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Bacillus subtilis biofilm formation and social interactions

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Abstract

Biofilm formation is a process in which microbial cells aggregate to form collectives that are embedded in a self-produced extracellular matrix. *Bacillus subtilis* is a Gram-positive bacterium that is used to dissect the mechanisms controlling matrix production and the subsequent transition from a motile planktonic cell state to a sessile biofilm state. The collective nature of life in a biofilm allows emergent properties to manifest, and *B. subtilis* biofilms are linked with novel industrial uses as well as probiotic and biocontrol processes. In this Review, we outline the molecular details of the biofilm matrix and the regulatory pathways and external factors that control its production. We explore the beneficial outcomes associated with biofilms. Finally, we highlight major advances in our understanding of concepts of microbial evolution and community behaviour that have resulted from studies of the innate heterogeneity of biofilms.

[H1] Introduction

Bacterial biofilms are a mode of collective living that confers emergent properties to the inhabitants of these communities[1]. A self-produced extracellular matrix that encapsulates the cells and facilitates their attachment to surfaces, among other functions, is a hallmark of biofilm formation[2]. The biofilm research field is fast moving due to the biological relevance of these multicellular consortia to an array of advantageous and detrimental effects in natural systems and human applications.

Bacillus subtilis is a soil-dwelling, non-pathogenic, Gram-positive bacterium that is commonly found in association with plants and their rhizosphere. *B. subtilis* is a highly tractable microorganism for which a broad suite of genetic and molecular tools is available that facilitate *in vitro* manipulation and study. Consequently, *B. subtilis* has been the major model organism for the study of Gram-positive bacteria for many decades. Furthermore, *B. subtilis* has been exploited for several industrial applications, including the production of hydrolytic enzymes, fermentation of food and, most recently, as a probiotic[3].

The ability of *B. subtilis* to switch from a motile to a sessile state has been utilized to study biofilm formation. The undomesticated, ancestral isolate NCIB 3610 is widely studied to explore the three types of well-structured, three-dimensional biofilms that *B. subtilis* typically forms in vitro: a pellicle biofilm that develops at an air–liquid interface, a colony biofilm that develops at an air–solid interface and a submerged surface-attached biofilm[4-6] (Figure 1). *B. subtilis* also forms biofilms on biotic surfaces, including fungal hyphae[7] and roots[8] and leaves[9] of plants. Furthermore, natural *B. subtilis* strains form pellicle biofilms on processed food products[10].

The *B. subtilis* extracellular matrix contains an exopolysaccharide (EPS) that is synthesised by the products of the 15-gene *epsABCDEFGHIJKLMNO* (*epsA–O*) operon[4], protein fibres of TasA encoded by the *tapA–sipW–tasA* operon (*tapA* operon)[11] and BslA (encoded by *bslA*), a hydrophobin-like protein that confers hydrophobicity to the community[12,13]. A role for extracellular DNA (eDNA)[14] and mineral deposits as components of the extracellular matrix has also been documented[15]. There is a strong correlation between the molecules that are needed for biofilm formation on biotic and abiotic surfaces, validating the utility of the laboratory model systems[16].

A substantial focus of many research groups has been to identify the regulatory mechanisms that underpin biofilm formation and characterize the properties of the extracellular matrix components of *B. subtilis* biofilms. Over the past 20 years it has become apparent that a *B. subtilis* biofilm formed by a single isogenic species is a remarkably heterogeneous community, making it ideal for the study of evolution within biofilm communities. Furthermore, *B. subtilis* exhibits emerging ecological properties that are dependent on biofilm formation, which affect social behaviours and interactions with hosts, resulting in a shift in the focus of research towards these as yet underappreciated aspects of *B. subtilis* physiology.

In this Review, we describe the recent advances in our knowledge of the principles governing biofilm formation, including transcription regulation and composition of the matrix. We explore biofilm formation within the context of microbial interactions and expand on interactions of the *B. subtilis* biofilm with hosts, such as plant roots and the intestinal tract. Finally, we highlight the progress that has been made in determining the evolutionary processes that occur in biofilm populations. By illuminating the versatile nature of *B. subtilis* biofilms, we extend the utility of *B. subtilis* biofilm formation beyond its usefulness as a laboratory model to a multifaceted and functional system with real-world applications in agriculture and human health.

[H1] The *B. subtilis* biofilm matrix

Great advances have been made in the characterization of *B. subtilis* biofilm matrix components, which include the secreted proteins TasA, TapA and BslA, as well as a mineral scaffold, eDNA and an exopolysaccharide. *B. subtilis* biofilms have been compared to colloidal hydrogels, with the matrix corresponding to the cross-linked gel[17].

[H2] Products of the *tapA* operon

The *tapA* operon encodes three proteins that are involved in *B. subtilis* biofilm formation: TapA, SipW and TasA.

[H3] TapA. TapA is a 253-residue multidomain secreted protein containing a predicted 43-amino-acid signal peptide. Tap A has been described as a TasA assembly and anchoring protein[18]. Secreted TapA contains three distinct structural domains with varying levels of intrinsic disorder[19,20]: an N-terminal domain (amino acids 44–75) that is necessary for pellicle and colony biofilm formation, a central stable domain (amino acids 75–191) that forms a β-sandwich fold (Protein Data Bank (PDB) identifier (ID) 6HQC and 6QAY) and a C-terminal domain (192–253) that is highly disordered in solution. As only residues 44–57 of TapA are

essential for biofilm structure, the central stable domain, which is also the mostly highly conserved region of TapA, currently has no known function[20]. Co-expression of the wild-type *tapA* allele and a variant with mutations in residues 50–68 (overlapping with the essential region) slowed pellicle formation, although the underlying mechanism is unknown[21].

In vivo, TapA localizes to foci on the cell surface that are proposed to anchor TasA fibres to the cell wall and accelerate TasA fibre formation[18]. However, TapA is not necessary for TasA fibre formation and does not affect fibre architecture *in vitro* but does enhance polymerization[21-23]. Interestingly, TapA forms β-sheet rich aggregates in isolation and associates with fibril structures *in vivo* even when TasA is absent[22]. Whether these Δ*tasA*-fibres consist solely of TapA or whether TapA just has a propensity to associate with fibril structures remains to be clarified. Nonetheless, function of TapA in the matrix is closely tied to that of TasA. Consistent with this, strains lacking TapA have lower levels of matrix-localized TasA, and full function of TasA and TapA in the matrix requires their concomitant expression within the same cells[18].

[H3] SipW. SipW is a type I signal peptidase that post-translationally modifies both TasA and TapA by cleaving their N-terminal signal peptides at the extracellular surface[24] (Figure 2). The cytoplasmic C-terminal domain of SipW additionally activates expression of the *epsA–O* and *tapA* operons in surface adherent biofilms[25], adding a supplementary function to this protein that is essential for biofilm formation.

