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1 **Biofilm dispersal for spore release in *Bacillus subtilis***

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11 **ABSTRACT**

12 The dispersal of bacterial cells from a matured biofilm can be mediated either by active or passive

13 mechanisms. In this issue of the *Journal of Bacteriology*, Nishikawa and Kobayashi demonstrate

14 that the presence of calcium influences dispersal of spores from the pellicle biofilm of *Bacillus*

15 *subtilis*. The authors propose that temporal heterogeneity in matrix production and chelation of

16 calcium by dipicolinic acid in spores weakens the biofilm matrix and causes passive dispersal.

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18 **KEYWORDS**

19 *Bacillus subtilis*, biofilm, dispersal, spore, development

20

21 **COMMENTARY**

22 Biofilm formation is a complex developmental process undertaken by microbes that is initiated by

23 attachment or aggregation of cells, advanced by production of an extracellular matrix, and

24   generally finalized by disassembly of the biofilm, a process called dispersal (1). The specific biofilm

25   life cycle depends on the microorganism, its ecological niche, and the encoded regulatory

26   pathways. In addition, environmental factors, including intra- and interspecies compounds may

27   influence the different steps of biofilm development. A detailed understanding of the different

28   stages of biofilm formation and disassembly could help us to prevent deleterious microbial

29   communities and promote beneficial ones.

30   *Bacillus subtilis* became a model organism to study bacterial differentiation processes due to its

31   ability to create a dormant cell structure, called a spore, that has remarkably resistance to heat,

32   pressure, and chemicals in addition to its capability to take up extracellular DNA and incorporate it

33   via recombination into its genome (2). These features, alongside other biotechnologically

34   beneficial properties stimulated robust probing of the physiology and genetics of this species in

35   the last century. The study of *B. subtilis* biofilm formation was initiated about two decades ago,

36   and has created a plethora of understanding since the first publication (3) regarding how gene

37   expression connects to biofilm initiation and matrix production and the identity and function of

38   the main biofilm matrix components (4, 5). Interest in *B. subtilis* biofilms is further stimulated by

39   the species being more than a laboratory model: biofilms are important for plant growth

40   promotion, probiotic impact, and biotechnological applications (6, 7).

41   The molecular details of *B. subtilis* biofilm development have been predominantly explored in two

42   laboratory systems, air-liquid interface floating biofilms, known as pellicles, and architecturally

43   complex colonies formed on agar surface (6). Dissection of gene expression in colonies revealed

44   that *B. subtilis* biofilm population is phenotypically heterogeneous; distinct cell types inhabit a

45   biofilm, including motile cells, matrix producers, extracellular protease producers, and in the later

46   stages of development, spores are formed on the upper layer of the colonies (8, 9). Efficient

47   initiation of pellicle development requires motility (10) and establishment of the floating biofilm at

48   the air-medium interface proceeds through distinct morphological changes (11). Importantly,

49   matrix gene expression in the nascent pellicle is temporally and spatially heterogeneous, after a

50   highly heterogeneous matrix production during initiation of the pellicle, the majority of cells

51   express the genes for matrix production in the middle of biofilm development (around 24 hours)

52   (12). In the later stages, the population becomes heterogeneous again, only a fraction of the cells

53   will produce the matrix (12), while spores also appear (13, 14). Such temporal heterogeneity is

54   mirrored by physical heterogeneity; during the initial and later stages of pellicle development,

55   next to a robust, highly matrix-expressing population, a fragile fraction is also present, within

56   which the cell-cell aggregation can be easily disrupted (12). The dynamic transcriptional landscape

57   of the developing pellicle has also been associated with variation in metabolism of the cells (15).

58   Additionally, it has been proposed that during biofilm colony maturation, the evolutionary

59   younger and more diverged genes are increasingly expressed toward later timepoints of colony

60   development (16).

61   While initiation and maturation of pellicle biofilm development is extensively investigated,

62   dispersal mechanisms are less explored in *B. subtilis* and the literature still perpetuates errors in

63   the understanding of biofilm dispersal with respect to norspermidine and D-amino acids that have

64   since been corrected (17, 18). In this issue of *Journal of Bacteriology*, the publication by Nishikawa

65   and Kobayashi (19) reveals a novel mechanism of *B. subtilis* dispersal and highlights a connection

66   between emergence of spores and biofilm disassembly (Fig. 1). Interestingly, *B. subtilis* grown in

67   variety of commonly used synthetic and complex biofilm media (e.g. MSgg and 2×SGG) does not

68   display typical dispersal. The pellicle remains robust for up to a week at 30°C, during which time

69   only a minor and very slow decay in the thickness is observed in MSgg grown pellicles. However,

70   pellicles that were cultivated at 37°C in a modified LBGM medium (lysogeny broth supplemented

71   with glycerol and manganese (20), but containing reduced amount of manganese compared to

72   previous publications) showed a rapid establishment within a day, fragmented structure on the

73   second day, and strong dispersal after 3 days. While removal of manganese prevents biofilm

74   development (20, 21), supplementation at lower concentration creates conditions that allows

