Thiopeptide antibiotics stimulate biofilm formation in *Bacillus subtilis*

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Bacteria have evolved the ability to produce a wide range of structurally complex natural products historically called "secondary" metabolites. Although some of these compounds have been identified as bacterial communication cues, more frequently natural products are scrutinized for antibiotic activities that are relevant to human health. However, there has been little regard for how these compounds might otherwise impact the physiology of neighboring microbes present in complex communities. *Bacillus cereus* secretes molecules that activate expression of biofilm genes in *Bacillus subtilis*. Here, we use imaging mass spectrometry to identify the thiocillins, a group of thiazolyl peptide antibiotics, as biofilm matrix-inducing compounds produced by *B. cereus*. We found that thiocillin increased the population of matrix-producing *B. subtilis* cells and that this activity could be abolished by multiple structural alterations. Importantly, a mutation that eliminated thiocillin's antibiotic activity did not affect its ability to induce biofilm gene expression in *B. subtilis*. We go on to show that biofilm induction appears to be a general phenomenon of multiple structurally diverse thiazolyl peptides and use this activity to confirm the presence of thiazolyl peptide gene clusters in other bacterial species. Our results indicate that the roles of secondary metabolites initially identified as antibiotics may have more complex effects—acting not only as killing agents, but also as specific modulators of microbial cellular phenotypes.

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atural-product antibiotics have proven to be exquisite tools for combating infectious disease. Increasingly, a more elaborate role is being realized for these molecules formerly called "secondary" metabolites: as key signaling molecules for interspecies and intraspecies bacterial communication (1–7). Antibiotics at subinhibitory concentrations can trigger nonlethal physiological responses in human pathogens, including quorum sensing, virulence factor production, or biofilm formation (8–12). In some cases this activity has been tentatively linked to the mechanism of antibiotic resistance of the small molecule. For example, quorum-sensing induction by the aminoglycoside tobramycin arises from sublethal inhibition of translation by Rhfl/R system components (13).

More broadly, many natural-product antibiotics trigger biofilm formation, which has been interpreted as evidence that forming biofilms might be a general mechanism of defense against competitors (14). Biofilms are communities of bacterial cells living in a sticky, self-produced extracellular matrix on either a liquid or solid surface (15). The formation of bacterial biofilms can be both beneficial—such as on plant roots (16–18) or in wastewater treatment plants (19)—or detrimental—such as on in-dwelling medical devices or during infection (20, 21). Thus, understanding the chemical signals that induce and inhibit biofilm formation in bacteria has broad relevance.

The ability to form biofilms, like many bacterial phenotypes, is a result of bacteria differentiating into transcriptionally distinct cell types (22–24). *Bacillus subtilis* is a model organism whose cellular differentiation capabilities have been well characterized (23). Other members of the genus *Bacillus* can stimulate *B. subtilis* to differentiate into biofilm-matrix–producing cells (25). We monitored these interactions using a fluorescent transcriptional reporter for matrix gene expression. This reporter (PAJH–yfp) consists of P_{tadA} [the promoter for the tapA operon (TasA anchoring/assembly protein A) that encodes TasA, the major protein component of *B. subtilis* biofilm matrix (26)] driving production of a yfp gene (encoding yellow fluorescent protein, YFP). Thus, when these *B. subtilis* cells are producing biofilm matrix, they also produce YFP. In particular, members of the *Bacillus* genus elicited *B. subtilis* biofilm formation via two distinct mechanisms (25). Some induced the matrix reporter via the sensor histidine kinase kinD (which activates the master transcriptional regulator for biofilm formation, SpolA); others induced the matrix reporter through a separate SpolA-dependent mechanism (25). This latter activity was linked to putative secreted metabolites present in conditioned medium that also had antibiotic activity (25).

Here, we pursue the identification of one of the biofilm-inducing metabolites secreted by *Bacillus cereus* ATCC 14579 that induced matrix formation in *B. subtilis* via both mechanisms (25). Using matrix-assisted laser desorption/ionization time-of-flight imaging mass spectrometry (MALDI-TOF IMS), we identified the thiocillins, members of the thiazolyl peptide class of natural products, as *B. cereus*-produced compounds that trigger biofilm formation in *B. subtilis* in a *kinD*-independent manner. The thiocillins are ribosomally encoded, posttranslationally modified.