[H3] TasA. Originally proposed as an antibiotic factor[26], TasA has various emerging roles, including functions in biofilm structure, sliding motility, signalling and plant colonization. TasA is a 261 amino acid protein with a 27-residue signal peptide[27]. TasA is found as fibres in the biofilm matrix and is required for the structural integrity of the biofilm[11,23]. TasA-fibres have been isolated directly from biofilms and also produced from recombinant protein. The source of the fibres influences the level of amyloid character that is detected[11,22,28,29]. TasA fibres isolated from *B. subtilis* pellicles show binding of thioflavin T, Congo red and the amyloid-specific antibody A11, which suggests that these isolated fibres have amyloid properties[11]. Subsequent biophysical and recombinant protein approaches have revealed that both amyloid and non-amyloid TasA fibres exist[22,28,29].

Recombinant TasA (which lacks the signal peptide and thus corresponds to the mature secreted protein) forms biologically active fibres that are non-amyloidogenic and have a similar secondary structure to those isolated from biofilms[28]. Unlike curli, the amyloid fibres that are produced by *Escherichia coli* and other enteric bacteria (reviewed elsewhere[23]), monomeric TasA in *B. subtilis* is globular in solution and adopts a jelly-roll-like fold with a flexible helix-

rich region[29]. At low pH, TasA forms dense gel-like networks that are comparable to colloidal aggregation[30]. Transmission electron microscopy (TEM) imaging of TasA fibres consistently reveals repeating units along the protofilament (smallest diameter fibres) axis that are estimated to be ~5 nm in length[22,28], similar to the size of the TasA monomer. Like amyloid fibres, biologically active TasA fibres are resistant to proteases but, unlike β-amyloid fibres, TasA fibres are susceptible to the detergent sodium dodecyl sulfate, suggesting key differences in fibre structure. NMR analysis of TasA fibres, after addition to Δ*tasA* cultures, showed some changes in structure between the monomeric and fibre forms, including increased β-sheet character[29]. By contrast, another study found that the CD spectra of the monomer structure were highly similar to those recombinant fibres and fibres purified from *B. subtilis* biofilms[28]. These studies also showed that the N-terminal region of processed TasA is important for polymerization, as addition of a single amino acid to the N-terminus blocks polymerization and locks TasA in a monomeric form. Thus, although the structure of TasA fibres is still unknown, the importance of their role in *B. subtilis* biofilms is well established.

TasA additionally affects and has a regulatory role in cell physiology[9,31]. The presence of TasA fibres within the biofilm matrix stimulates expression of motility genes and down-regulates matrix expression in subpopulations of cells, and thus contributes to colony spreading on surfaces[31]. Deletion of *tasA* has other pleiotropic effects, including the down-regulation of genes related to sporulation and an increase in expression of matrix and antimicrobial secondary metabolite-related genes[9]. Moreover, TasA is associated with the detergent-resistant fraction of the cell membrane and influences membrane fluidity. However, most strikingly, *tasA* deletion leads to a decrease in *B. subtilis* viability in biofilm-inducing conditions. These functions have been proposed to be separate from TasAfibre forming and structural roles, as a strain producing a biofilm-inactive TasA variant retained wild-type physiological traits[9]. However, the level of the variant TasA produced by this strain was lower than in the wild type, opening the alternative prospect that the level of TasA needed varies with different processes. Looking beyond *B. subtilis*, TasA orthologues also have multiple roles. For example, in *Bacillus cereus*, the TasA orthologue CalY acts as a cell-associated adhesion factor during early biofilm formation prior to its release from the cell by the SipW homologue[32]. It is safe to say that despite the many studies conducted to date on TasA and its orthologues, many questions remain about its active structure, biological functions and roles in different strains and species.

[H2] The biofilm surface layer protein BslA

BslA has two genetically separable functions, being required for both biofilm hydrophobicity and biofilm architecture[13,33]. BslA seems to act synergistically with TasA fibres and exopolysaccharide (EPS) to generate structural complexity in the biofilm, as production of these matrix components is unaltered in mutants lacking BslA but colony biofilm structure is compromised[34]. Whereas *bslA* is transcribed uniformly in the biofilm population, the protein primarily localizes to the biofilm periphery, forming a hydrophobic coating[12,13]. The crystal structure of BslA provides insight into this aspect of its function, as it reveals an immunoglobin G-like fold with a cap region that can exist in either a cap-in hydrophilic state or a cap-out hydrophobic state[35] (Fig. 2d). The protein forms a 2D lattice in vitro, creating an elastic film at interfaces, which has been studied by biophysical techniques (Box 1)[35]. It is conjectured that at the biofilm–air interface BslA transforms from cap-in to cap-out formation and this transition leads to hydrophobicity of the biofilm surface.

A C-terminal Cys-x-Cys (CxC) motif is required for BslA oligomerization in the oxygen-rich environment of the biofilm surface, and the motif is essential for conferring hydrophobicity to the community, but is not required for the complex architecture of the biofilm[33]. The importance of the CxC motif for hydrophobicity was further demonstrated when YweA, a BslA paralogue lacking the CxC motif, was used in genetic complementation studies. Native YweA was unable to reinstate either hydrophobicity or structural complexity in a *bslA*-deficient strain, whereas a YweA variant containing the CxC motif fully restored hydrophobicity and partially restored the complex architecture[33]. Biofilm hydrophobicity and the corresponding presence of BslA (and its dimerized form) confers resistance of the resident bacteria to chemical attack[33]. To date, BslA has been studied primarily in NCIB 3610 and its relevance in the non-laboratory environment or other isolates is yet to be elucidated.

[H2] Biofilm matrix carbohydrates

EPS is the main biofilm carbohydrate and is synthesised by the 15 gene products of the *epsA–O* operon[4], which is transcribed in propagating waves at the edge of the expanding colony biofilm[36]. In addition to its signalling function, EPS is required for complex colony structure, pellicle formation and water retention[25,37-41]. The protein tyrosine kinase EpsB and EpsA are predicted to regulate EPS production[42]. The operon also encodes a sugar dehydratase (EpsC), multiple putative glycosyltransferases (EpsD, EpsE, EpsF, EpsH, EpsI, and EpsJ), a putative pyruvyl transferase (EpsO) and a predicted polysaccharide transport protein (EpsK)[43,44,45] (Figure 2). Bioinformatic analysis predicts that EpsL is a sugar transferase that possibly transfers the first sugar unit onto a lipid carrier. EpsG is a predicted transmembrane protein of

unknown function. The function of a few of these proteins have been studied in more detail. EpsE is a bifunctional protein that is involved in EPS synthesis and directly inhibits motility by acting as a clutch on the flagella rotor[44]. EpsC has NAD^+-dependent UDP-*N*-acetylglucosamine 4,6-dehydratase activity[46]. EpsN can produce UDP-2,6-dideoxy 2-acetamido 4-amino glucose from of UDP-2,6-dideoxy 2-acetamido 4-keto glucose in a pyridoxal 5′-phosphate-dependent manner[47]. EpsM can transfer acetyl groups to UDP-2,4,6-trideoxy-2-acetamido-4-amino glucose to produce *N*,*N*′-diacetylbacillosamine. Based on sequence homology and the *in vitro* activities of EpsC, EpsM, and EpsN, it has been proposed that these proteins may have a role in the biosynthesis of *N*,*N*′-diacetylbacillosamine, a modified monosaccharide that is produced by some bacteria[48].

