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Bacillus cereus sensu lato biofilm formation and its ecological importance

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ABSTRACT

Biofilm formation is a ubiquitous process of bacterial communities that enables them to survive and persist in various environmental niches. The *Bacillus cereus* group includes phenotypically diversified species that are widely distributed in the environment. Often, *B. cereus* is considered a soil inhabitant, but it is also commonly isolated from plant roots, nematodes, and food products. Biofilms differ in their architecture and developmental processes, reflecting adaptations to specific niches. Importantly, some *B. cereus* strains are foodborne pathogens responsible for two types of gastrointestinal diseases, diarrhea and emesis, caused by distinct toxins. Thus, the persistency of biofilms is of particular concern for the food industry, and understanding the underlying mechanisms of biofilm formation contributes to cleaning procedures. This review focuses on the genetic background underpinning the regulation of biofilm development, as well as the matrix components associated with biofilms. We also reflect on the correlation between biofilm formation and the development of highly resistant spores. Finally, advances in our understanding of the ecological importance and evolution of biofilm formation in the *B. cereus* group are discussed.

1. Introduction

Biofilms are bacterial communities living in a collective form that confers various advantages on the inhabitants, and cells in biofilms represent a higher level of organization than solitary cells [1]. Bacterial biofilms are ubiquitous and widespread in both natural and artificial environments. Cells in biofilms are encased in a self-produced matrix typically comprising exopolysaccharides (EPS), fiber proteins, and frequently also extracellular DNA (eDNA) [2,3]. The driving forces of the transition from a unicellular to a multicellular lifestyle are a rapidly-growing field of research, especially the evolutionary and ecological factors.

Bacillus cereus sensu lato (s.l.) includes three main species; the foodborne pathogen *Bacillus cereus*, the biopesticide control agent *Bacillus thuringiensis*, and the anthrax-causing pathogen *Bacillus anthracis* [4,5]. High levels of genome similarity between these three species of *B. cereus sensu lato* makes their taxonomical classification difficult to discern [6]. Importantly, the ecological niches of *B. cereus s.l.* are widely distributed among soil, plant rhizosphere, and arthropod and nematode guts [7–11]. The highly diversified ecology of *B. cereus s.l.* is also reflected by the fact that both probiotic and pathogenic traits have been identified in the group [12]. Furthermore, besides being widely commercialized as pesticides, strains of the *B. cereus s.l.* group have also been exploited as plant growth-promoting bacteria (PGPB), suggesting an intrinsic ability to colonize plants [13,14].

B. cereus isolates vary in their physiological properties and survival abilities under different stress conditions. Nevertheless, the formation of biofilms by *B. cereus* strains is a universal trait that facilitates survival and persistence in harsh environmental conditions [5,15]. Most scenarios, such as colonization of plant rhizosphere and soil, are related to the sessile state of bacterial biofilms. For instance, *B. cereus* colonizes plant roots by forming biofilms. The *tasA* gene is an essential gene for *Bacillus subtilis* biofilms, and its paralog is needed for root colonization in *B. cereus* [16,17]. *B. cereus* biofilms are known to be the source of device contamination in clinical settings and in food industries [18]. Furthermore, the production of endospores during the late developmental stage complicates the removal of biofilms during the cleaning process due to the ability of spores to survive heating and irradiation processes [19]. Owing to the persistence of biofilms and the secretion of potential enterotoxins such as nonhemolytic enterotoxin (NHE), hemolysin BL (HBL), and cytotoxin K (CytK), a considerable amount of research has focused on strategies to prevent biofilm formation or remove mature biofilms, which has been systematically reviewed in other studies [20,21].

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The bacterial biofilm lifestyle is a cyclic process for most if not all species, involving at least five phenotypically distinct stages [22]; a complete biofilm cycle typically includes initial attachment, irreversible attachment, biofilm maturation, initiation of biofilm dispersion, and dispersal. Among these stages, studies on *B. cereus* biofilms have mostly focused on the first three stages, especially the involvement of biofilm matrix components, the role of flagella, and regulatory networks. Similarly, these developmental stages have been extensively explored for *B. subtilis* biofilms, and comparative studies have uncovered both shared and distinct molecular mechanisms between these two species [23]. For instance, EPS synthesized by the coded enzymes of the *epsA–O* operon in *B. subtilis* is one of the main extracellular matrix components, while its homolog has a minor role in *B. cereus* biofilms. The genomes of *B. cereus* lack paralogs of *bslA* and *tapA* genes in *B. subtilis*, whereas there are two paralogs of *B. subtilis tasA* [17,24]. Various in-depth studies into the *B. cereus* biofilm lifestyle are being driven by these variations in biofilm formation between the two species.