**Significance**

Thiazolyl peptides are known antibiotics produced by diverse bacterial taxa. It has been believed that antibiotics are deployed by bacteria as weapons, providing them with an evolutionary advantage over other microbes. We show here that these weapons can also act as chemical tools that elicit biofilm production in the model bacterium *Bacillus subtilis*. Importantly, the biofilm-inducing (and therefore signaling) properties of these compounds are independent of their killing activity. We go on to use this biofilm-inducing activity to identify and confirm the presence of thiazolyl peptide gene clusters in other bacteria. These results indicate that thiazolyl peptides, and potentially other antibiotics, have the ability to alter bacterial behavior in ways important both to the environment and to human health.

Author contributions: A.A.B. and E.A.S. designed research; R.B. and E.A.S. performed research; J.D.W. and P.C.D. contributed new reagents/analytic tools; R.B. and E.A.S. analyzed data; and R.B., A.A.B., and E.A.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1414272112/-/DCSupplemental.
peptide antibiotics that exert their activity by interfering with the interaction between the 23S rRNA and the protein L11 of the 50S ribosome (27, 28). The biosynthesis genes for the thiocillins have recently been elucidated (29), leading to the identification of related thiazolyl biosynthesis clusters in the genomes of diverse bacterial taxa. We show, using purified compounds and genetically modified variants of B. cereus, that the kinD-independent matrix-inducing activity of the thiocillins is independent of the antibiotic activity for which they are known. We further examine the structure–activity relationship of the thiocillins' matrix-induction ability, and use this activity to identify putative thiazolyl peptide gene clusters in other Bacillus species.

Results

Using IMS to Connect Phenotype to Chemotype. We indirectly monitored the response of B. subtilis to environmental signals that activated biofilm matrix production using the P_{tapA-yfp} transcriptional fluorescent reporter strain. We used this strain to examine the consequences of coculturing B. subtilis with B. cereus under conditions in which B. subtilis does not normally produce biofilms (Fig. 1A). The B. subtilis colonies closest to B. cereus fluoresced, indicating that tapA gene expression is activated in these cells (Fig. 1B; ref. 25). We hypothesized that this biofilm activation was due to compounds secreted by B. cereus that diffused through the agar of the assay plate and altered the cellular physiology of B. subtilis.

The striking visual distribution of this coculture interaction induced us to use MALDI-TOF IMS to connect this fluorescent signal with the molecular cue(s) responsible for inducing it. MALDI-TOF IMS involves collecting numerous mass spectra in a grid-like pattern across 2D samples, which can then be used to correlate chemical ion distributions with spatial biological phenotypes (30, 31). From the IMS data collected, we looked for ions whose spatial distributions corresponded to the region where the fluorescent signal for matrix was activated. One of these was an ion with m/z = 1,142, identified in linear negative mode (Fig. 1C and Fig. S1A). When we searched for known compounds produced by B. cereus with this molecular weight, we identified a variant of thiocillin, micrococcin P1 [M-H]− (Fig. 1D; ref. 29) as a potential match, suggesting that the thiocillins may be the cue produced by B. cereus that induces matrix production and P_{tapA-yfp} fluorescence in B. subtilis. During the normal expression of the tcl biosynthesis cluster in B. cereus, a number of structural variants of thiocillin of different molecular weight are produced (29); we observed peaks corresponding to the masses of many of these structural variants, supporting our hypothesis that these ions observed in our IMS data may represent the thiocillins; we observed m/z = 1,141 micrococcin P2, [M-H]−, 1,155 (thiocillin 3, [M-H]−), 1,181 (thiocillin 1, [M-H+Na]+), 1,193 (YM-266184, [M-H+Na]+), and 1,195 (thiocillin 2, [M-H+Na]+) (Fig. S1B). We will refer to the thiocillins by their generic name here, unless using a specific purified variant.

Thiocillins Are Partially Responsible for B. cereus’ Ability to Induce B. subtilis Biofilm Matrix. Using a mutant of B. cereus that does not contain the structural genes required to produce the thiocillins (ΔtclE-H) (32), we examined the ability of wild-type (WT) and ΔtclE-H B. cereus to induce P_{tapA-yfp} expression in B. subtilis (Fig. 2A). Both the visual fluorescence of these interactions and their quantification indicate that the thiocillins are responsible for approximately half of the matrix-inducing ability of B. cereus (Fig. 2A, Left).