The composition of EPS and whether it contains *N*,*N*′-diacetylbacillosamine is still unclear owing to conflicting findings from multiple studies. One analysis found that EPS contained glucose, *N*-acetylgalactosamine and galactose (with about 3:2:1 ratio)[49]. Further supporting this finding, the galactose metabolism pathway was found to be important for biofilm formation and regulation of this pathway was interrelated to regulation of the *epsA–O* operon[49]. By contrast, another study concluded that EPS is predominantly composed of mannose (88%) and glucose (12%)[50]. Furthermore, *B. subtilis* EPS is cross-reactive with an antibody raised against poly-β-1,6-*N*-acetyl-D-glucosamine (PNAG), a common biofilm exopolysaccharide[45]. Further investigation of the structure of EPS and monosaccharide components is necessary.

[H2] Other matrix components

eDNA is a common matrix component in bacterial biofilms and has been studied in diverse microbial systems[51]. The function of eDNA in *B. subtilis* biofilms is less explored than other biofilm systems but studies have found that eDNA is important for biofilm architecture and is required in the early stages of biofilm formation[14]. Treatment of *B. subtilis* biofilms with DNaseI during early biofilm development (12 h or earlier) led to an appreciable reduction in biofilm biomass but no difference was seen when the enzyme was added at later time points. Retention and localization of eDNA in the biofilm is also dependent on EPS production, a possible interaction that is further supported by isothermal titration calorimetry[45].

Another highly anionic polymer of the *B. subtilis* matrix is poly-γ-glutamic acid (γ-PGA), a linear polymer of L-glutamate and D-glutamate linked at the γ-carboxyl instead of at the α-carboxyl that is typically used in proteins. The levels of γ-PGA in the matrix are highly

dependent on the strain and growth conditions. Some strains, such as *B. subtilis* B-1, have been described as a γ-PGA dominant matrix former, whereas the common biofilm model strain NCIB 3610 produces very little γ-PGA[52]. Synthesis of γ-PGA is carried out by enzymes encoded by the *pgsBCAE* operon (previously known as *ywsC–ywtABC*): PgsB and PgsC are responsible for polymerization and PgsA and PgsE comprise the export machinery[53] (Figure 2b). The export function of PgsE has been proposed based on findings from the highly homologous system of *Bacillus anthracis*. The presence of γ-PGA in the biofilm matrix is correlated with water retention and resistance to ethanol-induced dehydration[54].

In addition to organic molecules, the biofilm matrix also contains inorganic compounds, such as metal ions and minerals. For example, *B. subtilis* induces calcite precipitation from calcium ions, which is correlated with increased colony biofilm wrinkling and complexity and may increase resistance to environmental assault by creating diffusion barriers[55, 15]. The conversion of calcium acetate to calcite crystals is linked to the increase in intra-colony pH during biofilm development, a process that is dependent on the *ureA–C* operon. The structure and morphology of calcium carbonate crystals are affected by TasA, TapA and EPS in vitro, suggesting that these molecules interact with calcium ions in the biofilm[56].

Although the most abundant constituents of the biofilm matrix in *B. subtilis* have been identified, the interaction among them is largely unexplored. Intriguingly, substitution of specific conserved residues in TasA with a cysteine residue allows pellicle biofilm development in the absence of EPS[57]. However, this process is dependent on BslA[57], suggesting that these TasA substitutions result in a novel inter-molecular interaction between these two components. Further investigation of matrix interactions and structures is required to fully understand this complex system.

[H1] Regulation of biofilm formation

Biofilm formation is an energetically expensive process that requires the transcription of a suite of genes to ensure timely production of the matrix molecules. Although the regulation of biofilm formation in *B. subtilis* has been extensively studied (reviewed elsewhere[58,59]), additional aspects of regulatory networks are still being discovered. Here, we briefly introduce the main components of these networks and review recent advances in the regulation of biofilm development mediated by intracellular circuitry.

[H2] Intracellular regulatory pathways

Biofilm formation and motility are described as mutually exclusive lifestyles, with cells in a bacterial population expressing genes that are necessary either for motility or for biofilm matrix production but not for both[60]. Consistent with this idea, *B. subtilis* exhibits heterogeneity in gene expression within the cell population (extensively reviewed elsewhere[59]), and transcription of the genes needed for motility is inversely correlated with that of the genes needed for biofilm matrix production. The transcription factor Spo0A is a critical regulator of *B. subtilis* biofilm formation[4,5], with this regulatory pathway serving as an integration 'hub' for many competing and overlapping signals to control the proportion of cells in the biofilm that transcribe genes linked to biofilm matrix production. Initiation of biofilm formation is promoted when moderate levels of phosphorylated Spo0A (Spo0A~P) are reached within a cell.

The sensor histidine kinases KinA, KinB, KinC and KinD are indirectly responsible for the phosphorylation of Spo0A via a phosphorelay, in which the phosphoryl groups are sequentially transferred from the kinases to the relay proteins Spo0F and Spo0B and finally to Spo0A[61,62]. The phosphorelay is affected by numerous Rap phosphatases and their corresponding Phr pentapeptides that together act as quorum-sensing systems[63]. These Rap–Phr systems affect biofilm development and evolution[64], especially the plasmid-encoded RapP in strain NCIB 3610[65]. At threshold levels, Spo0A~P directly represses transcription of *abrB*, a transition state transcription repressor of several operons involved in biofilm formation, and concomitantly promotes the expression of *abbA*, which encodes an anti-repressor of AbrB[66].

A second Spo0A~P-dependent anti-repressor pathway involves the transcription repressor SinR, a homotetramer that directly inhibits transcription of the matrix-encoding *epsA–O* and *tapA* operons[60]. Threshold levels of phospho-Spo0A trigger production of the SinR anti-repressor protein SinI and thereby stimulate transcription of the matrix-encoding operons. The first structures of full-length tetrameric SinR and dimeric SinI in solution shed light on how SinR binds to DNA and how SinI inhibits this function[67]. The formation of a flexible SinR homotetramer facilitates bending of the target DNA to enable repression of biofilm genes. SinI inhibits SinR from effectively binding DNA by forming SinR–SinI dimers that disrupt the formation of SinR homotetramers. SlrR is another antagonist of SinR which is induced by threshold levels of phosphor-Spo0A[68] and promotes transcription of the *epsA–O* and *tapA* operons, thus enabling the switch from a motile state to biofilm formation[69]. As levels of phospho-Spo0A~P are not elevated homogeneously in the population, the regulatory network

heterogeneously induces expression of matrix genes, creating subpopulations of matrix producer (ON-state) and non-producer (OFF-state) cells[70-72].

Other transcription regulation systems also influence expression of biofilm-related genes by altering Spo0A~P levels. For example, the phosphodiesterase YmdB influences the switch between biofilm and motility gene expression. YmdB deficiency has wide ranging effects on gene expression, resulting in reduced expression of biofilm genes and enhanced expression of motility genes, including *hag* (encoding flagellin) and autolysin genes[73,74]. By allowing the expression of SinR-repressed genes in a subpopulation of cells, YmdB acts as a regulatory protein that affects phenotypic heterogeneity in matrix gene expression[75].

