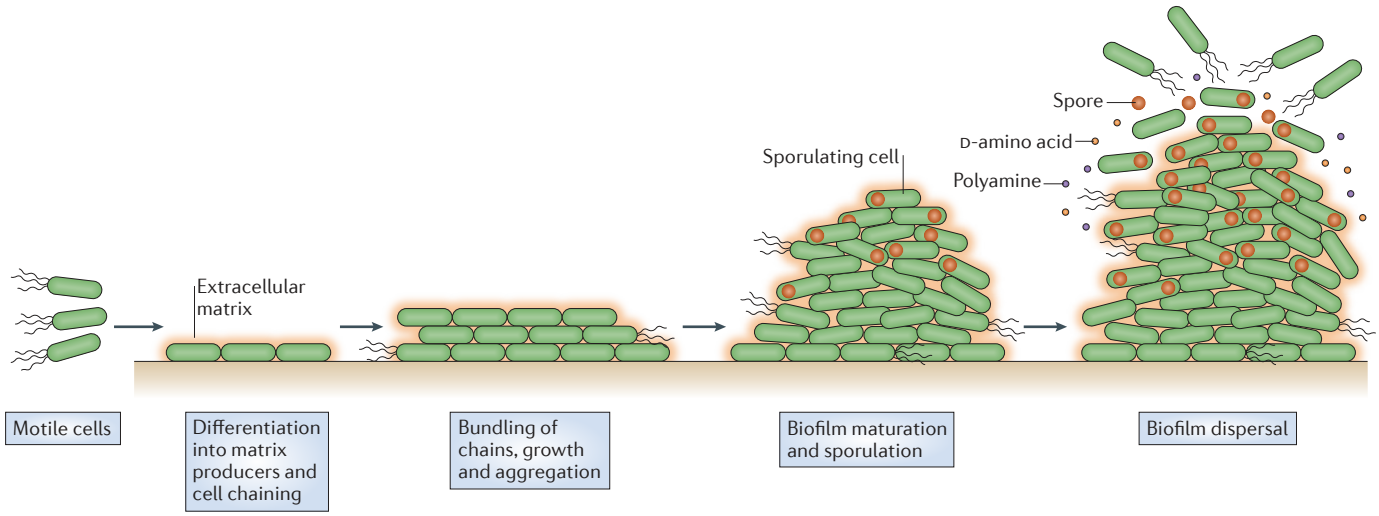


Figure 1 | **The life cycle of a *Bacillus subtilis* biofilm.** The formation of a biofilm occurs in several stages, comprising the development, maturation and disassembly of the bacterial community. At the initiation of biofilm formation, motile cells with flagella differentiate into non-motile, matrix-producing cells that stop separating and form chains that are surrounded by extracellular matrix. In mature biofilms, matrix-producing cells sporulate. In aged biofilms, some cells secrete small molecules such as D-amino acids and polyamines, which break down the extracellular matrix and allow the cells to disperse in the environment. It is important to note that although functionally distinct cell types exist within the biofilm, these cells are genetically identical, and differentiation into a specific cell type is not terminal and can be altered when environmental conditions change.

case of 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[20,22–25]. In laboratory conditions, biofilms have a limited lifespan, and they eventually disassemble in response to self-generated signals[26,27]. As a biofilm disassembles, 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, about the structural components that constitute the extracellular matrix and about 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 and induce biofilm formation. We also discuss recent findings relating to the secreted molecules that are produced by cells within the biofilm and target the extracellular matrix to disassemble the community. This research has shed light on the potential to control the growth of biofilms formed by 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. Different laboratory conditions lead to the formation of different types of biofilm, several of which have been used to study *B. subtilis* biofilm formation, including colony biofilms at the air–agar interface (FIG. 2), floating biofilms at the air–liquid interface (also termed pellicles) (FIG. 2) and, in the case of certain domesticated strains, submerged, surface-adhered biofilms at the liquid–solid interface. 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[14] (see Supplementary information S1 (movie)). Wrinkles form as a consequence of localized cell death coupled with the rigidity provided by the extracellular matrix[28]. The *B. subtilis* matrix is primarily composed of exopolysaccharide (EPS) and proteins[16,17] (TABLE 1), and 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[29]. By contrast, in liquid media, 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 and adhere to the side of the culture vessel, where they form a submerged biofilm. The particular type of biofilm formed 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* 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 the 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[21]. The role of these motile cells in *B. subtilis* biofilms varies depending on growth conditions. Motility-defective mutants that do

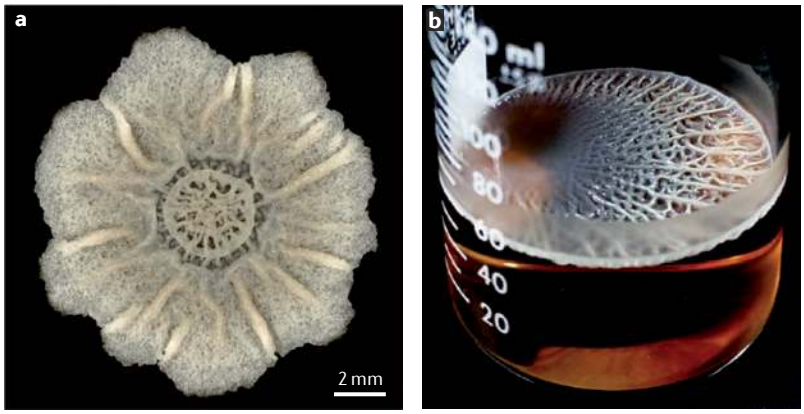


Figure 2 | **Laboratory-grown *Bacillus subtilis* biofilms.** **a** | Top-down view of a colony grown at room temperature on biofilm-inducing medium (MSgg medium) for 7 days. A time-lapse movie of the growth of this colony can be viewed in Supplementary information S1 (movie). **b** | Top-down view of a pellicle grown at room temperature for 5 days. Part **b** image is reproduced, with permission, from REF. 14 © (2001) National Academy of Sciences USA.

not have flagella are delayed in forming pellicle biofilms[15] and are defective in the formation of submerged, surface-adhered biofilms[30], but their colony morphology is akin to that of wild-type cells[21,31]. As a further morphological change, some cells in the biofilm eventually sporulate, although sporulation per se is not a requirement for biofilm formation[14,32].