B. cereus uses two distinct mechanisms to induce P_{tapA-yfp} fluorescence in B. subtilis: One requires the sensor histidine kinase KinD, and the other correlates with an antibiotic activity.

We previously proposed that this latter mechanism increases biofilm matrix production via a cell-type-specific killing of non-matrix-producing cells, thus increasing the subpopulation of matrix producers (25). To investigate which of these two B. cereus activities thiocillin might be responsible for, we examined the abilities of these B. cereus strains to induce P_{tapA-yfp} expression in a B. subtilis kinD strain. These data (Fig. 2A, Right) show that the B. cereus WT–B. subtilis kinD interaction has approximately half of the overall fluorescence observed in the B. cereus WT–B. subtilis WT interaction. This remaining signal is thus attributable to the activity produced by B. cereus that is acting via the kinD-independent pathway; it is virtually eliminated in the B. cereus ΔtclE-H–B. subtilis kinD interaction (Fig. 2A), indicating that the thiocillins are responsible for the kinD-independent activation of matrix induction in B. subtilis.

The Matrix-Inducing Activity of the Thiocillins Is Separable from Their Antibiotic Activity. To test whether the thiocillins were increasing P_{tapA-yfp} fluorescence in B. subtilis by specifically killing non-matrix-producing cells, we examined the activity of a B. cereus strain engineered to produce thiocillins containing a T4V mutation (Fig. 2B); T4V thiocillin has no antibiotic activity against B. subtilis (32) (Fig. S2). Remarkably, when we grew this B. cereus strain in our matrix-induction assay, it induced P_{tapA-yfp} fluorescence in B. subtilis to the same extent as WT B. cereus (Fig. 2A and B). B. cereus producing the T4V thiocillin variant also induced changes in B. subtilis colony morphology, where wrinkling correlates with biofilm production (Fig. S2). This finding was true for both the WT and kinD B. subtilis interaction (Fig. 2B). These results indicate that T4V thiocillin induces both P_{tapA} gene expression and changes in colony morphology associated with biofilm formation, and thus that the antibiotic activity of the thiocillins is separable from their matrix-inducing activity.

Fig. 1. IMS used to identify a matrix-inducing compound produced by B. cereus. (A) B. cereus grown as a colony on a microcolony lawn of B. subtilis. (Scale bar: 0.5 cm.) (B) This B. subtilis strain contains a fluorescent transcriptional reporter for biofilm matrix gene expression (P_{tapA-yfp}); the B. subtilis colonies closest to B. cereus are fluorescing, indicating that matrix gene expression is activated. (C) IMS data showing the distribution of an ion with m/z = 1,142 (linear negative mode) that corresponds to the area of fluorescence observed in B; relative scale is from 0.7 to 1.8. (D) Structure of micrococcin P1, a thiazolyl peptide antibiotic produced by B. cereus with a molecular weight consistent with the ion observed in C.
Biocidal Activity of Thiocillin at Low Concentrations.
We selected a concentration intermediate between these two concentrations (12.5 nM), and selected a time point (9 h) at which there was no difference in the OD600 of the DMSO- or thiocillin-treated cells. At this time point, we fixed the cells with 4% paraformaldehyde and measured the fluorescence of individual cells using flow cytometry. When B. subtilis PtapA yfp cells were grown in the presence of DMSO, only a small percentage of the population was fluorescent (11%), whereas a much larger percentage of the population (36%) expressed PtapA yfp when the cells were grown with YM-266183 (Fig. 3B). Interestingly, even at earlier time points (4 h), when B. subtilis growth is being inhibited, the percentage of the population that was expressing matrix was still higher in the thiocillin-treated cells than in those treated with DMSO (56% vs. 34%) (Fig. S4C). Thus, purified thiocillin has the ability to increase the proportion of matrix-producing cells when added to B. subtilis liquid cultures, irrespective of its antibiotic activity.