Another regulatory factor is the transcription termination factor Rho, an ATP-dependent RNA helicase/translocase that represses genome-wide transcription[76,77]. Rho both directly and indirectly regulates motility, biofilm formation and sporulation[78]. In Rho-deficient cells, *epsA–O* and *tapA* operons are transcribed at low levels owing to an increase in SinR-mediated repression of their promoters triggered by enhanced Spo0A phosphorylation.

The Spo0A mediated regulatory pathway is also modulated by the secondary messenger cyclic di-adenylate monophosphate (c-di-AMP). In addition to controlling growth, response to DNA damage and sporulation[79, 80], c-di-AMP also reduces expression of the *tapA* and *epsA-O* operons, leading to impaired biofilm formation[81]. While disruption of *sinR* restores biofilm formation under increased intracellular c-di-AMP concentrations, c-di-AMP accumulation does not affect the intracellular levels of SinR, suggesting that the nucleotide affects the activity of SinR. However, another study found that increased intracellular levels of c-di-AMP led to increased expression of the *tapA* operon. Furthermore, *B. subtilis* encodes two putative c-di-AMP transporters that are involved in c-di-AMP secretion and facilitate root colonization. Thus, *B. subtilis* can sense and respond to extracellular c-di-AMP, uncovering a potential role of this second messenger in inter-bacterial communication[82]. Given the differences in experimental results, it is possible that the effect of intracellular c-di-AMP levels or the ability to secrete c-di-AMP changes in response to environmental conditions. c-di-AMP is not the only secondary messenger molecule that affects cell differentiation in *B. subtilis*. Cyclic di-GMP (c-di-GMP) signalling directly inhibits motility in *B. subtilis* through the interaction of the putative c-di-GMP receptor YpfA with the flagellar motor protein MotA[83]. However, the effect of c-di-GMP levels on biofilm formation is disputed[83,84].

[H2] The Y-complex

The Y-complex comprises three proteins, RicA, RicF and RicT (also known as YmcA, YlbF and YaaT, respectively), which contribute to *B. subtilis* biofilm formation. Inactivation of these proteins results in impaired biofilm-forming ability[38,60], although the mechanism underpinning this phenotype remains unclear. A study reported that the Y-complex regulates biofilm formation by accelerating the phosphorylation of Spo0A[85], whereas another found that SinR mRNA and protein levels are increased in Y-complex mutants and demonstrated that the Y-complex interacts with and controls the activity of RNaseY, which destabilizes *sinR* mRNA[86]. However, more recent findings dispute the mRNA destabilization model and suggest that in addition to accelerating Spo0A phosphorylation, the Y-complex induces biofilm formation through an unknown Spo0A-independent pathway [87,88].

[H2] Sensing stress

Stress conditions influence biofilm formation in diverse bacteria[89]. In *B. subtilis*, the alternative sigma factor sigma B (σ^B) is activated by stressors, including heat, salt and starvation conditions[90]. Activity of σ^B manifests as a series of stochastic pulses within cells in the biofilm, showing maximal expression at the upper surfaces of the community[91]. Stochastic pulsing of σ^B appears to provide an adaptation mechanism for cells to either activate σ^B or sporulate, thus allowing cells in these different states to coexist in the same zone of the biofilm structure. Heterogeneous expression of biofilm components is linked with changes in biofilm structure and robustness over both space and time[71]. For example, during pellicle formation a substantial increase in biomass robustness is correlated with an increase in *epsA–O* expression, and with *tasA* expression being almost universally activated in the cells of the biofilm population[71]. These data also reveal heterogeneity between the transcription profiles from the *tapA* and *epsA–O* promoters. The genetically homogeneous population in the pellicle undergoes a phenotypic segregation into three phenotypically distinct subgroups producing different levels of EPS and TasA: matrix non-producers, EPS producers, and generalist cells that produce both major components. This differentiation of the population can be viewed as a 'phenotypic division of labour' where specialization leads to maximum population efficiency[92].

The repertoire of regulatory network components influencing biofilm development in *B. subtilis* is extensive. However, we still do not fully understand whether these pathways are active under all conditions or only become active in specific environmental conditions. Heterogeneous activation of transcriptional regulators further increases the phenotypic diversity of *B. subtilis* cells within the biofilms and enhances survival.

[H1] Impact of microbial interactions

The drive to understand microbial interactions and re-create simplified, ecologically relevant habitats has led to research on mixed biofilms. In the rhizosphere, *B. subtilis* cohabitates with many soil-dwelling bacteria and fungi, and interactions between these organisms and *B. subtilis* can modulate biofilm structure and robustness (comprehensively reviewed elsewhere[93]).

[H2] Biofilm modulation by other species

Numerous bacterial species modulate *B. subtilis* biofilm formation and structure[94-96]. The expression of *B. subtilis* biofilm genes is induced during co-culture with other members of the *Bacillus* genus[97]. *B. cereus* produces and secretes thiazolyl peptide antibiotics termed thiocillins, which trigger matrix production in *B. subtilis*[98]. Interestingly, abolishing the antibiotic activity of thiocillin did not affect its ability to induce biofilm gene expression, suggesting that secondary metabolites can have an alternative function in addition to antibiosis. By contrast, *Pseudomonas putida* and *Pseudomonas protegens* hinder biofilm-specific gene expression when co-cultured with *B. subtilis*, a behaviour that is mediated by secretion of the antimicrobial 2,4-diacetylphloroglucinol (DAPG) that delays *tapA* gene expression at subinhibitory concentrations[99]. DAPG alters *B. subtilis* biofilm growth and phenotypic differentiation adjacent to *P. protegens*. While various environmental signals are sensed by histidine kinases in *B. subtilis* that affect Spo0A~P levels, development of *B. subtilis* biofilms can also be affected in a Spo0A-independent manner[95]. The soil bacterium *LysiniBacillus fusiformis* induces wrinkle formation in *B. subtilis* colonies through a diffusible primary metabolite, hypoxanthine [95]. The induction of biofilm wrinkle formation was hypothesized to be mediated by localized *B. subtilis* cell death caused by increased intracellular hypoxanthine levels, as deletion of hypoxanthine transporters in *B. subtilis* abolished induction of wrinkles by *L. fusiformis*[95].

Mixed cross-kingdom biofilms are also observed. For example, *B. subtilis* colonization of the hyphae of both the filamentous black mould fungus *Aspergillus niger* and hyphae of the basidiomycete mushroom *Agaricus bisporus* depends on TasA and EPS[7]. *B. subtilis* also forms biofilms on the ectomycorrhizal fungus *Laccaria bicolor*, where eDNA has been suggested to contribute to this interaction[100]. The relationship can also be bidirectional; the plant pathogen *Fusarium culmorum* induces biofilm formation in *B. subtilis* through decreased expression of *sinR* and therefore enhanced *tasA* transcription[101]. In addition, the presence of fungal plant

pathogens can also induce the expression of fungitoxic secondary metabolites by *B. subtilis* to increase its competitive edge[102,103].