In the previous decade, a substantial amount of knowledge about biofilm formation in the. *B. cereus* group has been acquired through a wide field of research topics. This review summarizes recent advances in our knowledge of both the mechanisms and applications governing biofilm formation in *B. cereus s.l.* We explore advances in *B. cereus* biofilm formation within the context of global regulation and the components of the biofilm matrix, and expand on the heterogeneity within biofilm structures. Finally, we address advances in terms of ecological importance of several aspects including plant-associated biofilms and food industry contamination.

2. Global analysis of biofilm development

2.1. Genomic screens to identify biofilm-related genes

In contrast to *B. subtilis*, regulatory mechanisms that control biofilm formation in *B. cereus* are poorly understood, but recent progress advanced the characterization of biofilm-related genes in this group of *Bacilli* (Fig. 1). Random transposon insertion mutagenesis is an untargeted method that has been used extensively for bacterial genomics. Using this approach, Yan and colleagues (2017) identified 23 biofilm-related genes in *B. cereus* AR156, an environmental isolate that demonstrated promising biological control properties against plant fungal pathogens [25]. Among ~10,000 transposon insertion mutants, mutations within these 23 genes altered pellicle formation quantitatively. While most mutants exhibited reduced biofilm formation, mutants with either an in-frame deletion or a transposon insertion in the *clpY* gene enhanced pellicle biofilm (Fig. 1). The *clpY* gene encodes the ATPase substrate-binding subunit of the ClpY-ClpQ protease complex, and it is located in an operon with *clpQ*, *codY*, and *xerC* [26]. Mutations resulting in impaired biofilm formation were located among others in genes *comER*, *purD*, *purH*, *aad*, and *pepP*. According to functional prediction, these genes are related to key processes such as nucleotide biosynthesis, iron salvage, antibiotic production, ATP-dependent protease, and transcription regulation, suggesting that these activities are critical for biofilm formation. The function of *comER* was dissected and ComER was found to positively regulate both biofilm formation and sporulation, possibly by influencing the activity of Spo0A, the global regulator of sporulation and biofilm formation in most *Bacilli* [27].

Using a similar approach, Okshevsky et al. adapted an unbiased mariner transposon method to create a library of over 5000 transposon mutants of the model biofilm-producing strain *B. cereus* ATCC 10987 [28]. Screening for the lack of pellicle and submerged biofilm formation abilities identified 91 biofilm-related genes. Several of these were newly discovered including *dra* and those in the BCE_5583–5587 operon, while genes such as diguanylate cyclase-encoding BCE_0696, *gidAB*, and *dltB* were previously known to effect biofilm formation in other organisms. A key gene, *galE*, was identified as essential for biofilm formation in *B. cereus*. GalE governs galactose metabolism, which plays a critical role in biofilm formation of *B. subtilis* [29]. Similarly, a study on the evolution of plant-associated biofilms of *B. thuringiensis* further proved that *galE* is important for forming biofilms on plant roots [30]. Together, these reports indicated that metabolism of galactose, a common monosaccharide, is essential for extracellular matrix production in *Bacillus* species.