75   examination of the full pellicle biofilm life cycle in *B. subtilis*, including dispersal. These

76   observations highlight that biofilm dispersal might be more prevalent in the laboratory when slight

77   starvation is encountered, a condition that likely exists for microbes in nature. Interestingly, the

78   number of viable cells remained constant throughout the 3 days (19), suggesting the lack of active

79   lysis or cell death, but rather the presence of passive dispersal in *B. subtilis* succeeding the

80   previously reported reduced matrix gene expression at later stages of the pellicle development

81   (12, 15). Synthetic induction of genes involved in synthesis of the exopolysaccharides throughout

82   the cultivation and therefore prolonged exopolysaccharide production partially prevents biofilm

83   dispersal (19), thus the reduced matrix production only partially explains pellicle biofilm dispersal.

84   What could facilitate *B. subtilis* cells' dispersal from a biofilm in addition to reduced matrix

85   expression? *B. subtilis*, when colonizing plant roots under hydroponic conditions, first produces

86   the biofilm matrix followed by robust spore formation (22). Spore are anticipated to survive the

87   harsh conditions in the soil, including predation by protozoans, nematodes, or other microbes

88   (23–25). Therefore, pellicle dispersal could understandably be mediated by release of spores.

89   Nishikawa and Kobayashi (19) demonstrate that the induction of sporulation pathway, which

90   depends on a cascade of sigma factors activating specific gene expression either in the mother cell

91   (σ^E, and σ^K) or in the pre-spore (σ^F, σ^G), contributes to dispersal. Indeed, circumventing spore

92   formation by disrupting these sporulation-specific sigma factors, in addition to concomitantly

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93   synthetically prolonging exopolysaccharide production, prevents pellicle dispersion. Systematic

94   disruption of σ^K-dependent genes, the last downstream sigma factor within the activation

95   cascade, revealed that *spoVFA–spoVFB* operon is sufficient to explain biofilm dispersal in *B.*

96   *subtilis*. The *spoVFA–spoVFB* operon encodes a dipicolinic acid synthase that creates dipicolinic

97   acid (DPA). DPA after being produced in the mother cells is transported to the forespore

98   compartment where it starts chelating calcium ions contributing to dehydration and

99   mineralization of the spore (26). The direct connection between DPA mediated chelation of

100   calcium, and pellicle dispersal could be verified by addition of calcium to the biofilm medium,

101   which prevented dispersal (19). The impact of calcium on biofilm colony development has been

102   previously observed (27). Consistently, both Nishikawa and Kobayashi (19) and Mhatre *et al.* (27)

103   could demonstrate that calcium does not impact the expression of matrix genes in established

104   pellicle biofilms and under biofilm inducing conditions, respectively, besides both studies reported

105   larger biofilm colony size in the absence of calcium. The larger colony size observed in the absence

106   of calcium is likely connected to passive surface spreading, termed sliding, as the influence of

107   calcium on colony size was only apparent in the presence of matrix components and surfactin that

108   are all necessary for sliding (27–29). Production of the secondary metabolite surfactin, while not

109   being essential for biofilm development, alters the architecture of biofilm colonies (30). Calcium

110   influences self-assembly of surfactin (31), which might explain a possible impact of calcium on

111   surface tension and therefore biofilm colony size (27). Nevertheless, it remains to resolve the

112   direct connection between calcium level and surfactin functioning in colonies.

113   Thus, calcium has an important role both in spore maturation and also influences dispersal.

114   Nishikawa and Kobayashi (19) offer a plausible explanation how spore formation indirectly

115   influences biofilm weakening. Besides lower production of the matrix, the onset of spore

116   maturation and accompanying DPA production depletes the calcium in the extracellular matrix,

117   resulting in biofilm dispersal (Fig. 1). Calcium seems to impact the biofilm matrix and/or influence

118   regulation of biofilm in numerous bacteria (32–34). It remains to demonstrate whether and how

119   calcium directly interacts with the biofilm matrix in *B. subtilis*. Nevertheless, the elegant work by

120   Nishikawa and Kobayashi describes an intriguing example of passive dispersal and connects spore

121   formation with it release from the biofilms.

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123   **ACKNOWLEDGEMENT**

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213

214   **Figure 1**

215   **Schematic representation of the *B. subtilis* pellicle biofilm life-cycle.** After inoculation of

216   planktonic cells, oxygen depletion drives the motile cells to the air-medium interface, where

217   biofilm formation is initiated. At the start, part of the population produces the biofilm matrix.

218   During biofilm maturation, most cells expend energy making the biofilm matrix and calcium is

219   distributed across the biofilm, possibly stabilizing the matrix structure. Before dispersal, matrix

220   production diminishes, and spores are formed that chelate available calcium. Calcium depletion

221   and reduced matrix production allow passive dispersal of *B. subtilis* pellicle biofilms. Figure

222   created with BioRender.com

- ★ calcium
- biofilm matrix
- matrix producer cell
- matrix non-producer cell
- spore
- motile cell

planktonic state

initiation of biofilm

biofilm maturation

sporulation and biofilm dispersal

time