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# Translating metabolic exchange with imaging mass spectrometry

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## Abstract

Metabolic exchange between an organism and the environment, including interactions with neighboring organisms, is important for processes of organismal development. Here we develop and use thin-layer agar natural product MALDI-TOF imaging mass spectrometry of intact bacterial colonies grown on top of the MALDI target plate to study an interaction between two species of bacteria and provide direct evidence that a *Bacillus subtilis* silences the defensive arsenal of *Streptomyces coelicolor*.

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Historically, the study of microbial interactions has focused on an isolated signal or antibiotic activity, using detection methods founded on bioactivity-based assays. Penicillin (**1**) was discovered in this manner, as well as the majority of the natural products discovered to date. Thus, microbial interactions, including signaling and chemical warfare, have generally been considered in terms of an individual, predominant chemical activity. However, a single bacterial species is capable of producing many bioactive compounds that can alter the physiology of neighboring organisms. We have developed a mass spectrometry based method that enables us to visualize both the spatial and temporal production of numerous metabolites from a single bacterial species, and to observe the effects of multiple microbial signals in an interspecies interaction. The data reveal that chemical conversations between bacteria involve many signals that function simultaneously to direct the outcome of interspecies encounters. As evidenced by genome sequencing, bacteria dedicate up to 20% of their genome to the biosynthesis of secondary metabolites, underscoring the importance of these small molecules to the fitness of the organism in its native environment1,2.

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Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website

**AUTHOR CONTRIBUTIONS** Y.L.Y., Y.X. and P.C.D. prepared bacterial cultures and performed IMS. Y.L.Y., Y.X., P.S. and P.C.D. were involved in the data analysis and interpretation. P.S. prepared the mutant strains. Y.L.Y., P.S., P.C.D. wrote the paper.

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MALDI-TOF imaging is based on the control of the movement of the MALDI plate in an x-y direction, allowing the MALDI-laser to ionize the sample at predefined points3–4. The resulting collection 100s to 1000s of spectra, collected as a grid, create a 2 dimensional mass spectrometry profile. Any ion observed in these spectra can be spatially visualized as a false color and superimposed on an optical image of the sample. Here we adapt this technique to observe natural product production from bacteria cultivated on a thin layer of agar on top of the MALDI plate. We refer to the detection of these secondary metabolites as natural product MALDI-imaging mass spectrometry (IMS) to reflect the type of molecules that are discussed and observed in this paper5–6. The method and the requirements of the thin layer agar technique are described in more detail in the Supplementary Methods.

We used IMS to observe natural product production and chemical communication by *Bacillus subtilis* and *Streptomyces coelicolor*. Many natural products have been characterized from both organisms by standard mass spectrometry, making these bacteria good candidates for analyzing interspecies communication to validate the method7–18. The structures of the natural products from *B. subtilis* and *S. coelicolor* discussed in this paper are shown in **chemical compound information**, their characterization is provided in the Supplementary results. To apply IMS to an intact bacterial colony, a thin film of agar media was layered on top of the MALDI target plate and inoculated with *B. subtilis* (Fig. 1a). A digital image of the colony following incubation was captured to record bacterial growth. On the thin-film of agar media, the *B. subtilis* colony showed a morphological pattern similar to previously described biofilms19. The positions of ions that are detected by IMS were superimposed as false-colored images onto the surface of the colony image.