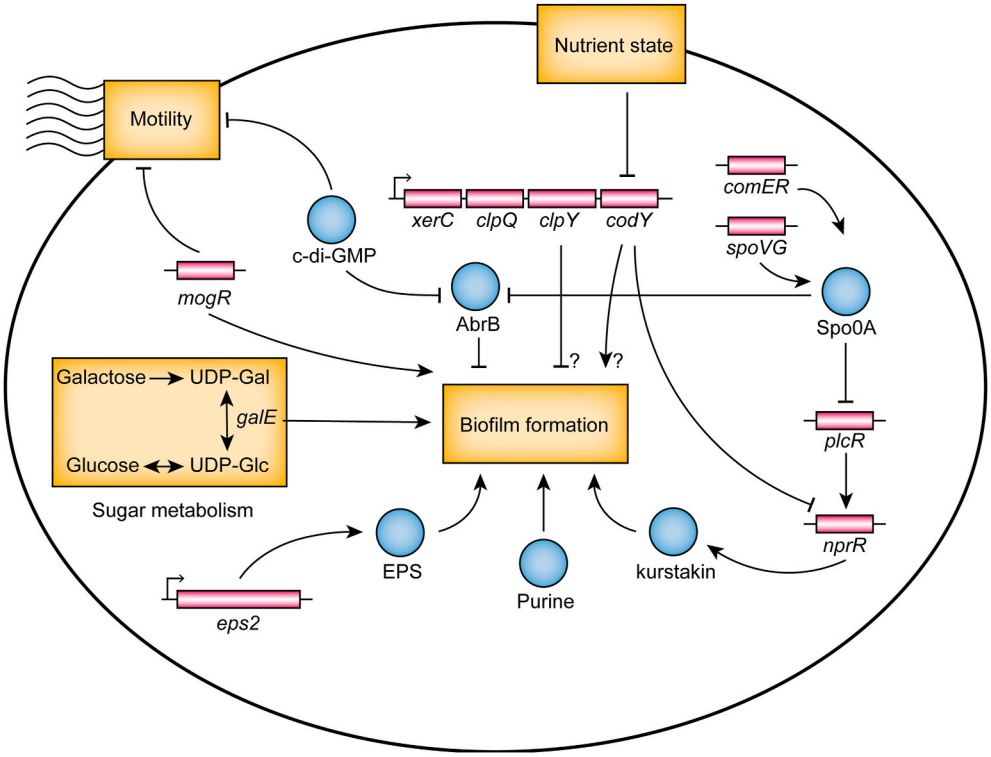

Fig. 1. Schematic diagram of the regulatory network of *B. cereus* group bacteria that controls biofilm formation. Circles represent protein products, pink rectangles represent open reading frames (ORFs), and yellow rectangles indicate physiological activities. Arrows represent activation and blunt lines denote repression. ClpY represses biofilm formation via an unknown mechanism. Two genes, *comER* and *spoVG*, reportedly promote *spo0A* transcription, which in turn affects biofilm formation. NprR promotes kurstakin synthesis, which itself positively regulates biofilm formation. Furthermore, EPS produced by enzymes encoded in the *eps2* operon is essential for biofilm formation, as well as purine biosynthesis. Similarly, *galE*, a gene encoding an enzyme related to galactose metabolism, is important for biofilm formation. The regulator MogR inhibits motility and promotes biofilm formation. Finally, c-di-GMP also regulates motility and biofilm formation, by repressing the biofilm repressor AbrB. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

By combining RNA sequencing and mass spectrometry-based proteomics, profound physiological changes in biofilm assembly of *B. cereus* ATCC14579 were revealed compared to planktonic cells [31]. These metabolic changes, such as reinforcement of the cell wall, elevated synthesis of secondary metabolites, and extracellular matrix in biofilm cells are part of an efficient strategy employed by *B. cereus* to adapt to changeable environmental conditions. Compared with planktonic cells, biofilm populations exhibit altered metabolism of nucleotides, sugars, amino acids, and energy. Moreover, virulence factors are expressed at substantially higher levels in planktonic cells, suggesting they have a metabolic proclivity to colonize new habitats, and are also more hazardous. In addition, two putative EPS gene clusters were identified; one (*BC5263-BC5279**, named *eps1*) homologous to the *eps* operon in *B. subtilis* is not responsible for biofilm development, while the other operon (BC1583-BC159, named *eps2*) is overexpressed in biofilm cells.

2.2. Motility and biofilm formation

Although flagella were not directly required for adhesion to glass in *B. cereus*, flagella-driven motility is involved in biofilm formation in various aspects, including biofilm initiation and recruitment of cells from the motile state [32]. The mechanisms through which motility influences biofilm formation in *B. cereus* have been reviewed previously [23]. In *B. cereus* ATCC 10987, the majority of mutants exhibiting a pellicle-deficient phenotype also displayed impaired motility, suggesting a positive correlation between biofilm formation and motility in the pellicle model [28]. That biofilm formation and motility are reciprocally regulated by the second messenger c-di-GMP is widely accepted not only in *B. cereus* group bacteria, but also in a number of other species [33–36]. However, a recent study showed that in *B. thuringiensis*, overexpression of MogR, a homolog of the transcriptional repressor MogR in *Listeria monocytogenes*, led to non-motile cells and a substantial increase in biofilm formation [37]. These results imply intimate cross-regulatory connections between motility and biofilm formation.

It was previously shown that a minor subpopulation of *B. thuringiensis* cells was able to swim in an axenic biofilm despite the presence of extracellular matrix [38]. Even short chains were able to migrate in the biofilm matrix by adopting a snake-like motion [38] that was also observed in the matrix of an exogenous biofilm of *Staphylococcus aureus* (Fig. 2).

Recently, Yu and collaborators [39] demonstrated that a strain of *B. cereus* was able to transport the bacteriophage PHH01 infecting *Escherichia coli* on their flagella. While the interactions between bacteriophages and bacterial biofilms are still poorly understood, hitchhiking phages were shown to increase infection of a preformed host biofilm of *E. coli*, creating a possible biotope for motile *Bacilli* carriers. Specifically, bacterial phages could adsorb onto the flagella of *B. cereus*, increasing phage motility and resulting in more efficient infection of *E. coli* biofilms. Furthermore, phage infection reduces interspecies competition and promotes *B. cereus* biofilm formation in the resulting populations.

2.3. Global regulators revisited

The major regulatory networks of biofilm formation in *B. subtilis* have been extensively studied [42–44]. Nevertheless, genetic networks are still being discovered in *B. cereus* group bacteria. In this section, we briefly revisit several major components of these networks, and cover recent advances in the networks governing biofilm development.