Matrix Induction Is a General Property of Thiazolyl Peptides. The thiopeptides are ribosomally synthesized and posttranslationally modified (RIPPs) in the class of bacteriocins known as thiazolyl peptides (33). We were therefore curious whether the biofilm-inducing activity of thiocillin was a generic property of this class of metabolites. We purified a range of structurally and functionally diverse thiazolyl peptides (thioestrepton, nosiheptide, berninamycin, and GE37468; Fig. S5), and tested whether they influenced PtapA yfp expression in B. subtilis. We spotted 450 ng of these purified compounds (comparable to the amount of thiocillin used in Fig. 2C) onto B. subtilis WT and kinD lawns and quantified the resulting fluorescence (Fig. 4). All of these thiazolyl peptides induced PtapA yfp expression. There was no significant difference between the fluorescence induction of any individual thiopeptide on the WT or on the kinD mutant lawns (P > 0.1), indicating that, similar to thiocillin, all of these thiopeptides activate biofilm gene expression in a kinD-independent manner. Both thioestrepton and berninamycin demonstrated antibiotic activity at these concentrations. These data and those from Fig. 2 and 3 demonstrate that thiazolyl peptides possess structural or functional features that allow them to increase the number of matrix-gene–expressing cells in B. subtilis in a kinD-independent manner, regardless of their antibiotic activity.

**Structure-Function Specificity of Thiocillin’s Activity.** We next investigated the specificity of thiocillin’s activity. We began by examining the matrix-inducing activity of structural mutants of thiocillin, including those with disrupted thiazolyl rings (C2A, C5A, C7A, and C9A), those with a larger ring (A78), and those unable to form a ring.

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**Fig. 2.** Thiocillin contributes to the ability of B. cereus to induce PtapA yfp gene expression in B. subtilis in a kinD- and antibiotic-independent manner. (A) Colonies of WT and ΔtapA-H B. cereus spotted onto a lawn of WT or kinD B. subtilis PtapA yfp microcolonies and quantification of the fluorescence (n = 3). (B) Colonies of the thiocillin T4V mutant of B. cereus spotted onto the same B. subtilis lawns, as well as quantification of the fluorescence (n = 3). A total of 450 ng of purified thiocillin or T4V thiocillin spotted onto a lawn of WT B. subtilis PtapA yfp microcolonies and quantification of the fluorescence (n = 3). The halos visible in C are not due to B. subtilis cells dying, but by being physically moved during spotting of the compound. *P < 0.1; **P < 0.05.

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**Fig. 3.** Purified thiocillin increases the proportion of matrix-producing B. subtilis cells in liquid culture, even when not inhibiting growth. (A) Growth curves of B. subtilis from growth in shaking liquid culture with 12.5 nM YM-266183 or an equivalent volume of DMSO (gray diamonds, WT with DMSO; black squares, WT with YM-266183; light green triangles, PtapA yfp with DMSO; dark green circles, PtapA yfp with YM-266183). (B) Flow cytometry of the fluorescence intensity of B. subtilis cells harvested from the 9-h time point from A (front, WT cells with DMSO; middle, PtapA yfp cells with DMSO; back, PtapA yfp cells with YM-266183). A total of 30,000 cells were quantified for each sample.