Localization patterns for ions ranged from coincident with the colony to apparent exclusion from the colony and diffusion into the surrounding medium (Fig. 1b). An ion with a mass of *m/z* 715, which localized exclusively to the colony, is a partially characterized polyglutamate compound that is possibly a component of extracellular matrix or cell wall material (Supplementary Fig.1)7. In contrast, ions of the surfactin lipopeptides (**2a–c**, *m/z* 1075) and plipastatin lipopeptides (**3a–d**, *m/z* 1545) diffuse into the medium. The surfactin (**2a–c**) and plipastatin (**3a–d**) signals are collections of ions that are separated by 14 Da, dependent upon different fatty acid chain lengths (Fig. 1b)7,10. The highest intensities of surfactin (**2a–c**) and plipastatin (**3a–d**) were found in the medium surrounding the colonies while the surfactin signal spanned the entire colony, with apparent concentrations in the center and at the edges. However, accumulation of plipastatin (**3a–d**) in the colony was at the limit of detection, at least within the surface 15–25 μm accessible to $N_2$ laser penetration and ionization. To confirm the identities of the ions, we analyzed strains carrying disruptions in the surfactin (**2a–c**) and plipistatin (**3a–d**) gene clusters (Fig. 1b). A strain mutant at *srfAA* and *ppsB* showed the predicted loss of both compounds. To further support the assignment of these molecular ions, TOF-TOF and FT-ICR-MS, MS-CPA and a de-replication tool were performed to confirm their identities (Supplementary Figs. 2 and 3)7,8,20,21. The temporal resolution of secondary metabolite production was also examined by IMS. In addition to polyglutamate (*m/z* 715), surfactin (**2a–c**), and plipastatin (**3a–d**), the *m/z* range was extended to include subtilosin (**4**, *m/z* 3443)7,9,10. This established that a major shift in secondary metabolism occurs at 22 hours of growth, when both plipastatin (**3a–d**), and

subtilosin (**4**) are produced prior to sporulation (Supplementary Fig. 4)9. Thus, in these experiments, IMS provided accurate identification of small molecule metabolites based on their mass with sufficient sensitivity to detect ions across the entire grid (Supplementary results).

Secondary metabolites play a vital role for survival of organisms in environments where other organisms or shifting environmental conditions demand specialized cellular functions or physiology. Aside from signals coordinating developmental transitions within a single-species population, the activity of many secondary metabolites is unclear when produced under standard bacterial culture conditions. To observe the scope of chemical communication between two organisms, we co-cultured *B. subtilis* and *S. coelicolor* and followed small molecule production from both bacteria (Supplementary Fig. 5). *B. subtilis* produces a hybrid polyketide, nonribosomal peptide, bacillaene (**5**), that was identified previously in a co-culture screen with *S. coelicolor*22,23. In the absence of bacillaene (**5**), which is encoded by the *B. subtilis pksA-S* gene cluster, *S. coelicolor* produced prodiginines. We employed IMS to investigate this unusual interspecies interaction (Supplementary Fig. 5). The bacillaene (**5**) ion itself was not directly detectable by IMS, either as a result of inherent light-instability23 or poor ionization. The prodiginines are detected as ions of *m/z* 392 (streptorubin B, **6**) and *m/z* 394 (undecylprodiginine, **7**) and were confirmed by TOF-TOF (Supplementary Fig. 6)12. In comparisons of the wild type strain and the Δ*pks* strain, which harbors a deletion of the *pksB-R* ORFs, we observed production of prodiginines and three unknown metabolites of *m/z* 407, 641 and 812, only in the absence of bacillaene (**5**) (Δ*pks*). The *sfp* gene encodes a 3'phosphopantetheinyl transferase required for activation of the bacillaene (**5**), surfactin (**2a–c**), and plipastatin (**3a–d**) synthesis enzymes24. A mutation in *sfp* blocks production of these molecules, producing a similar co-culture phenotype as the Δ*pks* mutation. We sought to determine if bacillaene (**5**) was solely responsible for the inhibition of secondary metabolites by co-cultured *S. coelicolor*. Because the *m/z* 392, 407, 641, and 812 ions, produced by *S. coelicolor* were not detected in interactions with *B. subtilis* that carried *srfA*$^{-}$ or *ppsB*$^{-}$ only, we conclude that the inhibitory effect on *S. coelicolor* secondary metabolism is specific to the activity of bacillaene (**5**). Thus we refer to the *S. coelicolor* metabolites as bacillaene repressed molecules (BRMs).