In *B. subtilis*, Spo0A-AbrB and SinI-SinR are the central genetic circuits regulating biofilm formation [45,46]. The acrystalliferous strain *B. thuringiensis* 407 Cry-shares similar regulatory networks related to biofilm development including Spo0A, AbrB, and SinI/SinR [47]. A homologous gene to *spo0A* of *B. subtilis* was characterized and demonstrated to be crucial for biofilm formation in *B. cereus* 905 [48]. Growing evidence suggests that Spo0A acts as a general key regulator for biofilm formation in *B. cereus* group bacteria. For example, experiments using mutagenesis, heterologous expression, and transcription profiling indicated that *B. cereus* AR156 harbors a highly similar genetic circuit (*BcspoA-BcsinI-BcsinR*) [49]. A more recent study explored the multiple functions of SpoVG, another important sporulation regulator, in *B. cereus* 0–9 [50]. In *B. subtilis*, SpoVG is involved in sporulation via an unexplored mechanism, while studies on B. cereus 0–9 revealed that SpoVG controls biofilm formation by activating the transcription of Spo0A. Furthermore, SpoVG was also reported to influence both AbrB and SinI/SinR networks, and therefore biofilm development [50]. Thus, it was speculated that SpoVG is positioned upstream of Spo0A in the regulatory pathway, which expanded our knowledge of the *spo0A-sinI-sinR* genetic circuit in *B. cereus*. Additionally, researchers dissected the role of the YmdB protein, which shares 72.35% identity with the corresponding protein in *B. subtilis* 168, and found that deletion of the *ymdB* gene greatly inhibited biofilm formation, which is likely achieved through the repressor SinR [51], similar to the *B. subtilis* YmdB protein [52,53].

In another study, the Wang group described the SinI/SinR system and the CalY protein in *B. cereus* 0–9, and demonstrated their roles in regulating biofilm formation [54]. They also reported an important role for GapB, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) involved in gluconeogenesis, in biofilm formation. Specifically, GapB is responsible for extracellular DNA release and biofilm formation through modulating the expression of *lrgAB*, which encodes an autolysis regulator, rather than the SinI/SinR system.

Central metabolism is regulated by both specific and global

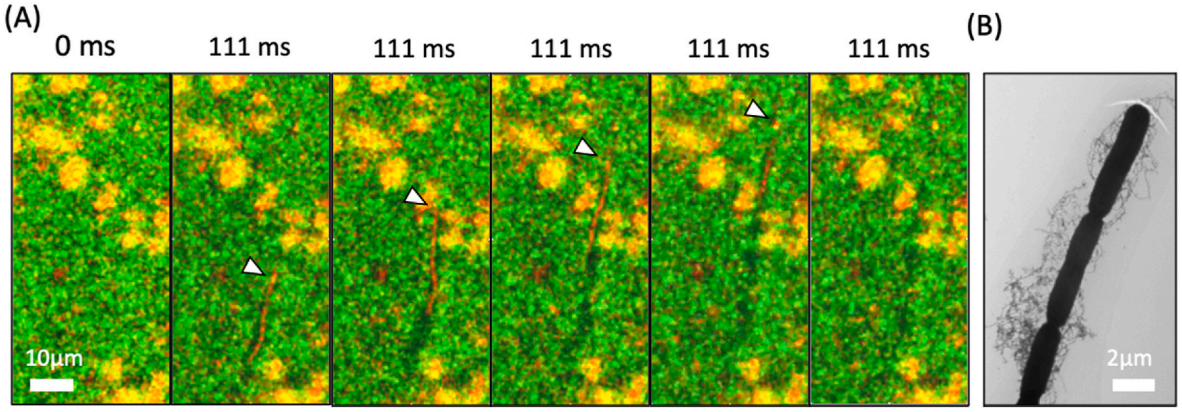

Fig. 2. (A) *B. cereus* 8D1a chains (colored red) swimming in the matrix of an exogenous *S. aureus* biofilm (green). (B) Hyperflagellated chains of *B. cereus* 8D1a observed in transmission electron microscopy. See methods in Refs. [40,41]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

regulators. CodY, a GTP-binding protein and pleiotropic transcriptional regulator, affects genes associated with nitrogen and carbon metabolism in *Bacilli* [55,56]. Specifically, CodY represses the transcription of numerous genes related to competence, sporulation, motility, and biofilm formation in *B. subtilis* [26,57]. In *B. cereus* group bacteria, CodY also regulates pathogenesis and biofilm formation, albeit with some differences. For example, in the *B. cereus* ATCC14579 strain CodY represses biofilm formation, while in the *B. cereus* UW101C strain biofilm formation is promoted by CodY [58,59]. This difference could be explained by strain-specific polar effects of the antibiotic marker in UW101C, which may result in differential expression of genes downstream of *codY*. By contrast, in strain ATCC14579, a marker-less mutant was utilized, and CodY was found to be required for basal level *clhAB2* expression, which encodes two membrane proteins involved in cell shape, chaining, and autolysis in *B. cereus* ATCC 14579 [60].