[H2] Synergism in multispecies biofilms

Dual-species biofilms can gain unique characteristics as compared to their respective monoculture colonies[96]. The soil dwelling and rhizospheric bacterium, *Pantoea agglomerans* has increased antibiotic resistance when it cohabitates with *B. subtilis*[94], which requires *B. subtilis* TasA and a *P. agglomerans* exopolysaccharide. Similarly, *B. subtilis* adherence to a *Streptococcus mutans* biofilm also depends on TasA[96]. Interestingly, production of *B. subtilis* biofilm components is induced by *S. mutans*, suggestive of a feedback loop. In addition to synergistic mixed-species communities, the *B. subtilis* matrix has a protective role in encounters with other microbes. On the leaf surface, *Pseudomonas chlororaphis* invades the *B. subtilis* population when the biofilm matrix is absent, while the type VI secretion system of *P. chlororaphis* additionally stimulates sporulation of *B. subtilis*[104]. In the presence of the biofilm matrix, *B. subtilis* co-exists with *P. chlororaphis*, forming a community that collectively promotes plant growth and protects from pathogens. The *B. subtilis* matrix is also needed to create a spore-filled biofilm megastructure upon interaction with *Myxococcus xanthus*[105]. The emergent properties of the *B. subtilis* biofilm can also affect *B. subtilis* and other microbes in the surrounding environment. Potassium ion channel-mediated electrical signals generated by a *B. subtilis* biofilm alter the membrane potential of distant cells and modulate their motility[106]. This communication mechanism extends beyond the cells within the *B. subtilis* population; for example, *Pseudomonas aeruginosa* cells are attracted to the electrical signal released by the *B. subtilis* biofilm[107].

[H2] Interspecific competition

Engagement with a microbial neighbour can also be less amicable, as *B. subtilis* expresses toxins and other effectors to dominate a niche. The build-up of the extracellular iron-chelating molecule pulcherriminic acid in the region surrounding colony biofilms initiates growth arrest of the producing community while concomitantly preventing invasion by neighbouring microorganisms[108,109]. Other bioactive compounds, including surfactins, the cannibalism toxin SDP (cannibalism in this context refers to inducing lysis of conspecific cells and cells of other species to release nutrients for biofilm growth), sporulation killing factor and various other secondary metabolites, are of interest owing to their potential agricultural use as biocontrol agents. For example, *B. subtilis* biofilms outcompete *Serratia plymuthica* both *in*

vitro and on plant roots by producing bacillaene, a non-ribosomally synthesized antibiotic[110]. *B. subtilis* biofilms on plant roots also form a zone of exclusion that prevents *E. coli* colonization, by an unidentified mechanism[111]. Competition is also present between *Bacillus* spp., as *B. subtilis* biofilms expand and subsume neighbouring *Bacillus simplex* colonies, by secreting SDP and the lipopeptide toxin surfactin, which induce *B. simplex* cell death[112]. Of note, *B. subtilis* produces a biofilm-specific antibacterial toxin, YIT, which contributes to its competitiveness against sensitive strains or species[113]. YIT is produced during biofilm formation from an operon paralogous to that of SDP but unlike SDP can permeate the biofilm matrix. Depending on their concentration, many of these compounds can also affect 'kin' (or conspecific) strains, leading to nuance in interspecific and intraspecific microbial interactions[114-116]. Imaging mass spectrometry provides a powerful novel approach to study how specific metabolites and peptides are spatially distributed in the above-mentioned interactions and will contribute to understanding of how interspecific and intraspecific interactions modulate *B. subtilis* biofilm development.

[H1] Biofilm formation on plant roots

B. subtilis is a well-known biocontrol agent that is widely used in agriculture[117]. However, the underlying mechanism of these plant–microbe interactions and how they contribute to preventing pathogenic microorganism colonization has only recently been explored[118]. Advances in model systems and experimental designs have allowed visualization of *B. subtilis* biofilm dynamics in the rhizosphere over longer time scales. Although these studies primarily examined *Arabidopsis thaliana*, other plant species are beginning to be used[8,111] (BOX 2). A large diversity of *B. subtilis* strains have been isolated from the rhizosphere[119,120 16]. For example, 50 unique isolates were collected from the rhizosphere of cocoa trees (*Theobroma cacao*), of which 90% were able to form robust biofilms[119]. Similarly, another study found variation in the ability of rhizosphere *B. subtilis* isolates to form biofilms *in vitro* and on tomato plant roots[16]. The variation among *B. subtilis* strains is not only observed in their ability to form biofilms but also in additional plant growth-promoting and biocontrol traits, such as mineral solubilization, indole acetic acid production, siderophore secretion and surfactin production[16 120].

[H2] Colonization of plant roots

Before biofilms can form, motile cells need to find and adhere to the root surfaces. Indeed, both motility and chemotaxis are required for *B. subtilis* colonization of *A. thaliana*

roots[121]Of the 10 known chemoreceptors encoded in the *B. subtilis* genome, McpB, McpC and TlpC mediate attraction to *A. thaliana* root exudates[121] (Figure 3). Interestingly, colonization of growing roots is not solely dependent on these chemoreceptors, suggesting that redundancies are present in the chemotaxis and other sensing systems that respond to root-derived signals[121]. *B. subtilis* preferentially colonizes the root differentiation and elongation zone, as shown for *A. thaliana* and cotton plants[111, 122]. Microfluidic imaging techniques showed that accumulation of *B. subtilis* at the elongation zone began within 20 min of inoculation[111]. Intercellular signalling between *B. subtilis* cells (that is, secretion of c-di-AMP) is important for successful attachment to the root, as the c-di-AMP permeases YcnB and YhcA are necessary for efficient colonization of *A. thaliana* roots[82].

B. subtilis matrix components are required for colonization and biofilm formation on plant roots[16,123,124]. Deletion of the *epsA–O* operon or *tasA* coding region result in defective biofilm formation on tomato seedling roots[16,124]. Interestingly matrix-deficient *B. subtilis* mutants only establish dense biofilms on *A. thaliana* roots when co-inoculated with wild-type cells, suggesting that matrix components are shared[92,123]. Overproduction of γ-PGA also contributes to root colonization and persistence of some *B. subtilis* strains, although it is not essential[125,126, 52]. The role of surfactins in *B. subtilis* biofilm formation on root surfaces is unclear[120,127, 128]. Although surfactin production alters the structure of colony and pellicle biofilms *in vitro*, the most recent data suggest that surfactin does not affect biofilm formation on plant roots[120, 128].

[H2] Ecological interactions

Our molecular understanding of plant signalling and interactions with *B. subtilis* continues to advance. Plant polysaccharides, including xylan, pectin, and arabinogalactan, have been found to have a crucial role in stimulating *B. subtilis* biofilm formation[123,129]. These molecules perform this function in two distinct ways; first, they induce biofilm matrix gene expression by stimulating the activity of histidine kinases that phosphorylate the master regulator Spo0A and, second, they are processed and incorporated into the biofilm matrix[123]. Digestion and utilization of galactose might occur through the galactan utilization pathway, as *B. subtilis* binding to plant roots or β-1,4-galactobiose leads to derepression of the galactan utilization (*gan*) operon[129].