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**Fig. 4.** Normalized average integrated intensity shows the proportional increase in gene expression in this structural mutant. (A) Comparison of the integrated fluorescence intensity of microcolonies of B. subtilis mutants spotted onto the same B. cereus WT lawn. The colonies of the thiocillin T4V mutant of B. cereus were spotted onto the same B. subtilis lawns (WT and kinD). A total of 30,000 cells were quantified for each sample. The asterisks indicate a significant increase in expression, with *P < 0.1, **P < 0.05.
Bacteria with Cryptic Thiazolyl Biosynthesis Genes Induce Matrix in *B. subtilis*. Other *Bacillus* species also have the ability to activate *P*$_{tapA}$-$yfp$ expression via an antibiotic-associated, kinD-independent activity (25). Here we quantified the *P*$_{tapA}$-$yfp$ fluorescence induced by these strains in *kinD B. subtilis* and compared it to the fluorescence they cause in WT *B. subtilis* (Fig. S7B). These data indicate that three of these strains induce *P*$_{tapA}$-$yfp$ fluorescence in *B. subtilis* via an entirely *kinD*-independent mechanism (*Bacillus atrophaeus*, *Bacillus villisporus*, and *Bacillus mojavensis*); unfortunately, there are only partial genome sequences available for these strains, making it impossible to determine whether they possess cryptic thiazolyl biosynthesis genes that could be responsible for this activity. However, we used the sequences of the biosynthesis genes required for thiazolyl production [the lantibiotic dehydratase genes (shown in red in Fig. 6A)] (29), as well as the program antiSMASH (36) to search all available *Bacillus* bacterial genomes to identify strains containing cryptic thiazolyl peptides. We identified 10 *Bacillus* strains that contained these putative biosynthetic gene clusters, consistent with being capable of producing thiazolyl peptides (37). In addition to the lantibiotic dehydratase genes, these gene clusters included a YcaO homolog (a cyclodehydratase believed to catalyze production of thiopetides’ thiazoles and oxazoles from cysteines and serines) and one to five copies of a putative structural gene, as well as other transporters and potential modification enzymes. Two of these bacterial strains were publicly available, and we tested them for their ability to induce *P*$_{tapA}$-$yfp$ gene expression in WT and *kinD B. subtilis* (Fig. 6B and Fig. S8). *B. atrophaeus* 1942 and *Bacillus* sp. 107 both induce matrix gene expression at levels comparable to *B. cereus* WT, but only *B. atrophaeus* 1942 does so in a *kinD*-independent manner (Fig. 6B). Interestingly, the biosynthetic gene cluster of *Bacillus* sp. 107 appears unique in that it does not contain a dehydratase; it is possible that it does not produce a typical thiazolyl peptide. Our data suggest that numerous *Bacilli* can induce expression of the *P*$_{tapA}$-$yfp$ biofilm reporter in *B. subtilis* via a *kinD*-independent manner and that some of these bacteria also contain putative thiazolyl-producing biosynthetic gene clusters.

**Discussion**

Antibiotics have frequently been considered merely weapons in a chemical arms race between soil microbes. Here we show that some of these killing agents also act as interspecies signals, altering bacterial gene expression and thus the development of their neighboring microbes. Specifically, we used a combination of coculture, fluorescent reporter assays, and MALDI-TOF IMS to identify and characterize the thiolcillin—thiazolyl peptide natural products produced by *B. cereus*—as inducers of biofilm-matrix gene expression in
**B. subtilis.** Antibiotic-mediated biofilm formation is not a new phenomenon, but in many instances, it has been thought to be a nonspecific response to antibiotic challenge (i.e., stress) that confers increased resistance. Indeed, we initially proposed cell-type–specific killing as a mechanism for the kinase-independent interaction between *B. subtilis* and *B. cereus* (25). However, using the T4V mutant of thiocillin, here we showed that thiocillin’s biofilm-activation activity can be divorced from its antibiotic activity.

This and previous work (25) indicate that *B. cereus* and the thiocillins activate matrix gene expression independently of the five sensor histidine kinases (KinA–KinE) known to control the activity of the master transcriptional regulator Spo0A in *B. subtilis*. Thus, thiocillins do not appear to fall into the class of small molecules that affect potassium ion concentration (like the lipopeptide surfactin), which activate matrix through a KinC-dependent pathway (38). We also found that thiocillins mutants with significant disruptions to the thiazolyl peptide backbone and major macrocycle were no longer biofilm-active, whereas structurally diverse thiazolyl peptides from several different bacterial species all induced matrix production in *B. subtilis*. These data suggest that biofilm induction may be generalizable to thiazolyl peptides as a class and that the potential sensor for these molecules may be capable of responding to general structural features of this diverse family of compounds. An example of a receptor for such largely varied structures is provided by the master transcriptional regulator Spo0A in *B. subtilis*. At present, no homolog to the Tip transcription factors could be identified in the genome of *B. subtilis* (36). Putative thiazolyl-like biosynthetic gene clusters may be capable of responding to general structural features of this diverse family of compounds, as evidenced by the biofilm-inducing activity of the thiazolyl peptide tapA.

**Materials and Methods**

**Strains.** Strains used in this work are listed in Table S1. The *B. subtilis* strain was NCB13610 from our laboratory collection. The *B. cereus* strain 14579 was obtained from ATCC. Mutant *B. cereus* strains were constructed as described in ref. 32.