The absence of bacillaene (**5**) itself did not fully explain the effect on BRM upregulation in *B. subtilis-S. coelicolor* co-culture, as the molecules are produced during *S. coelicolor* growth in isolation. In the presence of *B. subtilis* Δ*pks*, however, BRMs are produced earlier. We suspected that a second metabolite produced by *B. subtilis* could stimulate upregulation of BRMs by *S. coelicolor*. One candidate molecule was bacilysin (**8**), a dipeptide inhibitor of glucosamine biosynthesis that could activate a generalized stress response mechanism, leading to upregulation of BRM synthesis. *B. subtilis sfp* strains carrying an additional mutation, *bacB*, to prevent synthesis of bacilysin (**8**) induced BRM production in these assays with high efficiency, indicating that bacilysin (**8**) is not the primary inducer of BRM synthesis. Using this methodology additional interesting correlations can be made. For example, it was noted that the detection of the *m/z* 655 molecule is sustained throughout the time-course of co-culture only by the *bacB*$^{-}$/*sfp*$^{-}$ strain but not in the wild type strain (Figs. 2a and 2b).

In addition to the BRMs, ions corresponding to SapB (**9**) and CDA (**10a–b**), are observed earlier in the interaction of *S. coelicolor* with the *bacB*$^{-}$*/sfp*$^{-}$ strain than the wild type (Fig. 2a). SapB (**9**), a lanthionine-bridged peptide, is required for the formation of hyphae and sporulation in *S. coelicolor*16. Furthermore, the time-course data indicates that mature SapB (**9**) production is inhibited by surfactin (**2a–c**) (Fig. 2c). This suggests that surfactin (**2a–c**) disrupts aerial development and sporulation as previously described25, and that the inhibition is due to prevention of SapB (**9**) biogenesis. Indeed co-culturing *S. coelicolor* with purified surfactin (**2a–c**) confirmed that SapB (**9**) production was inhibited, in agreement with this hypothesis. Additionally, using IMS we found that CDA (**10a–b**), an antibiotic that targets gram-positive organisms, was inhibited as well (Supplementary Fig. 7), providing an indication that one natural product, in this case surfactin (**2a–c**), not only has an effect on morphology but that it also silences the defensive arsenal of a second bacterial species. These examples highlight the richness of information that the thin-layer agar IMS provides.

This analysis of the interspecies interaction between *B. subtilis* and *S. coelicolor* by IMS demonstrates the complex chemical and dynamic metabolic exchanges involving multiple molecules: BRMs, bacillaene (**5**), CDA (**10a–b**), SapB (**9**) and the m/z 655 ion. This IMS approach provides an additional tool for exploration of interspecies interactions and symbiont-or pathogen-host communication of organisms grown in culture.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