Root-associated biofilms also represent an environment for kin discrimination (differential interaction with organisms based on relatedness) among *B. subtilis* strains or isolates. The ability of *B. subtilis* strains to create mixed-isolate biofilms on plant roots directly

correlates with the phylogenetic distance of the strains, suggesting that there is antagonism between non-kin strains during rhizoplane colonization of the rhizoplane (the region of the rhizosphere in which the root is in contact with the soil)[114]. Microbes that are potentially valuable to plants must compete with other organisms in the ecosystem to occupy this space. Tomato plant exudates induce bacillaene production by *B. subtilis* during colonization, which leads to increased killing efficacy of the rhizoplane competitor *S. plymuthica*[110]. In addition, superior *B. subtilis* colonization compared with *S. plymuthica* also mediated increased plant systemic resistance against the plant pathogen *Pseudomonas syringae* [110]. This interplay suggests that a complex symbiosis exists between *B. subtilis* and the plant host, which is driven by evolutionary adaptation.

[H1] Biofilms in the intestinal tract

Commensal bacteria are pivotal to human health, as they prompt the host's immune system to induce protective responses that prevent colonization and invasion by pathogenic species. Interest is growing in the identification of the genes and mechanisms that are utilized by beneficial microbes to limit disease caused by invading pathogens. Although *B. subtilis* strains are mainly found in the soil, they have also been isolated from human skin and the gastrointestinal tract, showing that the bacterium has adapted its physiology to survive under diverse conditions[130,131]. *B. subtilis* is one of the predominant microorganisms used in probiotic products, despite the underlying mechanisms by which the bacterium can restore and maintain a healthy gut flora being largely unknown. Studies have begun to link the protective effect of B. *subtilis* to its biofilm production (Figure 4).

In a mouse model, wild-type *B. subtilis* confers protection from *Citrobacter rodentium*, an enteric pathogen that causes acute colitis, unlike an EPS-deficient *B. subtilis* strain[132]. Protection by *B. subtilis* EPS is a result of host immune modulation rather than prevention of pathogen colonization or disruption of the epithelium. EPS prevents colitis in a TLR4-dependent manner that requires myeloid cells[50]. EPS induces the formation of anti-inflammatory M2 macrophages that produce TGFβ and PDL1 to broadly inhibit the activation of T cell responses[133]. Furthermore, *B. subtilis* EPS also protects against *Staphylococcus aureus* systemic infection. Macrophages from EPS-treated mice exhibited an M2 phenotype and also restricted growth of internalized *S. aureus* through the production of reactive oxygen species[134]. Altogether, these data suggest that *B. subtilis* EPS can induce the differentiation of immune

cells that have antibacterial and anti-inflammatory properties, which ultimately contribute to increased host survival.

Caenorhabditis elegans is a nematode that is widely used to study bacterial pathogenicity and host immunity. *B. subtilis* was first found to be beneficial to *C. elegans*, as substituting *E. coli* OP50 for *B. subtilis* as a food source extended the longevity of the *C. elegans daf-2* and *age-1* long-lived mutants[135]. Biofilm formation by *B. subtilis* was later identified as one of the factors that prolong *C. elegans* longevity. Two molecules produced by *B. subtilis* that extend *C. elegans* lifespan are the quorum-sensing molecule CSF and nitric oxide. Synthesis of both molecules is enhanced in *B. subtilis* biofilm-forming conditions, compared with planktonic growth[136]. *B. subtilis* biofilm formation in *C. elegans* also confers resistance against different types of stress, such as heat and oxidative stress[137]. The protective role of the *B. subtilis* biofilm was additionally demonstrated against the causative agents of two detrimental neurodegenerative diseases, α-synuclein in Parkinson disease and amyloid-β in Alzheimer disease. A probiotic *B. subtilis* strain inhibits aggregation of α-synuclein and cleared α-synuclein aggregates in a *C. elegans* synucleinopathy model. Biofilm-deficient strains exhibited higher rates of α-synuclein aggregation than in a wild-type biofilm-proficient strain[138]. Similarly, *B. subtilis* biofilm formation in the gut contributes to the role of *B. subtilis* in protecting against amyloid-β-related toxicity in a *C. elegans* Alzheimer disease model[139]. Research is currently ongoing to further investigate the potential of *B. subtilis* to treat or prevent neurodegenerative diseases. Future developments in the field, such as imaging technologies, chemical profiling, and using genetically modified host systems will create opportunities to identify the underlying mechanisms involved in host–microbe interaction during *B. subtilis* biofilm formation.

[H1] Experimental evolution in the biofilm

An emerging topic in biofilm research is mapping the principles that govern evolution in biofilm populations. Studies have uncovered novel insights into genetic and phenotypic diversity and interactions between matrix elements that would otherwise have remained undetected. Intriguingly, repeated cultivation of *B. subtilis* in shaking planktonic conditions results in rapid genetic differentiation, discernible as derivative isolates with distinct colony morphotypes[140]. The variability in colony biofilm structures was connected to mutation of *sinR* and the resultant alteration in expression of biofilm-related genes. Similarly, pellicle biofilms formed by *B. subtilis* can be exploited for studying experimental evolution of *B. subtilis*

biofilms (Figure 5), primarily because of the degree of heterogeneity observed within the otherwise robust biofilm population. *B. subtilis* can diversify into distinct colony variants that exhibit differences in their biofilm formation abilities and expression of biofilm-related genes[141]. The evolved colony morphotypes also display differences in surface complexity and hydrophobicity. Interestingly, the co-cultivation of derived morphotypes increases the population yield (that is, the abundance of cells) compared with the ancestral strain NCIB 3610[141]. The increased biofilm dimensions with a mixed population are driven by the shareability of the secreted matrix components[71,92]. Indeed, a mixture of *tasA* and *epsA–O* mutant derivative strains rescue pellicle biofilm development by 'public goods' sharing[92]. Intriguingly, a mixture of two genetic derivative strains, one lacking EPS and the other TasA, enhances pellicle productivity (the number of cells residing in the biofilm) and plant root colonization compared with that of a strain that produces both components, revealing a 'genetic division of labour'[92]. Similarly, both EPS-containing supernatant and purified TasA protein fibres rescue biofilm formation by the respective mutants[11,18,22,23,92]. However, the degree of sharing of the two matrix components differs and TasA cannot be shared as widely in the population as EPS[71]. Such an imbalance creates a collapse of genetic division of labour when the mixture of *eps* and *tasA* mutants is evolved for a few hundred generations[57]. Eventually, *tasA* and *eps* mutants lacking one of the matrix components enhances the air–medium interface colonization ability, either by enhancing production of EPS or by altering TasA protein properties, respectively. In the EPS-deficient strain, introduction of cysteine residues at specific residues in TasA generates thicker fibres than those produced by the wild-type ancestor[57]. However, in the presence of EPS, strains with cysteine-substituted TasA are at a disadvantage, as hydrophobicity is hampered, possibly due to disulphide bond formation between the newly substituted cysteine in TasA and the cysteine residues of BslA[33,141]. Importantly, experimental evolution studies using strains that lack robust biofilm formation ability have helped to uncover novel features of TasA.