It is widely accepted that in Gram-negative bacteria, elevated levels of the second messenger cyclic diguanylate (c-di-GMP) increase biofilm formation and decrease motility [61,62]. However, the influence of c-di-GMP has been less studied in Gram-positive bacteria. In *B. subtilis*, c-di-GMP-signaling seems to regulate swarming motility but not biofilm formation [63,64]. The link between c-di-GMP signaling and biofilm formation has only recently been examined in detail. By applying bioinformatics tools, 11 proteins were suggested to contain domains (GGDEF/EAL) associated with c-di-GMP synthesis or breakdown in *B. thuringiensis* 407 [34]. Several of the corresponding genes were demonstrated to influence biofilm formation, motility, toxin production, and sporulation in this strain. Among these proteins, CdgF acts as a master diguanylate cyclase essential for biofilm formation. Similar results were reported for *B. thuringiensis* BMB171, verifying that high levels of intracellular c-di-GMP can induce cell-cell aggregation and biofilm formation [36]. Following the identification of CdgF, a collagen-binding protein, CbpA was revealed to be downregulated in the *cdgF* deletion mutant, suggesting that this gene was induced by CdgF [65]. Although no correlation could be identified between the absence of CbpA and biofilm formation or motility, overexpression of CbpA led to reduced biofilm formation and motility in *B. thuringiensis* 407 [65]. The authors speculated that this might be due to physical disruption of biofilm and motility properties, whereas the true biological function remains to be further explored.

Bacteria use quorum sensing (QS) to coordinate gene expression with cell density. The quorum sensing systems that are the best-studied in *Bacillus* include Rap, NprR, and PlcR, identified as the first members of the novel RNPP protein family. *B. cereus* cells use QS to regulate various crucial biological functions such as virulence, sporulation, and biofilm formation [66,67]. CodY regulates the expression of PlcR and concomitantly the PlcR regulon, which controls most of the known virulence factors in *B. cereus* [58,68]. Further studies showed that CodY controls the expression of virulence genes through the import of PapR [69], which acts as a QS effector that activates PlcR [70]. Presumably, PlcR also plays an important role in biofilm formation. PlcR was found to repress the production of an unknown biosurfactant that contributed to biofilm formation under low-nutrient conditions [71]. Nevertheless, whether this biosurfactant-related gene is directly controlled by PlcR or other genes under PlcR regulation remains to be explored. In addition, PlcR promotes the transcription of NprR, which positively regulates the transcription of kurstakin, a lipopeptide that additionally also promotes biofilm formation [72].

A recent study explored the Rap-Phr QS systems (receptor-signaling peptides) in *B. cereus* group bacteria [73]. Rap-Phr systems in *B. subtilis* have been shown to influence biofilm formation and plant attachment under laboratory conditions [74]. In *B. thuringiensis* Bt8741, four Rap-Phr systems (RapC, RapK, RapF, and Rap-like) inhibit sporulation, two of which (RapK and RapF) also inhibit biofilm formation [73]. Furthermore, the production of extracellular molecules (public goods), including matrix components, is likely regulated by Rap proteins in Bt874 [73].

3. Biofilm components: regulation of matrix production

In *B. subtilis*, exopolysaccharide is the main biofilm carbohydrate, and it is synthesized by enzymes encoded by the *epsA–O* operon [45]. However, despite high similarity, *B. cereus* homologs of *B. subtilis epsA-O* play only a minor role in biofilm formation [31]. For instance, deletion of the *eps* locus in *B. cereus* does not influence pellicle formation [48]. Li and colleagues provided the first evidence of an exopolysaccharide consisting of two amino sugars, GlcNAcA (N-Acetylglucosamine) and XylNAc (2-N-acetylamino-1,2,4-trideoxy-1,4-iminoxylitol), that contributes to biofilm formation in *B. cereus* group bacteria [75]. The Pel polysaccharide was initially discovered in the Gram-negative bacterium *Pseudomonas aeruginosa*, and its synthesis is associated with the *pelABCDEFG* operon. However, a recent study using a systematic pipeline identified this gene cluster in many Gram-positive species as well [76,77]. Strikingly, one of the *pel* gene clusters, *pelDEADAFG* in *B. cereus* ATCC 10987, is involved in the biosynthesis of a Pel-like polysaccharide which is essential for biofilm formation [77]. This was the first report that a Pel-like polysaccharide is involved in matrix production in *B. cereus*, making Pel one of the most prevalent biofilm polysaccharides studied to date [78]. Importantly, in line with one previous study [34], CdgF and CdgE were demonstrated to reciprocally regulate the production of Pel in *B. cereus*.