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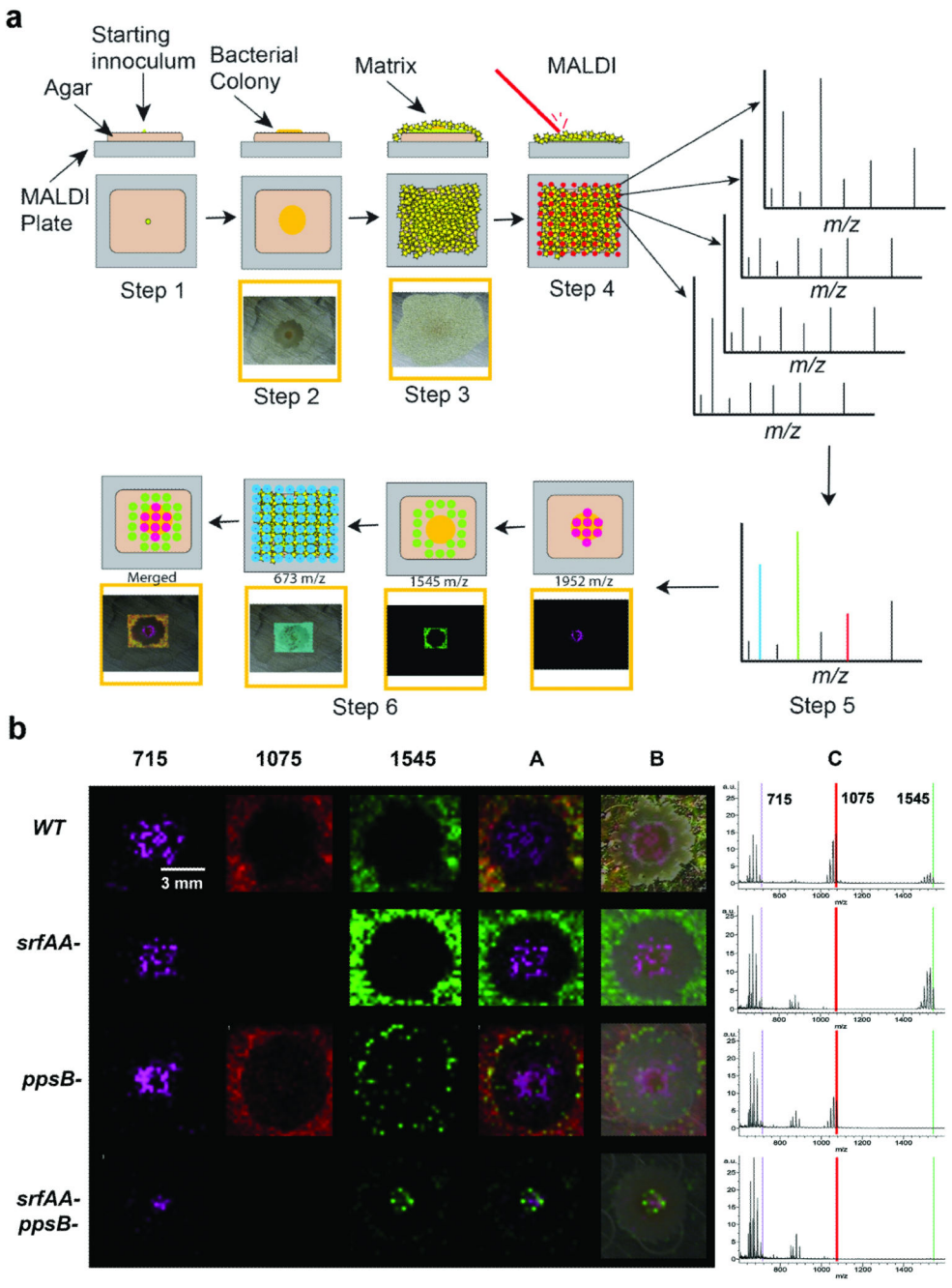


**Figure 1.**

(a) Outline of the thin layer agar npMALDI-I on schematic of the thin layer agar npMALDI-I approach. Step 1, cover the MALDI plate with a thin layer of growth media and then inoculate with a small sample containing the bacteria. Step 2, allow the bacteria to grow. Step 3, cover the sample with matrix. Step 4, subject the sample to MALDI imaging. Step5, average all the spectra obtained in the imaging run. Step 6, display all the ions of interest as a color. (b) IMS of *Bacillus subtilis* 3610 and the *srfAA*, *ppsB* mutants and the *ppsB* and *srfAA* double mutant. A is the merged signal of *m/z* 715, 1075, and 1545. B is the merged

signal with an optical image of the colony. The right side of this figure shows the average signal from *m/z* 600–1600 of all the spectra obtained in the imaging runs.

