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Kalamara, Margarita; Stanley-Wall, Nicola R.

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1 Margarita Kalamara ¹ and Nicola R. Stanley-Wall ^{1*}

2 ¹ Division of Molecular Microbiology, School of Life Sciences, University of Dundee, Dundee, UK

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

4 * Corresponding author: n.r.stanleywall@dundee.ac.uk

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6 **ABSTRACT** (75 words limit)

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

13 **KEYWORDS**

14 *Bacillus subtilis*, biofilm, spore, differentiation, specialised metabolites

15

16 **COMMENTARY**

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

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

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

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

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

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

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

101

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