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The intertwined roles of specialised metabolites within the *Bacillus subtilis* biofilm

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ABSTRACT (75 words limit)

Bacteria produce specialised metabolites with a range of functions. In this issue of the *Journal of Bacteriology* Schoenborn *et al.* study the production and role of secondary metabolites during biofilm development and sporulation in *Bacillus subtilis*. Most metabolites studied are produced during differentiation and six are required for the development of biofilms and/or spores. The authors propose a model for the timing of production and role in differentiation exerted by each secondary metabolite.

KEYWORDS

Bacillus subtilis, biofilm, spore, differentiation, specialised metabolites

COMMENTARY

Social interactions in the microbial world are incredibly complex, but we are slowly starting to understand some of the intricate mechanisms that underpin them. Specialised metabolites, which are a diverse range of molecules with a broad range of functions, are major players in defining these social dynamics. These molecules can impact populations of microbes through killing or inhibiting growth, triggering differentiation between physiological states, or manipulating nutrient availability in the environment (1). The prevalence of these molecules, and their pervasive impact on many aspects of microbial life, means that they have crucial roles in defining the composition and emergent properties of inter-kingdom, inter-species, intra-species, and single strain communities of microbes.

Biofilms are highly heterogeneous structures where microenvironments and gradients in nutrient availability, oxygen levels, and cells types develop (2) (Figure 2A). *Bacillus subtilis* is a Gram-positive soil bacterium that has been extensively used for the study of social interactions in the context of biofilm formation. Examples of the cell types that make up a *B. subtilis* biofilm include motile cells, biofilm matrix producers, exoprotease producers, and endospores, facilitating the division of labor and the sharing of public goods between the community members (3). The regulatory processes leading to differentiation of cells into these physiological states is highly complex and relies on input from a variety of environmental signals, some of which are specialised metabolites produced by the cells in the biofilm themselves (3).

B. subtilis is well known for the plethora of specialised metabolites it produces. Most of these specialised metabolites are primarily linked with their antimicrobial properties and include

bacilysin (4), bacillaene (5), subtilosin A (6), plipastatin (7), surfactin (8), sporulation killing factor (9) and sublancin 168 (10). Other specialised metabolites produced by *B. subtilis* include the iron-chelating molecule pulcherriminic acid (11) and the siderophore bacillibactin (12) (Figure 2B). The specialised metabolites with a known impact on cell state differentiation in *B. subtilis* communities are surfactin and the pheromone ComX, which act as extracellular signals to induce the differentiation of cells into biofilm matrix producers and “cannibals” (13). Cannibals are a subpopulation of cells that produce the specialised metabolites sporulation killing factor (SKF) and sporulation delaying protein (SDP), which function to lyse sister cells in the community to use them as a nutrient source and delay sporulation (9, 13). The siderophore bacillibactin has also been found to be involved in the development of *B. subtilis* biofilms (14). However, while we know that some specialised metabolites are crucial for cell fate differentiation and biofilm formation, there has not been a comprehensive systematic study of the interplay between secondary metabolite production and differentiation until now.

In this paper, Schoenborn *et al.* tested the role of nine specialised metabolites during biofilm formation and sporulation by examining the expression, production, and impact of deletion mutants on differentiation (they deleted biosynthetic genes necessary to produce specialised metabolites; these genes are often referred to as "clusters" based on their genomic structure). The authors demonstrated that most clusters (those needed for surfactin, subtilosin A, ComX, SDP, SKF, bacilysin, and bacillaene production) are expressed at a higher level under differentiation-inducing conditions, except for the plipastatin and bacillaene clusters. Largely mirrored by this analysis, all metabolites examined, including plipastatin and bacillaene, were produced in significantly higher amounts under conditions that promote biofilm formation and

differentiation. The higher production of specialised metabolites during cell fate differentiation points to these molecules having a role during these processes. Interestingly however, the ability of cells to produce most of these molecules was not essential for biofilm formation, at least individually, as deletion of genomic regions required for biosynthesis of the specialised metabolites did not impact biofilm structure. The exception was surfactin, the absence of which resulted in a biofilm deficient strain when analysed by the pellicle biofilm model. These findings are in contrast to a recent study showing that lack of surfactin does not impact pellicle biofilm development, but consistent with surfactin being required for architecturally complex colony biofilms to form (15). In line with an impact to colony biofilm formation, in this study, the lack of surfactin caused a decrease in the expression of the biofilm matrix protein encoding gene *tapA*. The reduction in *tapA* expression was also found to be the case for the mutant lacking the ability to produce ComX, which is consistent with both ComX and surfactin being important for differentiation of cells into biofilm matrix producers. Another two molecules, subtilosin A and bacillaene, impacted matrix gene expression, but this was at the later stages of the pellicle biofilm formation, after around 16 hours of growth. At this point the increase in expression of the biofilm matrix protein starts to level off in the wild type but continued to increase in the subtilosin A and bacillaene mutants. Looking at sporulation dynamics, the authors showed that lack of surfactin, plipastatin, bacilysin, subtilosin A, ComX, and bacillibactin all impacted sporulation. At 16 hours, the number of spores was significantly lower for the strains incapable of producing the specialised metabolites compared to the wild type, suggesting that these molecules are required for triggering spore formation.

We are gaining more and more understanding about the multifaceted nature of specialised metabolites and some of the *B. subtilis*-produced specialised metabolites are now known to be multifunctional. For example, bacillaene protects *B. subtilis* from predation by other bacteria (16), can modulate production of secondary metabolites by competing bacteria (17), impacts the composition of mixed species bacterial communities (18), inhibits biofilm formation by other bacteria (19), and has been suggested to impact biofilm development of *B. subtilis* biofilms at subinhibitory concentrations (20). This paper by Schoenborn *et al.* reveals an additional role for some of the less widely explored specialised metabolites produced by *B. subtilis* in differentiation. Plipastatin (which has been studied for its ability to inhibit the growth of multiple plant pathogenic fungal species (21)), the bacteriocin subtilosin A (6), and bacillibactin (which is a siderophore), are now known to also function as signals that regulate sporulation in *B. subtilis* mixed communities. Therefore, it is clear that these molecules have a function in both competition against others, either through their antimicrobial functions or in limiting available nutrients in the environment by sequestering them, and in impacting cooperative dynamics in a single species biofilm. One can speculate about the multipurpose role for the molecules. Bacteria produce a relatively limited number of molecules with which they need to navigate an incredibly complex world. *B. subtilis* can be found in the gastrointestinal tract of animals, in association with plant roots, in bulk soil, and in marine environments and is likely to have to interact with its eukaryotic hosts, other species of microbes, and members of its own species. It therefore makes sense, from an evolutionary perspective, for bacteria to ensure that the limited molecules they produce have a variety of functions to help them thrive in an ever-changing environment.

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Figure 1: Secondary metabolites and *B. subtilis* biofilms. (A) Vertical cross-section of a colony biofilm formed by *B. subtilis* NCIB 3610 with a schematic representation of the concepts covered in this work. The cross-section was prepared and imaged by Dr. Sofia Arnaouteli while visiting the laboratory of Prof. Lars Dietrich. (B) Schematic of the genome of *B. subtilis* strain NCIB 3610 showing the locations on the chromosome of the secondary metabolite biosynthesis clusters and other explored molecules. The secondary metabolite biosynthesis clusters were predicted using AntiSMASH version 6.0 (22) and the genome map was constructed using GCView (23).