The rapid evolution of mutants with altered biofilm formation kinetics also highlights adaptation pathways in *B. subtilis*. A strain deficient in the phosphodiesterase YmdB does not form biofilms, although the ability to form pellicles in biofilm-inducing medium is rapidly recovered due to acquisition of mutations in *sinR*. Similarly, mutants lacking functional propelling flagella in *B. subtilis* rapidly attain mutations in *sinR* and increased expression of biofilm genes, an adaptation that increases competitiveness compared with its ancestor[142,143]. Of note, the range of mutations in *sinR* overlap in *ymdB* and motility-deficient *hag* background, highlighting a general evolutionary adaption in strains lacking biofilm-gene expression.

Experimental evolution can also be exploited to investigate how matrix-deficient derivative strains affect biofilm-proficient wild-type strains. Strains deficient in both EPS and TasA are unable to exploit the matrix nor do they incorporate into the pellicles formed by the wild-type strain[144]. However, after *de novo* evolved competition mediated by recombination of cryptic phi3T phage with the SPβ prophage region of *B. subtilis*, the double-mutant strain partially increases in the biofilms[144,145]. However, it remains unclear why the matrix-deficient double-mutant strain benefits more from this competition than the wild-type strain. By contrast, *epsA–O* or *tasA* single-mutant strains can readily exploit the matrix and benefit from this 'public good', as the cells disengage from the metabolic burden of producing the respective matrix component and therefore act as 'cheaters'[146].

The spatial structure of biofilms is a parameter that can affect bacterial adaptation. The ability of *B. subtilis* to form biofilms has been exploited to study metabolic adaptation in comparison with planktonic growth or other differentiation processes, such as sporulation [147]. It will be interesting to uncover distinct bacterial evolutionary and adaptation processes during biofilm development. Ongoing research is currently being performed to expand our understanding of the evolution of *B. subtilis* biofilms directly on the plant root, connecting experimental evolution with plant host-associated biofilms.

[H1] Applications of biofilms

B. subtilis is recognized as a superb model system to study the matrix components, gene regulation and social interactions during biofilm development. Systematic understanding of how *B. subtilis* biofilms can be harnessed for use in probiotics has only recently been explored. Intriguingly, humans have a long history of exploiting microorganisms and the use of *Bacillus* spp. can be traced back to the production of traditional fermented foods, including the Japanese soybean breakfast food natto and the Chinese delicacy pidan (Century egg). Today, a diverse range of processes exploit *Bacillus* spp. across many sectors of the economy, and more recently several of the innovations are both informed and inspired by knowledge of the processes and materials that underpin biofilm formation. The secreted matrix molecules have unique features that lend themselves to novel biotechnological or biomedical purposes. BslA undergoes a limited structural metamorphosis at an interface such that a surface-active region becomes exposed[35], which enables BslA to stabilize a variety of multiphase formulations[148]. Surface-active proteins have widespread uses in both nature and bioengineering, thus recombinant BslA has been proposed as a tool for stabilization of ice cream to slow down the melting process[149].

Novel industrial uses that require the direct use of *B. subtilis* biofilms have also emerged over the last decade, including the proposed use of *B. subtilis* biofilms for calcite precipitation[150]. Looking beyond the use of *B. subtilis* as a probiotic or biological control agent, *B. subtilis* biofilms can be repurposed as a protective coating for other probiotic bacteria in the gut[151]. Building on the viscoelastic properties of the whole biofilm, *B. subtilis* biofilms have been postulated as 'living materials' that can be used for a variety of purposes[152].

[H1] Conclusions and future perspectives

The future of *B. subtilis* research will undoubtedly be driven by next-generation experimental approaches, ecological relevance and applications triggered by a basic understanding of the molecular components of biofilm formation. Recent developments in single-cell transcriptome analysis should help to reveal heterogeneity in global gene expression profiles within both single- and mixed-species biofilms, while progress in imaging mass spectrometry at high spatial resolution will allow understanding of the chemical language that influences biofilm gene expression heterogeneity within microbial communities. Furthermore, increased resolution and sensitivity of imaging approaches applied to analyse biofilms of *B. subtilis* and co-inhabiting microbes directly within the host at continuous time scales can be applied to investigations of diverse environments ranging from the plant rhizosphere to the animal digestive system. While we have learnt a lot from dissecting *B. subtilis* biofilm formation in the laboratory, the discovery of *B. subtilis* biofilms in situ in nature still requires further technological and conceptual approaches. The first imaging of a naturally formed *B. subtilis* biofilm in situ in the soil is still awaited.

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Competing interests

The authors declare no competing interests.

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Display items

Figure legends

Figure 1. Formation of *Bacillus. subtilis* biofilms in vitro. a | Schematic of the three modes of biofilm growth in *B. subtilis*, colony, pellicle and submerged, surface-attached biofilms. **b** | A 48-hour-old colony biofilm formed by NCIB 3610 (90% founding cells) and an isogenic variant that constitutively expresses green fluorescent protein (GFP; 10% founding cells) after growth at 30°C on an MSgg agar plate. Heterogeneity and patches of clonal derivatives are evident when the biofilm is examined for GFP fluorescence. **c** | Confocal microscopy image of the central rugose region of an NCIB 3610 colony biofilm grown for 48 hours on MSgg agar at 30°C. **d** | Scanning electron microscopy image of individual cells of *B. subtilis* 168 in a biofilm. **e** | NCIB 3610 pellicle biofilm at the air–liquid interface. A water droplet placed on top of the pellicle in the central region (arrow) reveals the hydrophobicity of the biofilm. **f** | Confocal microscopy image of a submerged biofilm formed by *B. subtilis* isolate JH642 that is constitutively expressing GFP. Image in part **b** courtesy of Michael Porter, University of Dundee. Image in part **c** reprinted with permission from ref.[162], Wiley. Image in part **d** courtesy of Anna Dragoš and Paul J. Kempen, Technical University of Denmark. Image in part **f** courtesy of Eisha Mhatre, University of Pittsburgh.

Figure 2. *Bacillus subtilis* biofilm matrix components and biosynthetic systems. a | The biofilm matrix composition is complex and can contain self-produced molecules, including BslA, TasA fibres, extracellular DNA (eDNA), poly-γ-glutamic acid (γ-PGA) and exopolysaccharide (EPS). **b** | A cartoon schematic of the γ-PGA (red), EPS (yellow), and TapA (blue/purple) systems showing predicted cell membrane-embedded and cytosolic protein components based on bioinformatics and experimental evidence. The predicted glycosyl transferases (GT) of the EPS system have been labelled based on their GT family membership. SipW (dark blue) post-translationally cleaves TapA (light blue) and TasA (purple) at the cell surface with the arrows representing the location of cleavage. **c** | Transmission electron microscopy image of a recombinant BslA film

with the inset showing the Fast Fourier Transform supporting high order of the lattice. **d |** Cartoon representation of two monomers from the BslA crystal structure (Protein Data Bank (PDB) identifier (ID): 4BHU)[12], showing the 'cap in' and the 'cap out' conformation[35]. **e |** Recombinant TasA forms fibres *in vitro* that can be visualized by transmission electron microscopy. **f |** Cartoon representation of the crystal structure of TasA (PDB ID: 5OF1) with β-strands in purple and α-helices in yellow. Part c adapted with permission from ref.[35], National Academy of Sciences.