Exopolysaccharide and polymer components. The major EPS component of all *B. subtilis* biofilms is synthesized by the products of the *epsABCDEFGHIJKLMNO* operon (EPS synthesis operon; hereafter referred to as the *eps* genes or *eps* operon)[14,33,34]. Mutations in the *eps* genes result in defective biofilm formation, as do mutations in *pgcA* (formerly known as *yhxB*; encoding α-phosphoglucomutase) and *gtaB* (encoding UTP-glucose-1-phosphate uridylyltransferase) — two genes that are involved in the production of nucleotide sugars which probably feed into the *eps* pathway[35,36]. Indeed, mutants that are defective in the synthesis of UDP-galactose, a precursor metabolite required for EPS biosynthesis[37], are defective in biofilm formation. UDP-galactose is a toxic intermediate product in galactose metabolism and is normally converted to the non-toxic molecule UDP-glucose by UDP-glucose 4-epimerase (GalE). When *galE* is mutated, growth on galactose is toxic because UDP-galactose accumulates. Interestingly, *galE* mutants that are grown in biofilm-inducing conditions or overexpress the *eps* genes can survive even in the presence of galactose because the UDP-galactose is shunted into the EPS pathway[37].

Of the 15 genes in the *eps* operon, only a subset has been studied individually[14,38–41]. The best studied protein encoded in this operon, EpsE, is a bifunctional protein that coordinates the production of EPS with the cessation of motility[41]. In addition to possessing the glycosyltransferase activity that is required for EPS synthesis, EpsE functions as a molecular clutch that inhibits flagellar rotation by interacting with the flagellar motor switch protein, FliG[41,42]. EpsE-mediated inhibition of motility occurs independently of the glycosyltransferase activity of the protein. This remarkable mechanism of regulation ensures that cells shut off motility when matrix production occurs to initiate 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[31]. This could explain the growth defect observed in colonies of mutants that are unable to produce EPS[43].

Another extracellular polymer, γ-poly-DL-glutamic acid (PGA), is produced in copious amounts by some

Table 1 | **Genes involved in extracellular matrix production**

Gene or operon	Role of encoded proteins in matrix production	Mutant phenotype		
		Colonies	Pellicles	Submerged biofilms
eps operon	Produces exopolysaccharide	Flat colonies (deletion of the operon[13], *epsE*[42], *epsG*[14] or *epsH*[14]; other genes not tested)	Thin pellicles (deletion of the operon[13], *epsE*[42], *epsG*[14] or *epsH*[14]; other genes not tested)	Defective submerged biofilms (deletion of *epsG*[34]; other genes not tested)
tapA	Anchors TasA fibres to the cell	Flat colonies[13]	Thin pellicles[13]	Wild-type submerged biofilms[51]
	Minor component of TasA fibres			
sipW	Signal peptidase required for TapA and TasA processing and secretion	Flat colonies[13]	Thin pellicles[13]	Defective submerged biofilms[51]
	Required for *eps* gene expression in submerged biofilms			
tasA	Major protein component of TasA fibres	Flat colonies[13]	Thin pellicles[13]	Wild-type submerged biofilms[51]
pgsB	Produces γ-poly-DL-glutamic acid (together with several other *pgs* operon enzymes)	Wild-type colonies[13]	Wild-type pellicles[13]	Overexpression enhances growth of submerged biofilms[45]
bslA	Provides surface hydrophobicity	Flat colonies[56]	Thin pellicles[53,56]	Untested in the submerged biofilm assay

bslA, biofilm surface layer; *eps*, exopolysaccharide synthesis; *pgsB*, γ-poly-DL-glutamic acid synthesis B; *sipW*, type I signal peptidase W; *tapA*, TasA anchoring and assembly; *tasA*, translocation-dependent antimicrobial spore component.

Box 1 | **Domestication of laboratory strains**

When 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.* analysed a common laboratory strain (*Bacillus subtilis* str. PY79) and a 'less domesticated' strain (*B. subtilis* subsp. *subtilis* str. NCIB 3610), which is a close relative of strain PY79 (REF. 110). They found that strain NCIB 3610 forms robust wrinkled colonies (a hallmark of biofilm formation) and floating pellicle biofilms in a defined medium (FIG. 2; Supplementary information S1 (movie)), but adheres poorly to glass surfaces[13,14]. At the same time, Hamon and Lazazzera developed a submerged, surface-adhered biofilm assay with the commonly used laboratory strain *B. subtilis* subsp. *subtilis* str. JH642 (REF. 32). They found that this strain forms submerged, surface-adhered biofilms on polyvinylchloride (PVC) and glass surfaces, but it and its close relatives (*B. subtilis* subsp. *subtilis* str. 168 and *B. subtilis* str. PY79) do not produce robust wrinkled colonies[14,111]. 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 the 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* (exopolysaccharide synthesis C), *degQ* and *swrA* (swarming motility A) — 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 strain NCIB 3610 (REF. 111). *sfp* encodes a phosphopantetheinyl transferase that is required for surfactin production, and the point mutation in strain 168 impairs surfactin production. *epsC* is in the *epsABCDEFGHIJKLMNO* operon, and the point mutation results in a decrease in exopolysaccharide production. Both surfactin and exopolysaccharide 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[45,53]. SwrA is a regulatory protein that is important in swarming motility and the synthesis of γ-poly-DL-glutamic acid[45,53]. 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[112]. Thus, it is possible that the effect of RapP is mediated by interactions with a regulatory protein. More specific details regarding the genomic differences between *B. subtilis* strains have been discussed in several articles[110,113].