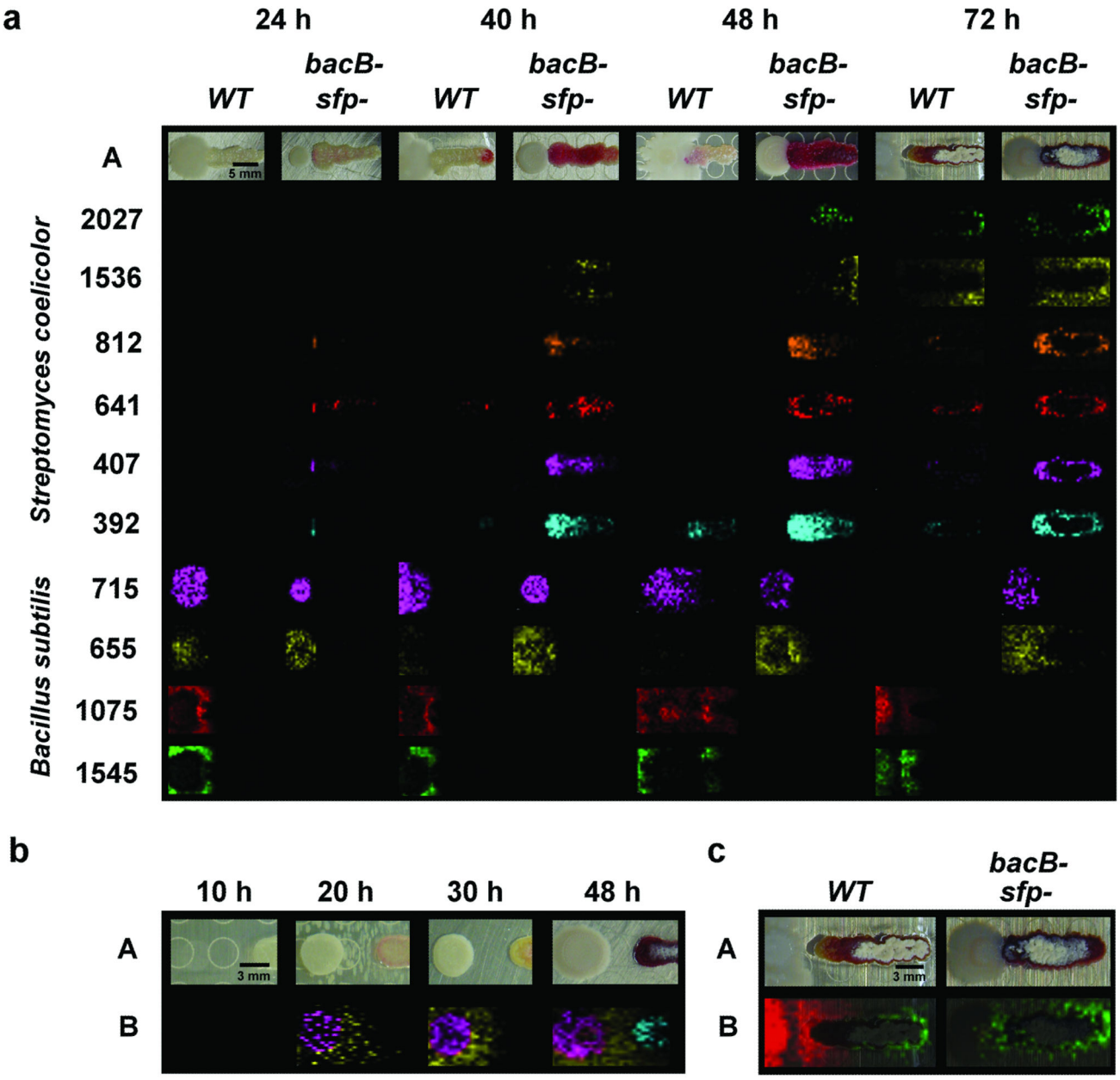


**Figure 2.**

Time course IMS of *Bacillus subtilis* strains and *Streptomyces coelicolor* cohabitation. (a) *Bacillus subtilis* strains were spotted 5 mm away from *Streptomyces coelicolor*. A is optical photograph of colonies. *m/z* 392 is a representative prodiginines (**6** and **7**) $[M+H]^+$. *m/z* 1075 is surfactin representative (**2a–c**) $[M+K]^+$. *m/z* 1545 is plipastatin representative (**3a–d**) $[M+K]^+$. *m/z* 1536 is CDA representative (**10a–b**) $[M+H]^+$. *m/z* 2027 is sapB (**9**) $[M+H]^+$. *m/z* 407, 641, 655 and 812 are unknown compounds. (b) *bacB/sfp* double deletion *Bacillus subtilis* strain were spotted 8 mm away from *Streptomyces coelicolor*. A is optical photograph of colonies. Purple is *m/z* 715. Yellow is the unknown ion at *m/z* 655. Blue are prodiginines (**6** and **7**). (c) The aerial hyphae formation and SapB (**9**) production by