A follow-up study characterized the roles of *eps1* and *eps2* in multicellularity of *B. cereus* ATCC14579 [79]. Interestingly, EPS2, putatively responsible for the synthesis of a capsular polysaccharide, is indeed involved in adhesion to surfaces, cell aggregation, and biofilm formation. By contrast, EPS1 does not contribute to biofilm formation, but it is important for colony spreading on the surface of agar medium. Thus, EPS1 and EPS2 likely play different but complementary roles in the multicellular lifestyle of *B. cereus* [79].

Regarding proteins assembling in the biofilm matrix, while there are no homologs of *bslA* or *tapA* genes in the *B. cereus* genome, there are two homologs of *tasA* [23]. The first one is *tasA*, located in the *sipW-tasA* operon, and the other is *calY* [17]. CalY can polymerize and form protein fibers that are similar to TasA fibers of *B. subtilis* [17]. Subsequent studies demonstrated that CalY is a bifunctional protein that contributes to matrix composition and adhesion to host tissues [80]. Deletion of *calY* led to a dramatic decrease in biofilm biomass and a significant reduction in adhesion to HeLa cells, suggesting its role as a major virulence factor in *B. thuringiensis*.

Recent efforts have focused on identifying the molecular architecture underlying the biofilm extracellular matrix. Combining multiscale approaches like attenuated total reflection Fourier transform infrared spectroscopy (FTIR), solid-state NMR (SSNMR), dynamic light scattering, and electron microscopy, the molecular architecture of functional amyloids in *B. subtilis* (TapA) and *B. cereus* (CalY) were analyzed [81]. TapA and CalY share striking similarities both in their 2D [^{13}C]-[^{13}C] fingerprints in SSNMR, and in their intense signal at 1630 cm^{-1} in FTIR, suggesting a shared structural fold. Although biofilms of *B. cereus* and *B. subtilis* differ in local structure and assembly kinetics, functional amyloids of TasA-bc are now proven to be similar to TasA-bs in both fold and shape [81].

4. Biofilm heterogeneity: sporulation and biofilm formation

Spatial differentiation in biofilms is accompanied by phenotypic heterogeneity. This differentiation is due to limited exchange of biofilm matrix, thus creating structured microenvironments within the biofilm. In *B. subtilis* biofilms, aerial architectures or 'fruiting bodies' serve as preferential structures for sporulation [45]. In bacterial colonies, cells in specific regions within the biofilm tend to express genes involved in certain functions such as matrix production and motility [82,83].

Bacterial biofilms retain high levels of heterogeneity, and at least five subpopulations (virulent, necrotrophic, virulent and necrotrophic, necrotrophic and sporulation, and an undefined subpopulation) were

identified concurrently in *B. thuringiensis* [66]. Such phenotypic heterogeneity is finely tuned by intertwined regulatory pathways, including PlcR, NprR, and Spo0A pathways. Successive differentiation in virulence, necrotropism, and sporulation can be observed within a single cell lineage, suggesting the possibility of successive activation of these differentiation phenotypes [66]. However, activation of these intertwined regulatory pathways can follow different patterns in different media, confirming the strong influence of available nutrients, and therefore environmental conditions [84]. In a structured biofilm, swimmer cells, which are highly mobile, can create tunnels in the biofilm matrix to increase nutrient flow, thereby improving overall bacterial fitness [38]. During biofilm growth, dynamic exchange occurs between planktonic and sessile populations. Firstly, planktonic cells grow until the biofilm formation is initiated. Thereafter, while the biofilm continues to grow, planktonic cells rapidly decrease in number, and eventually the entire planktonic cell population integrates into the biofilm. Interestingly, freshly recruited planktonic cells are mainly located in specific areas of the biofilm, where few sessile cells are originally present, suggesting spatial heterogeneity between the two populations [85]. In the case of floating biofilms of *B. thuringiensis*, two main structures have been described (ring and pellicle). Despite the similar growth of the two parts, cells in the ring structure sporulate 24 h earlier than those in the pellicle, as determined by monitoring the expression of *spoIID* and testing the relative number of spores [86]. Potentially, the ring enters starvation earlier, and dryness could also speed up the initiation of sporulation. A better understanding of the regulatory network controlling heterogeneity in these biofilms systems could help prevent *B. cereus* contamination on various surfaces and interfaces.

5. Ecological importance of B. *cereus* biofilms

5.1. Biofilm control in food and industrial settings

In general, *B. cereus* is considered as a soil-dwelling bacteria that is often isolated from food products including rice, milk, and meat [87]. *B. cereus* is also known to produce biofilms and is regarded as a source of contamination in artificial surfaces such as storage tanks, stainless steel pipes, and conveyor belts [88,89]. On these materials, *B. cereus* can form biofilms with diverse spatial organization (Fig. 3). In the following section, we systematically review the potential effects of *B. cereus* biofilms in food and industrial settings, and their associated control methods.