B. subtilis strains and can enhance the formation of submerged biofilms[44,45]. However, PGA is not required for the wrinkled-colony morphology or for pellicle formation[13,15].

Protein components. In addition to several uncharacterized proteins that are present in the matrix, two structural proteins have been described for *B. subtilis* biofilms: translocation-dependent antimicrobial spore component (TasA) and biofilm surface layer protein (BslA; formerly known as YuaB). TasA was the first described protein component of the extracellular matrix of colony and pellicle biofilms[13]. TasA assembles into long amyloid-like fibres that are attached to the cell wall by TapA (TasA anchoring and assembly protein; formerly known as YqxM)[46,47]. TapA is found in the cell wall fraction of cells grown as pellicles or colonies, and it not only anchors the amyloid TasA fibres to the cell, but also has a role in their assembly[47]. In addition to its cell wall localization, TapA can be purified as a minor component of the amyloid fibres[47]. These proteins are encoded by the *tapA–sipW–tasA* operon, which also encodes type I signal peptidase W (SipW), the enzyme that processes both TapA and TasA[48–50]. SipW-mediated processing occurs through recognition of an amino-terminal signal sequence and cleavage of the proteins as they are secreted, so that they can be released from the membrane and become cell wall-associated fibres.

Although TasA and TapA are essential in colony and pellicle biofilms, these proteins are not required in submerged biofilms[51]. However, mutation of *sipW* in certain domesticated strains results in defective attachment to glass or polyvinylchloride surfaces[13,51]. This is because SipW is a bifunctional protein that possesses not only a signal peptidase activity that can process TasA and TapA, but also a carboxy-terminal domain which 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[34].

In addition to TasA, another secreted protein, BslA, is important for surface hydrophobicity, complex colony morphology and pellicle formation[52–55]. The biofilm-defective phenotype of a *bslA* mutant can be extracellularly complemented by mixing this mutant with a mutant lacking *eps* and *tasA*[56], presumably because EPS and TasA are provided to the mixed population by the *bslA* mutant, whereas BslA is provided by the *eps tasA* mutant[56]. BslA forms a hydrophobic layer on the surface of the biofilm[52] and it has amphiphilic properties. When purified, BslA forms polymers in solution when the air–surface interface is increased by the addition of bubbles[52]. However, it is currently unclear exactly how BslA functions to confer hydrophobicity to the biofilm surface.

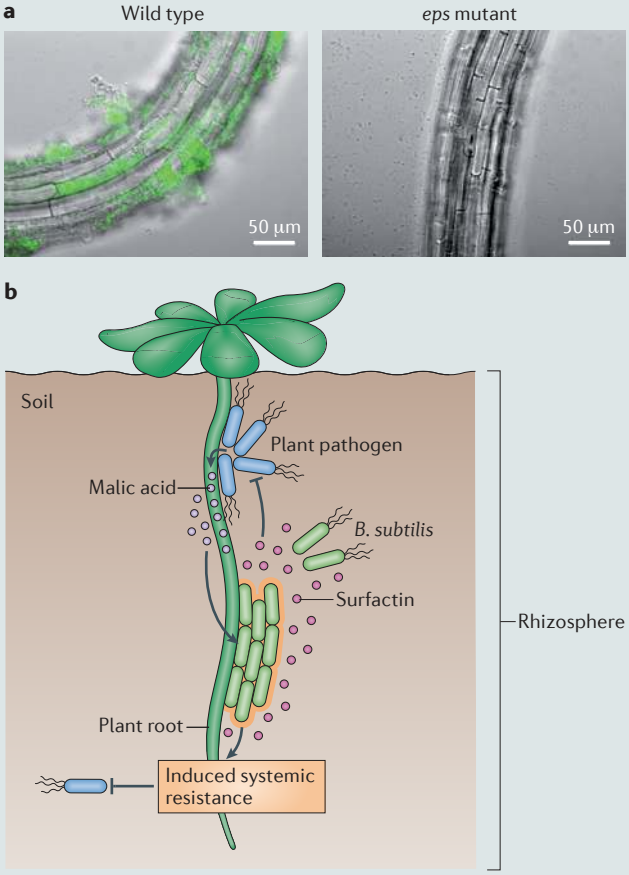
Regulatory pathways that control biofilm formation

Given the number of components that are necessary to produce the matrix, how does *B. subtilis* regulate their production and assembly? Indeed, the bacterium has a complex regulatory network to coordinate the expression of matrix genes in response to the shifting environmental conditions that it encounters in its natural habitat. FIGURE 3 is a simplified schematic of this network, and the four numbered subnetworks represent four integrated 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 more than 100 genes, including those necessary for biofilm matrix gene expression and sporulation[57,58] (FIG. 3a, subnetwork I). The activity of this protein is regulated by phosphorylation of a single aspartate residue, and both the 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 of that cell, and changes in the Spo0A-P concentration facilitate differential gene regulation[57]. For example, intermediate levels of Spo0A-P result in matrix gene expression, and higher levels induce the sporulation genes. Thus, when Spo0A is initially phosphorylated, biofilm formation is induced as a result of matrix gene expression, and 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