Figure 3. Formation of *Bacillus subtilis* biofilms in the rhizosphere. a | Schematic showing the rhizosphere (orange; the area around the growing plant root) with *B. subtilis* biofilms (teal) growing in the elongation zone. These biofilms influence plant growth and behaviour (green arrow) and directly protect against microbial plant pathogens through the section of active compounds. **b |** Magnified view of plane root cells, showing the three steps of *B. subtilis* colonization, namely attraction (step 1), attachment (step 2) and biofilm formation (step 3). Signals such as plant exudates and bacterially produced cyclic di-adenylate monophosphate (c-di-AMP) are important for attraction and attachment. **c |** Magnified view of bacterial cells, showing the molecular details of each step of plant root colonization. The chemoreceptors McpB, McpC, TlpC and others (pink) sense plant signals during attraction (step 1). The c-di-AMP permeases YcnB and YhcA (orange) are involved in intercellular signalling during attachment (step 2). The kinases KinC and KinD (green) sense plant polysaccharides and plant produced L-malic acid, which leads to matrix production. Binding of plant cell wall polysaccharides (green hexagon) to an unknown receptor also leads to upregulation of galactan (*gan* operon), which can lead to EPS production. Biofilm formation (step 3) occurs with the digestion of galactan and the production of matrix components.

Figure 4. Effects of *Bacillus subtilis* biofilms in the intestinal tract. a | Treatment of mice with the enteric pathogen *Citrobacter rodentium* (green) causes acute colitis (part **aA**). Pre-treatment of mice with *B. subtilis* NCIB 3610 spores (red) prior to inoculation with *C. rodentium* protects against acute colitis (part **aB**). Pre-treatment of mice with *B. subtilis* NCIB 3610 purified EPS (oval dots) and subsequently with *C. rodentium* also protects against acute colitis (part **aC**). Model of EPS modulation of immune responses. *B. subtilis* and purified EPS induce differentiation of M2 macrophages, which inhibit $CD4^+$ and $CD8^+$ T cells through the production of TGFβ and PDL1 (part **aD**). **b |** Inhibition of α-synuclein aggregation (green triangles) by *B. subtilis* biofilms in a *Caenorhabditis elegans* synucleinopathy model. Using *E. coli* strain OP50 (blue) as a food source leads to accumulation of α-synuclein aggregates (part **bA**), whereas using *B. subtilis* NCIB 3610 (red) as a food source results in reduced formation of α-synuclein aggregates (part **bB**). Using *B. subtilis* NCIB 3610 Δ*tasA* (yellow) as a food source removes the protective effect of *B. subtilis* biofilm formation on α-synuclein aggregation (part **bC**).

Figure 5. Evolution of social interactions in *Bacillus subtilis* biofilms. a | Experimental evolution of *B. subtilis* biofilms revealed genetic differentiation discernible by the appearance of versatile colony morphotypes with altered genetic background. **b |** Genetic division of labour leads to increased biofilm productivity compared with transcriptional division of labour. **c |** The presence of cheaters alters the intrinsic phenotypic heterogeneity during experimental evolution. **d |** An increase in cheater frequency ultimately leads to the tragedy of the commons (depletion of a shared resource through unhampered use driven by self-interest).

Boxes

BOX 1. Biophysical techniques in *Bacillus subtilis* biofilm research

From a biophysics perspective, biofilms are a form of soft matter — a material that can easily be deformed by external forces and behaves differently to solid and liquids. Biophysical techniques have been used to probe biofilm properties at both the whole biofilm and macromolecular level, yielding novel insights into their form and properties (comprehensively reviewed elsewhere[153]).

[b1] Sessile drop methods

These methods are used for direct measurement of the contact angle between a liquid and a surface and revealed that the upper surface of the *Bacillus subtilis* colony biofilm is non-

wetting[39], a finding that led to molecular analyses of the mechanisms underpinning this remarkable feature[12,13,33,35,141,154,155].

[b1] Pendant drop analysis

This type of analysis allows the measurement of surface and interfacial tension and was used to show that BslA is a surface-active protein that forms a stable film at an interface[12].

[b1] Rheology

Rheology is the study of how soft matter deforms and flows. Rheological analysis in the presence and absence of specific matrix elements has revealed that biofilms are viscoelastic structures with properties that change over time[156] or in evolved strains[57] and dual-species biofilms[94] and that the BslA surface layer adds stiffness to the biomass[54].

[b1] Profilometric imaging

This technique has been used to analyse surface topology at the macro- and meso-scale, revealing how the biofilm surface complexity affects wetting behaviour[154] and susceptibility to chemical and mechanical stresses[155], the effect of metal ions on biofilm surface wetting[157], and the disassembly of experimentally evolved colony morphotypes.

[b1] Langmuir trough

This apparatus allows the effect of a compressive force on molecules (across a range of scales) on the surface of a subphase to be determined. Studies with the Langmuir trough revealed that the elastic properties of the whole pellicle biofilm are conferred by the extracellular matrix and that the wrinkled morphology is derived from compression by growth in a confined space[158, 159]. On a smaller scale, the properties of the stable film of BslA assembled at the air–liquid interface have been probed[160].

[b1] Atomic force microscopy

A form of scanning probe microscopy that has been used to examine *B. subtilis* biofilms on clay particles[161] and to investigate the interaction between extracellular DNA and exopolysaccharide to probe the space between cells and uncover detail of the microstructure of the biofilm surface[14].

BOX 2. Imaging techniques to study plant colonization

Plant–microbe interactions are complex and challenging to investigate at high resolution in real time. Root colonization and biofilm formation in the rhizosphere have been studied primarily using endpoint assays in which seedlings or plants are exhumed from the growth media or soil. Advances in microfluidic setups have enabled the visualization of biofilm formation on the roots of thale cress (*Arabidopsis thaliana*), cotton plants and aspen trees. These studies customized microfluidic chip-based plant cultivation experiments (see the figure) to investigate root–microbe interactions (RMIs) over time. RMI chip systems have been designed to contain an array of isolated growth chambers with individual media inlets and outlets, allowing for a comparison of conditions and replicates in the same microfluidic device[8,111]. Colonization and biofilm dynamics at single-cell resolution can be investigated with RMI chip systems using confocal microscopy, such as investigating *Bacillus subtilis* preference between different *A. thaliana* genotypes and the interplay between *B. subtilis* biofilms and *Escherichia coli* at the root surface[111]. These experiments are somewhat limited by the small chamber size required for microfluidics, as fast-growing plants such as *A. thaliana* out-grow the chamber in about 10 days, whereas slower-growing plants such as tree seedlings could be visualized for much longer[8].

ToC blurb

In this Review, Stanley-Wall and colleagues provide an overview of biofilm composition and formation in *Bacillus subtilis* and how this research is informing microbial evolution and ecology and aiding in the development of beneficial applications for biofilms.