Foodborne illness is caused by toxins including hemolytic and non-hemolytic enterotoxins (NHE), cytotoxin K (CytK), and the emetic toxin cereulide. Usually, there are two types of poisoning symptoms; diarrhea is caused by enterotoxins, while emesis is caused by emetic toxins in food [91]. Various non-gastrointestinal diseases have been also reported [92]. Spores and biofilms of *Bacilli* are capable of contaminating most of the surfaces found in food processing industries, including inert surfaces like stainless steel [93], rubber or plastics, and vegetables [94]. Biofilm formation under these settings is widely affected by a variety of environmental factors including nutrient availability and osmolality [95]. Specifically, in food related environments, carbon sources, minerals, and food residues can substantially influence biofilm formation by *B. cereus* [96]. In addition, biofilm formation appears to be affected by environmental temperature and surface properties [88]. Biofilms with increased robustness are formed by *B. cereus* ATCC 14579 on stainless steel at 30 °C compared with plastic and glass surfaces at 25 and 30 °C [88]. In fact, providing amino acids such as those derived from vegetables in food can promote bacterial adaptability [15].

Although developing biofilms are mainly comprised of vegetative cells, spores are formed within the established mature biofilm during the later stages, suggesting that biofilms act as a reservoir for highly resistant spores [87]. *B. cereus* biofilms are highly resistant to disinfectants [97], and up to 90% of biofilm cells are spores in matured biofilms [98]. While sporulation is required for the survival of *B. cereus* on leaves, where biofilms are challenged by adverse conditions, when colonizing vegetables such as endives, sporulation is dispensable for *B. cereus* [99]. Strikingly, the non-sporulating *B. cereus* strain DSM 2302 can survive in foodstuff products due to high nutrient availability compared to plant leaves. Contrary to the assumption that spores reach the stomach and germinate, these food products increase stomach pH, thus allowing vegetative cells to survive through stomach passage and reach the intestine to induce poisoning [99]. A greater understanding of the synergy between biofilm formation, sporulation, and toxin production could improve risk assessment of *B. cereus*.

Clean-in-place procedures have been widely employed to control biofilm contamination in food plants. Typically, the application of NaOH (1%) and HNO_3 (1%) has been reported for biofilm control, albeit with relatively low efficiency [100,101]. In general, combination treatments are more effective than single treatments. A recent study suggested that biofilms of *B. cereus* can be efficiently reduced with simultaneous application of 200 ppm NaClO (10 min) and 7% citric acid (10 min) [102]. Citric acid occurs naturally in citrus fruits, which is a safer than NaClO for biofilm control. Another effect agent, peracetic acid, is more effective against spores, possibly by altering the interim layer of spores [103]. However, peracetic acid and sodium hypochlorite were not effective for removing *B. cereus* contamination on stainless-steel surfaces in contact with milk [104].

5.2. B. cereus plant-associated biofilms

In addition to the above-mentioned biofilm examples, *B. cereus* can also be isolated from the mycorrhiza and rhizosphere of plants. For both soil-borne pathogens and beneficial rhizobacteria, colonization of plant roots is an essential step that follows a pattern in which rhizobacteria form biofilms at preferred sites of root exudation [105]. *B. cereus* likely colonizes plant roots by forming biofilms, since an analog of *tasA*, an essential gene for *B. subtilis* biofilms, is necessary for root colonization by *B. cereus* [17]. A biofilm-defective mutant of *B. cereus* strain 0–9 obtained by random mutagenesis was inefficient at colonizing wheat roots and antagonizing fungal pathogens [106]. *B. thuringiensis* 407 cry-readily forms biofilms on the roots of *Arabidopsis thaliana* in hydroponic conditions (Fig. 4) [30]. Directed laboratory evolution of *Bacillus* root colonizers in planta has additionally provided a robust methodology for studying root-associated biofilms and the evolutionary path connected to this colonization setup [30,107,108]. This approach

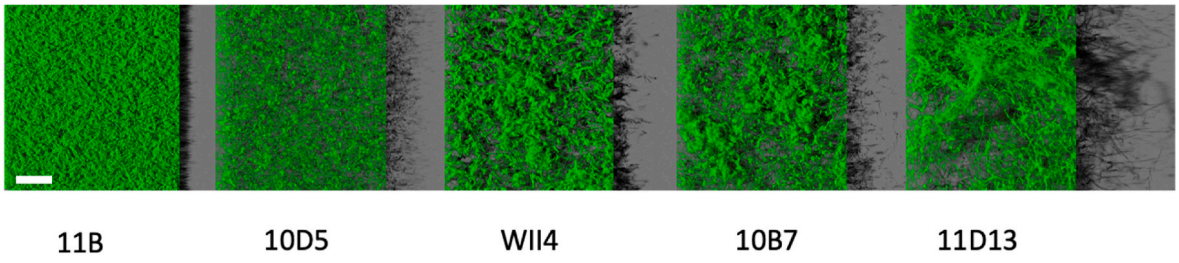

Fig. 3. Structural diversity of biofilms formed on an inert surface by five environmental *B. cereus* isolates. The white bar represents 30 μm. See methods in Ref. [90].