Box 2 | **Plant roots as a natural habitat for *Bacillus subtilis* biofilm formation**



In order to proliferate in the soil, *Bacillus subtilis* requires a nutrient source such as decaying organic material or plant roots[114]. The rhizosphere is rich in plant secretions that can provide bacteria with nutrients[115–117]. Bacteria in the rhizosphere can benefit the plant, and *Bacillus* spp. — including *B. subtilis* — are sold commercially as biological control agents for agriculture[116,118,119]. *Bacillus* spp. 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 the plant (in response to *B. subtilis*), which enhances the capacity of the plant to resist various pathogens[120–124].

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[125,126]. In addition, several other *Bacillus* spp. form biofilms on plant roots[127–129]. Biofilm formation on plant roots parallels *in vitro* biofilm formation in that the matrix exopolysaccharide (EPS) is required (REFS 95,126 and P.B., unpublished observations). Part **a** of the figure shows the roots of 6-day-old *Arabidopsis thaliana* seedlings 24 hours after inoculation with wild-type or EPS synthesis (*eps*)-mutant *B. subtilis* constitutively expressing YFP; overlays of fluorescence (false-coloured green) and transmitted light (grey) images are shown. Similarly, the master regulator Spo0A and the antirepressor SinI are required for root colonization (REF. 126 and P.B., unpublished observations). In many wild *B. subtilis* isolates, the presence of these genes, and thus the capacity of the organism to form a biofilm on the root, is also required for the strain to exert its maximal biocontrol effect[126].

B. subtilis colonization of *A. thaliana* roots also requires the production of surfactin, a lipopeptide antimicrobial that is also important for biofilm formation *in vitro*[127] (see the figure, part **b**). The production of surfactin and other lipopeptides by *Bacillus* spp. cells is one of the main mechanisms for plant biocontrol because these molecules can induce systemic resistance as well as strongly inhibit the growth of common plant pathogens such as *Pseudomonas syringae*[126,127,130].

To recruit *B. subtilis*, plants secrete small molecules. For example, when *A. thaliana* is infected with *P. syringae*, the plant secretes malic acid, and this enhances *B. subtilis* biofilm formation on the root[128] (see the figure, part **b**). Furthermore, root exudates from *P. syringae*-infected plants, or purified malic acid induce matrix gene expression in *B. subtilis*[128]. This phenomenon is not specific to *A. thaliana*; malic acid is also found in tomato root exudates (it is constitutively secreted in the rhizosphere by tomato plants) and, at high concentrations, can stimulate matrix gene expression and biofilm formation *in vitro*[95]. 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[95].

Part **a** images courtesy of P. Beauregard, Harvard Medical School, USA.

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.

Biocontrol
The protection of plants against diseases by using biological control agents such as beneficial bacteria.

KinD) that act either directly on Spo0A or indirectly via a phosphorelay[59]. The phosphorelay starts with Spo0F, which is phosphorylated by 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, that can also feed into this pathway, but it does not seem to have a role in matrix gene expression[60]. There are many levels of regulation within the phosphorelay, and this topic has been reviewed previously[61,62]. 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 analysed[32,60,63]. 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 controlling the activity of the master regulator SinR, a repressor of the *eps* and *tapA–sipW–tasA* 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* (see below and FIG. 3a, subnetwork I)[33,64,65].