*Streptomyces coelicolor* is inhibited by surfactin (**2a–c**). A is an optical photograph of colonies. Red is surfactin (**2a–c**). Green is sapB (**9**).

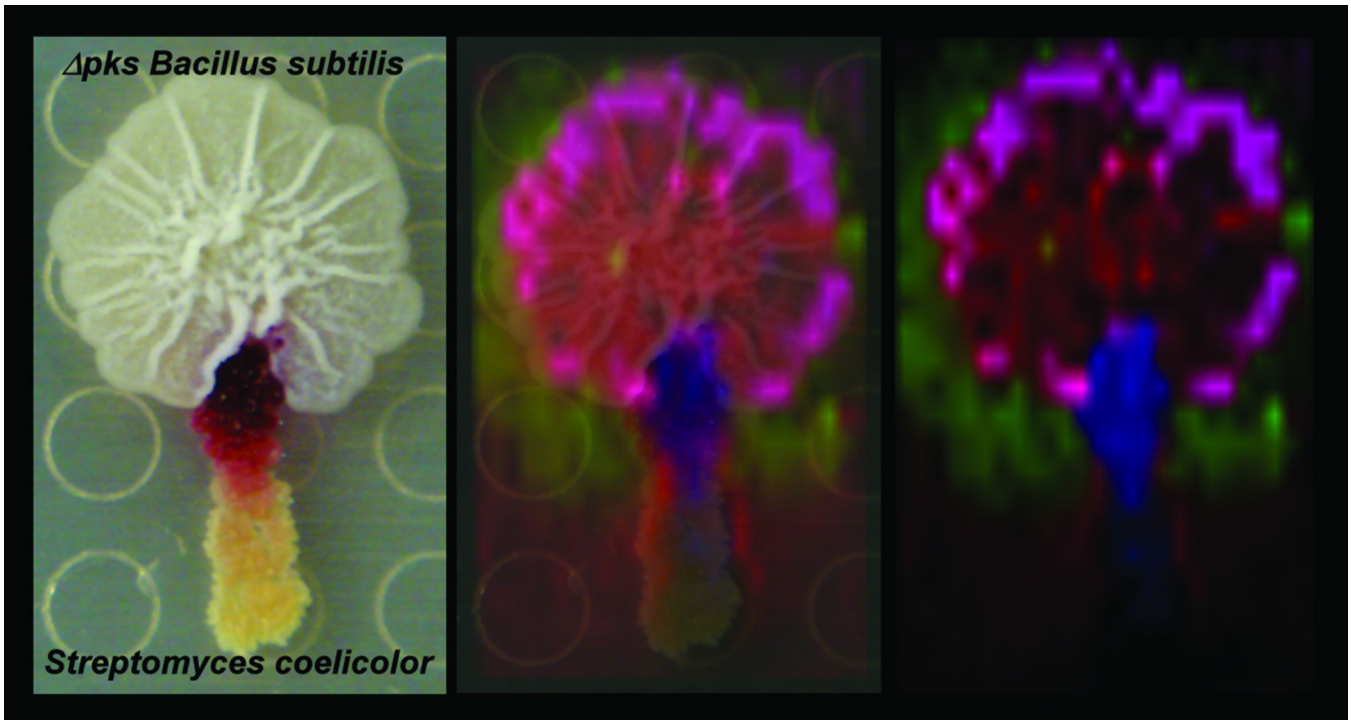


**Figure 3.**