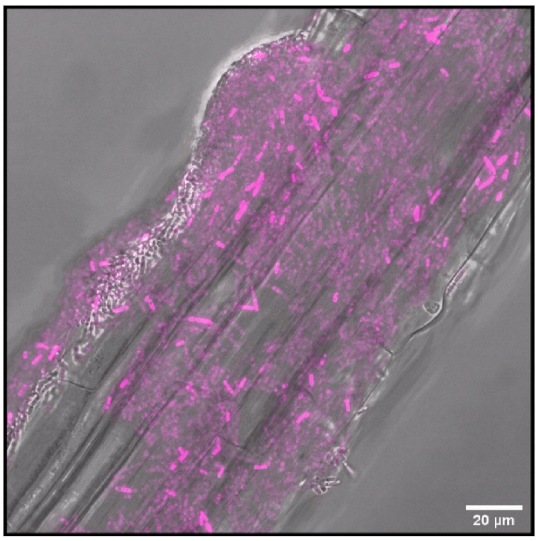

Fig. 4. Confocal laser scanning microscopy image of a fluorescently labeled *B. thuringiensis* 407 biofilm formed on the roots of *A. thaliana* after 48 h in hydroponic conditions. See the methods in Ref. [30].

allowed the identification of several genes related to efficient root colonization by *B. cereus*. For instance, a loss-of-function mutation in the transcriptional terminator Rho was critical for enhancing carbohydrate metabolism, thus influencing root-associated biofilm formation *in vitro*. While routine selection for root attachment is expected to increase root colonization by *B. thuringiensis*, the potential mutations arising in the experimental process might not necessarily benefit the proper use of plant-promoting bacteria, since trade-offs might occur, such as reduced motility or altered timing of sporulation. Thus, as highlighted, *B. cereus* and *B. thuringiensis* growing and forming biofilms in topsoil might colonize germinating plants, and develop biofilms on the rhizosphere and phylloplane, followed by sporulation to maintain survival [23], and therefore create a source of plant-based food product contamination.

6. Conclusions and future perspectives

While extensive studies have been conducted addressing the mechanisms and applications of *B. subtilis* biofilms, much less attention has been paid to *B. cereus* group bacteria. This could be due to the complex phylogenetic relationship that creates diverse genotypes and phenotypes among *B. cereus* group bacteria. For example, the basic building blocks of biofilm matrix are still being debated, as well as the regulatory networks influencing biofilm production. For example, the role of *epsA-O*, the major polysaccharide locus in *B. subtilis*, seems to be unrelated to biofilm formation in *B. cereus*. Furthermore, the lack of genetic accessibility for most *B. cereus* isolates has delayed progress in this field. Nevertheless, some studies have focused on how to control biofilm contamination, while few studies have investigated the underlying mechanism underpinning biofilm formation. To develop highly efficient cleaning procedures, a deeper understanding of how *B. cereus* biofilms are regulated may be needed, especially during the dispersal period. As phage-mediated competition can influence biofilm structures, further understanding of how phages influence biofilm formation and evolution in the *B. cereus* group may be critical given the potential of phage treatment as an alternative antibacterial method.

Regarding the pathogenic traits of *B. cereus*, one of the most critical issues is uncovering the relationship between biofilms, spores, and toxins. Although sporulation of the *B. cereus* group within biofilms has been documented, further study is needed to explore whether biofilms directly influence sporulation. Additionally, in *B. cereus* there is limited evidence of a relationship between toxin synthesis and biofilm development. Furthermore, the existence and evolution of biofilms *in vivo*, as well as their precise contribution to bacterial pathogenicity, have yet to be determined. Toxin production is vital for a foodborne pathogen, and sporulation or biofilm formation are likely to increase the risk of food poisoning. Investigations on the correlations between biofilms, spores, and toxins are needed, with a focus on biofilm evolution and gene expression.

In terms of plant-associated biofilms, although it has been demonstrated that pre-engineered bacteria may rapidly turn into a plant endosymbiont, laboratory-based guided evolution of root colonization has been established only recently. Our understanding of plant-microbe interactions will be further facilitated by future studies on multispecies setups in these evolution experiments. Importantly, evolution experiments based on field trails should be conducted to gather data from natural settings, providing huge potential for optimizing biofertilizers based on *B. cereus* group isolates.

In summary, the *B. cereus* group is a large group of bacteria with diverse phenotypes that form biofilms. Further knowledge in this area will help resolve problems in food contamination, and facilitate bioresource optimization in a strain-specific manner.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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