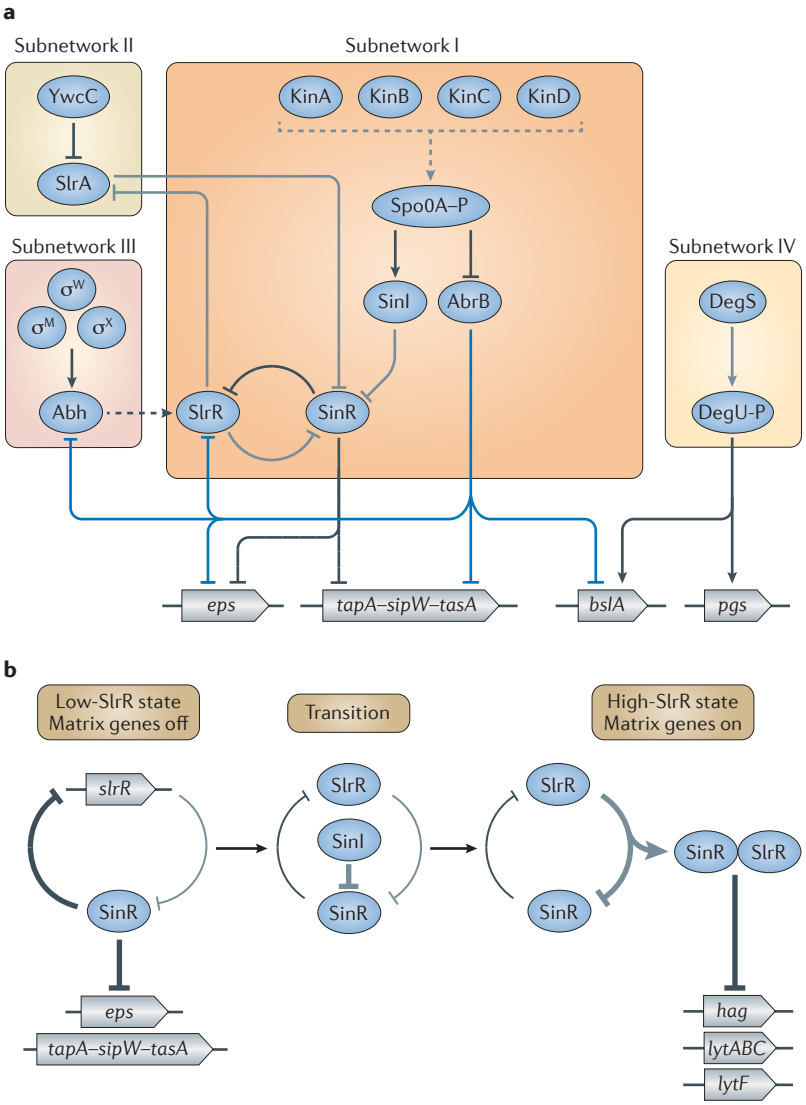


Figure 3 | **Simplified schematic of the regulatory network that controls biofilm formation in *Bacillus subtilis*.** **a** | Several subnetworks (I–IV) are integrated to activate or repress matrix gene expression depending on the environmental conditions. Details are discussed in the main text. The genes and operons encoding components of the extracellular matrix are shown: the *eps* (exopolysaccharide synthesis) operon, the *tapA–sipW–tasA* (TasA anchoring and assembly–type I signal peptidase W–translocation-dependent antimicrobial spore component) operon, the *bslA* (biofilm surface layer) gene and the *pgs* (γ-poly-DL-glutamic acid synthesis) operon. Dark grey and blue lines indicate transcriptional regulation, and light grey lines indicate protein–protein interactions; solid and dashed lines indicate direct and indirect regulation, respectively. **b** | A double-negative feedback loop involving the *slrR* gene, the SlrR protein and the SinR protein forms an epigenetic switch to regulate the expression of not only *slrR*, but also matrix genes (the *eps* and *tapA–sipW–tasA* operons), autolysin genes (*lytABC* and *lytF*) and a motility gene (*hag*; encoding flagellin). This switch adopts either a low-SlrR or a high-SlrR state. In the low-SlrR state, SinR represses *slrR* (bold inhibitory arrow), and this keeps the levels of SlrR low. In the high-SlrR state, SlrR binds to SinR (bold inhibitory arrow), 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 that maintains high SlrR levels. At the same time, SlrR re-purposes SinR in that the SinR–SlrR complex represses autolysin and motility genes. The double-negative loop is epigenetic in that both the low-SlrR and high-SlrR states are self-reinforcing and are stable for many generations. During biofilm formation, SinI is produced under the control of Spo0A-P (see main text for details) and drives the switch into the high-SlrR state by binding to and inhibiting SinR. σ, RNA polymerase σ-factor. Part **b** image is modified, with permission, from REF. 73 © (2010) Wiley and Sons.

However, when SinI is expressed, it blocks SinR-mediated repression through the formation of a SinI–SinR complex that renders SinR incapable of binding to DNA[66]. SinR is produced in all cells but is inactivated by SinI in only a fraction of cells, and thus only a subpopulation of cells expresses the *tapA–sipW–tasA* and *eps* operons[21,67].

In addition to determining which cells express matrix genes, Spo0A-P levels determine the duration of matrix gene expression in these cells. The promoter of *sinI* contains both a high-affinity activator and multiple low-affinity operators for Spo0A-P[67]. 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 low-affinity operators are also occupied and further *sinI* expression is curtailed[67]. Meanwhile, sporulation genes become activated by the high levels of Spo0A-P[57]. In addition, a second and embedded mechanism exists to turn off matrix genes once sporulation commences. The functions of SinI and SinR are remarkably sensitive to gene dose: a mere doubling of the *sinI* and *sinR* genes completely blocks matrix production[68]. Although actively dividing cells do not maintain two chromosomes (that is, double the gene dose) for very long, in early sporulation the presence of two copies of the chromosome in the mother cell is prolonged, resulting in higher levels 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 extracellular matrix.

Spo0A-P also represses a second matrix gene repressor, AbrB[69]. Like SinR, AbrB represses both the *tapA–sipW–tasA* and *eps* operons[33,51,64,65,70]. Furthermore, AbrB represses expression of the matrix protein BslA[55] and the regulatory proteins SlrR[65] and Abh[70] (see below). The presence of two Spo0A-regulated repressors, SinR and AbrB, with highly overlapping targets is likely to be a means of fine-tuning the regulation of biofilm formation and ensuring the coordinated expression of all the matrix genes.

The SlrR–SinR epigenetic switch. As mentioned above, SinR and AbrB inhibit the expression of the regulatory protein SlrR[65,71,72]. 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 the matrix gene promoters (of the *eps* and *tapA–sipW–tasA* operons) and the *slrR* promoter. This results in a self-reinforcing double-negative feedback loop involving *slrR*, SlrR and SinR (FIG. 3b), whereby SlrR expression, by blocking SinR activity, results in the *slrR* gene remaining derepressed. When SlrR levels are high, the matrix genes are also derepressed because free SinR levels are low. Conversely, when SlrR levels are low, SinR is not inhibited, so *slrR* is repressed and the matrix operons are also switched off. The second role of SlrR in the control of biofilm formation involves SinR–SlrR-mediated repression of the promoters for *hag* (which encodes flagellin and is required for motility) and genes involved in cell separation (*lytABC* and *lytF*,

which encode autolysins)[73,74] (FIG. 3b). Thus, SlrR activity blocks SinR-mediated matrix gene repression and re-purposes SinR (in the form of the SinR–SlrR complex) to become a repressor of autolysin and motility genes.

As mentioned above, cell chaining is essential at the onset of biofilm formation in *B. subtilis* (FIG. 1). This cell chaining is achieved by the SinR–SlrR-mediated repression of autolysins, which are required to separate chains of cells. SlrR is a member of the LexA family of autopeptidases; it 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, thus allowing the chains of cells to separate[73]. Inhibiting the separation of chains by using a non-cleavable mutant of SlrR[73] or by generating a triple mutant lacking three of the autolysins (LytC, LytD and LytF) required for cell separation[15] 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[73].

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). But what controls the switch from the low state to the high state? This switch is accomplished by SinI, which is produced under the control of Spo0A-P and, like SlrR, is an antagonist of SinR. Thus, the production of SinI inhibits SinR activity, leading to derepression of *slrR*. This results in the accumulation of SlrR to high levels and further inhibition of SinR by SlrR, driving the switch into a high-SlrR state. Because the switch is self-reinforcing, it persists in the high state for many generations and can be said to be an epigenetic switch.

The components of the epigenetic switch are subjected to additional regulation from another pathway comprising YwcC and SlrA (FIG. 3a, subnetwork II). SlrA is paralogous to SinI and thus functions as a SinR antirepressor[71]. The *slrA* gene is repressed by YwcC, a TetR-type transcriptional repressor[71,72]. When YwcC receives an as-yet-unknown signal, *slrA* is derepressed and the matrix genes are induced by SlrA-mediated inactivation of SinR[71]. However, 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[71].

The transcription of *slrR* is also indirectly activated by the regulatory protein Abh[75] (FIG. 3a, subnetwork III). The *abh* gene is itself repressed by AbrB[70], and its transcription is controlled by several extracytoplasmic function (ECF) RNA polymerase σ-factors, including σ^M, σ^W and σ^X (REFS 15,75–79). ECF σ-factors are activated by a variety of external stimuli, including cell wall stress and specific antibiotics[80], thereby providing a Spo0A-independent mechanism for responding to changes in external conditions.

The expression of *slrR* is positively regulated by several other proteins aside from Abh. For example, YmdB, a putative phosphoesterase, is needed for high levels of *slrR* expression[81]. In addition, *slrR* expression requires two small proteins, RemA and RemB[82]. Genetic analyses have shown that RemA and RemB activate expression of the *eps* and *tapA–sipW–tasA* matrix operons both via SlrR and in a manner that is independent of SlrR. The exact mechanism by which these small proteins function remains to be determined, but it seems that they act in parallel with SinR, AbrB and DegU (see below), the other known matrix gene regulatory proteins[82].

Other regulatory pathways. A fourth pathway that regulates the expression of only *bslA* and the PGA synthesis (*pgs*) operon involves the DegS–DegU two-component system[45,53,55,83] (FIG. 3a, subnetwork IV). In this system, DegS is the sensor histidine kinase that phosphorylates DegU, the response regulator. In *B. subtilis*, DegU is a global regulator that is involved in the control of a variety of cellular processes, such as competence, motility and secretion of degradative enzymes[84]. In addition, a *degU* mutant is defective in submerged biofilm formation, which requires the polymer PGA[45]. Furthermore, a *degU* mutant is defective in colony biofilm formation, owing to the loss of the surface hydrophobicity protein BslA[52,53,55,83].

Finally, in addition to the various transcription factors that are described above, the *eps* 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 *eps*-associated RNA (EAR) element and is conserved among a subset of bacteria from the order Bacillales. This element is thought to act as an antiterminator and increases *eps* gene expression by interacting with RNA polymerase[85].

The list of regulators described above underscores the remarkably complex and multilayered regulatory mechanisms that control biofilm formation in *B. subtilis*. These particular regulators are specific for this organism, and it is highly unlikely that we will find homologues with similar roles in other bacterial species. However, the take-home message is that such complex regulatory mechanisms probably evolved to facilitate the induction of the appropriate response to changing environmental conditions, thereby ensuring that biofilm formation occurs 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 σ-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. These known mechanisms involve two of the sensor kinases that phosphorylate Spo0A: KinC and KinD. At present, the mechanisms by which the other

Chaining
An event that occurs when daughter cells divide but remain connected by polar cell wall peptidoglycans.

Epigenetic switch
A regulatory switch that imposes heritable changes in gene expression by a mechanism that does not involve genetic mutation.

TetR-type transcriptional repressor
A two-domain protein with a DNA-binding domain and a small ligand-binding domain. Normally, repression is relieved when a specific small molecule binds to the ligand-binding domain.

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 lipopeptide that is produced by constituent cells of the biofilm[63]. Surfactin has been studied for its potent surfactant and antimicrobial activities, as well as its role in surface motility in *B. subtilis*[86–88]. Surprisingly, surfactin also acts as a signal that triggers phosphorylation of Spo0A via the sensor kinase KinC and thus positively regulates matrix gene expression[63]. Interestingly, surfactin is produced by only a subpopulation of cells, and the cells that respond to the molecule (that is, those that express matrix genes or *sinI*, which are also produced by only a subset of biofilm cells) are not from the subpopulation of surfactin-producing cells[89]. This concept represents a new way of thinking about self-generated quorum sensing signals in bacteria and has been referred to as paracrine signalling, to mean that the signal is unidirectional and the signal producer does not respond to the signal that it makes. This is in contrast to previously described quorum sensing systems, in which every cell in a population is thought to produce and respond to the signalling molecule (reviewed in REFS 19,90).

The 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 subsequently expressed[63]. Other compounds that cause potassium leakage but have different structures to surfactin, such as the fungicide nystatin and the antibiotic valinomycin[63], 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, as well as by *B. subtilits* (in the case of surfactin); 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, affects signalling in *B. subtilis* led to the finding that *B. subtilis* harbours membrane microdomains analogous to the lipid rafts of eukaryotes[91] (BOX 3). Sublethal concentrations of chlorine dioxide, which is a potent biocide at high concentrations, also induce matrix gene expression in a KinC-dependent manner[92]. Unlike surfactin, chlorine dioxide is thought to trigger KinC activation by collapsing the membrane potential of the cell. Thus, it seems 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 might be beneficial for *B. subtilis* survival; in the case of chlorine dioxide, the presence of EPS provides protection against the lethal effects of the molecule[92].

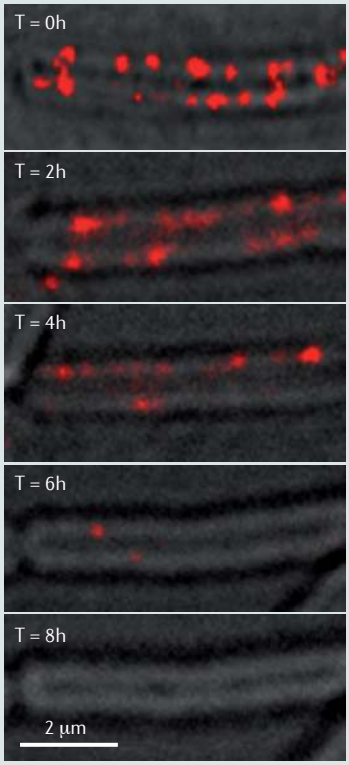
KinD-mediated matrix gene expression. Matrix itself appears to regulate matrix gene expression, and mutants that are unable to produce EPS and TasA have prolonged expression from the promoters of the *eps* and *tapA–sipW–tasA* operons (as observed using transcriptional reporters) and delayed sporulation in biofilm conditions[21,43]. This effect is at least partially due to the activity of KinD. Like many other two-component sensor kinases[93], KinD displays both kinase and phosphatase activity. KinD seems 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[43]. Therefore, a checkpoint exists in which differentiation into spores during biofilm conditions relies on the production of an extracellular matrix. KinD also activates matrix gene expression in response to compounds produced by soil microorganisms[94] and (as discussed in BOX 2) compounds in tomato root exudate[95].

Cannibalism to increase the matrix-producing subpopulation. 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 induce the expression of two cannibalism operons that encode secreted toxin peptides: sporulation killing factor (SkfA) and sporulation-delaying protein C (SdpC). Furthermore, these cannibal cells also express the resistance machinery for the toxins[96,97]. In the case of SkfA, the exact mechanism of resistance is unclear, but requires an ABC transporter (encoded within the cannibalism operon) that pumps the toxin out of the cell[97]. SdpC resistance requires the membrane-associated immunity protein SdpI, which is encoded by a gene that is divergently oriented relative to the toxin gene. SdpI is induced in the presence of the toxin, but only in those cells that have high enough (although still reasonably low) levels of Spo0A-P[98]. Thus, the cannibal cells secrete toxins that kill siblings which are not expressing the toxin or resistance genes. Because the cannibalism genes and matrix-producing genes are both activated by low levels of Spo0A-P, the population of matrix-producing cells and cannibal toxin-producing cells is highly overlapping[57,99]. Therefore, most of the matrix-producing cells also secrete toxins (and the resistance factors), and this effectively decreases the population of non-matrix producers. This system ultimately results in a population consisting of an amplified number of matrix-producing cells[99].

The cannibalism toxins produced by *B. subtilis* are not specifically active against only *B. subtilis* siblings. In fact, they preferentially kill different species when *B. subtilis* is grown in mixed cultures[100,101]. Consistent with this idea of non-self-specificity, cannibalism-like toxins secreted by close relatives of *B. subtilis* probably have a role in increasing the *B. subtilis* matrix-producing subpopulation in mixed-species soil communities[94]. This phenomenon was discovered in a screen for natural inducers of matrix gene expression; the screen used a *B. subtilis* strain that harboured a fluorescent reporter for matrix gene expression, and this strain was co-cultured with soil organisms. Surprisingly, despite the diversity

Cannibalism
In the context of *Bacillus subtilis*: killing of siblings through the secretion of a toxin. In a genetically identical population, some cells will secrete a toxin, but these cells also express the genes that confer resistance. Siblings that do not express the toxin or resistance genes will be killed.

Box 3 | **Lipid rafts coordinate signalling molecules in bacterial membranes**



Bacteria have been shown to possess membrane microdomains analogous to the cholesterol-rich lipid rafts found in eukaryotic cells. *Bacillus subtilis* membranes do not contain sterols, but lipids that are synthesized from 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[89,125]. The kinase KinC, which is important for controlling biofilm formation, localizes in these microdomains along with a variety of other proteins involved in signalling[91]. In addition, FloT (formerly known as YuaG) and YqfA, two homologues of the eukaryotic lipid raft protein flotillin 1, are associated with the detergent-resistant microdomains and localize in a punctate pattern throughout the membrane[91,131,132]. A mutation in the gene *yisP*, which encodes a squalene synthase that is required for the production of the detergent-resistant lipids, results in bacteria that are defective in biofilm formation. Moreover, known inhibitors of squalene synthases, such as zaragozic acid, disrupt these domains and inhibit biofilm formation. The series of images shows a FloT–YFP protein fusion localized in puncta throughout the *B. subtilis* membrane, and dissipation of these puncta over time after treatment with zaragozic acid. By 8 hours after treatment, all the FloT–YFP puncta have dispersed[91]. A fluorescence image (false-coloured red) is overlayed on the transmitted light image. This knowledge led to studies in other bacteria, in which membrane microdomains were disrupted using molecules that inhibit lipid biosynthesis, and this also inhibited biofilm formation and blocked the production of several virulence factors.

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 (a cannibalism-like toxin) that preferentially killed non-matrix-producing *B. subtilis* cells, resulting in an increase in the matrix-producing population[94]. The other inducing molecules required a functional KinD kinase 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 thus far on the process of building a biofilm. However, as the biofilm matures, resource limitation and waste product accumulation might mean that it becomes beneficial for the constituent cells of the biofilm to disperse[102,103]. One mechanism that *B. subtilis* has exploited to escape biofilms is the release of D-amino acids, a stationary phase phenomenon that occurs naturally in a number of organisms[27,104]. 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[27]. Furthermore, the accumulation of D-amino acids seems to be regulated by racemase enzymes (which catalyse the stereochemical conversion of L-amino acids to D-amino acids), as mutations in the racemase-encoding genes result in significantly delayed biofilm disassembly[27]. D-amino acids disrupt biofilm formation by becoming incorporated into peptidoglycan and thereby altering the association of certain proteins, including TapA, with the cell wall. Thus, D-amino acids result in the release of the TasA amyloid fibres from the cell[27,47].

In addition to D-amino acids, the supernatant of ageing *B. subtilis* biofilms harbours the polyamine norspermidine, which efficiently disperses biofilms[26]. The inhibitory activity of norspermidine is synergistic with that of D-amino acids, suggesting that these molecules act by different mechanisms. Indeed, norspermidine does not affect the association of TasA with the cell wall; instead, it interacts directly and specifically with the EPS component of the matrix[26]. This interaction results in the collapse of EPS, a process that has been visualized by microscopy, and a change in polymer size, as visualized using light scattering[26]. This biofilm-disrupting activity is also a feature of other polyamines such as norspermine, which are (like norspermidine) composed of repeating units that harbour three methylene groups flanked by two amino groups. Similar molecules such as spermidine and spermine, which contain an additional methylene group in one of the units, do not exhibit inhibitory activity, and it was proposed that the additional methelyne group alters the interaction with EPS[26]. Disrupting genes to interfere with norspermidine production results in more robust biofilms[26]. 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[105]. Although spermidine and norspermidine are similar in a chemical sense, different genes are required for the biosynthesis of each compound, and it is currently unknown how 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, cross-species biofilm inhibitors. For example, D-amino acids are also able to disrupt biofilm formation in the pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*[27]. Similar to the observations

made for *B. subtilis*, in which D-amino acids disrupt the association of TapA with the cell wall, D-amino acids prevent the surface localization of proteins in *S. aureus*[106]. The molecular basis for the inhibitory effect of D-amino acids on biofilm formation in Gram-negative bacteria is currently unknown. A recent study that analysed *P. aeruginosa* str. PAO1 flow cell biofilms showed that D-amino acids had a lethal effect on cells; however, in this strain, extracellular matrix production was increased, and biofilm formation was not inhibited. This would suggest that, at least in *P. aeruginosa*, the inhibitory effect of D-amino acids is dependent on the experimental conditions or is strain specific (the inhibitory activity of D-amino acids was shown using *P. aeruginosa* str. PA14 in static submerged biofim conditions)[27,107].

The polyamines norspermine and norspermidine are also potent inhibitors of biofilm formation in *S. aureus* and *Escherichia coli*[26], but studies are needed to identify the targets of the polyamines in these systems. However, it is likely that the EPS component of the matrix is altered in the presence of polyamines.

Inhibitors of lipid synthesis enzymes (such as the squalene synthase inhibitor zaragozic acid (BOX 3) and cholesterol-lowering statins) disrupt the formation of *S. aureus* biofilms. These molecules also inhibit the production of virulence factors such as proteases and the carotenoid staphyloxanthin[91], and this inhibition might contribute to the ability of cholesterol biosynthesis inhibitors to block *S. aureus* virulence[108].

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 it means that their use should not impose a selection pressure for resistant mutants, as is associated with traditionally used antibiotics. Many nosocomial infections are associated with biofilm formation, and these molecules might therefore represent promising alternatives to antibiotics[102,109]. Moreover, using these compounds to disrupt biofilms before treatment with other antimicrobials could provide a more effective means of eliminating harmful bacteria.

Concluding remarks

A multitude of genetic and cell biology tools have been developed for *B. subtilis*, and this species has proved 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, the 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 matrix-producing cells, and this also occurs 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. It is possible that only a subset of the matrix genes is needed for biofilm formation in certain environmental conditions (for example, TasA is dispensable in submerged biofilms) and that this is why such complex regulation has evolved.

Unlike the abundance of information that has amassed concerning the regulation of biofilm assembly, studies of 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 the production of these compounds regulated by even more external stimuli? What are the exact mechanisms by which these molecules exert their inhibitory effects?

Many features apply to the formation of all bacterial biofilms, such as the requirement for extracellular matrix comprising EPS and proteins. 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 achieved 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 the potential of combinatorial therapy, such as the coupling of these molecules with improved antibiotics, for more successful eradication of detrimental biofilms.

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Competing interests statement
The authors declare no competing financial interests.

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