**Culture Conditions and Matrix Induction Assay.** Bacterial cultures were grown in microcolony lawns assays were performed as described in ref. 25. Briefly, *B. subtilis* colonies were spread on dilute agar medium to form microcolony lawns, and the fluorescence intensity induced by WT *B. cereus*, *B. atrophaeus*, and *B. subtilis* sp. 107 WT and WT and *B. atrophaeus* 1942 induction of *P*<sub>tapA-yfp</sub> WT and *kinD* *B. subtilis* microcolony lawns (n = 3). *B. subtilis* sp. 107 induction of *P*<sub>tapA-yfp</sub> WT and *kinD* *B. subtilis* lawns are significantly different (P = 0.0094), but *B. atrophaeus* 1942 induction in these two strains is not significantly different, indicating a *kinD*-independent mechanism of induction. **P < 0.01.**
B. cereus was spotted onto them for coculture growth. See SI Materials and Methods for details.

Fluorescence Quantification. Typhoon data files were loaded into Metamorph (Version 7.1), and brightness and contrast were linearly adjusted. Quantification was performed as in ref. 25, except that thresholding was not used. See SI Materials and Methods for details.

Flow Cytometry. B. subtilis liquid cultures with DMSO or Thioellin (final = 12.5 nM) added were grown at 37 °C with shaking. Cells were fixed in 4% (wt/vol) paraformaldehyde, sonicated, and stored at 4 °C until analyzed by flow cytometry. See SI Materials and Methods for details.

MICS and Growth Curves. B. subtilis cultures were initiated by diluting a mid-log culture to OD(600) = 0.004 in 1 mL of LB in 24-well plates (Falcon). Equiv-alent volumes of DMSO or thioellipetides were added to separate wells, and the plates were covered with Aeroseals and grown at 37 °C with shaking. OD(600) measurements were taken by using a Tecan GENios plate reader.

IMS. IMS was performed as described in ref. 31. Briefly, agar coculture samples were grown at 37 °C with shaking. Cells were fixed in 4% (wt/vol) paraformaldehyde, sonicated, and stored at 4 °C until analyzed by flow cytometry. See SI Materials and Methods for details.

LC-MS. LC-MS data were acquired on an Agilent 6520 Accurate-Mass Q-TOF mass spectrometer with an electropray-sonication source in positive ion mode. The drying gas temperature was 350 °C, and the fragmentor voltage was 250 V. The thiazoyl peptides were separated by using a reverse-phase kinetex column; acetonitrile with 0.1% formic acid was run as a gradient from 2% to 100% over 15 min and held at 100% for 2 min against water with 0.1% formic acid.

Thioellin Purification and Concentrations. Thioellin, its mutants, and other thioellipetides were all purified as reported in ref. 32. Purified thiazoyl peptides were maintained as stocks at a concentration of 250 ng/μL in DMSO. For matrix induction assays, 1.8 μL (containing 450 ng) was spotted onto a dried plate freshly inoculated with a B. subtilis reporter microcolony lawn.

Statistics. All P values were calculated with a Tukey’s honest significant difference pairwise analysis using JMP software.

ACKNOWLEDGMENTS. We sincerely thank Roberto Kolter [Harvard Medical School (HMS)], in whose laboratory this work was initiated. We thank Hera Vlamakis (HMS) for insightful manuscript suggestions and Kirk Grubb [University of North Carolina Chapel Hill (UNC-CH)] for assistance with the bioinformatic analyses. P.C.D. was supported by UNC-CH Start-Up Funds. R.B. was supported by a UNC-CH Rosetty Society Fellowship. Part of this work was supported by NIH Grants GM094802, AI095125, and S10RR029121 (to P.C.D.).
Supporting Information

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SI Materials and Methods

Culture Conditions. All bioassay plates contained 20 mL, poured using a Wheaton Unispense liquid dispenser, of 0.1x LB (BD Difco), 100 mM Mops, pH 7 (Sigma), and 1.5% (wt/vol) agar. Liquid LB broth contained no agar. All IMS plates contained 10 mL of 0.1x LB, poured by hand.

Reporter Preparation. *B. subtilis* reporter strains were prepared as described in ref. 1. They were cultured in LB broth to midlog, diluted to an OD_{600} of 0.02, and regrown to midlog at least twice more to reduce background fluorescence. At midlog after these final dilutions, glycerol was added [15–20% (vol/vol) final], and aliquots were frozen at −80 °C. Before use, the cfu/mL of the aliquots was determined by plating serial dilutions on LB agar plates.

Matrix Induction Assay. Reporter aliquots were thawed and diluted in LB broth to a concentration of 5 × 10^5 cfu/mL, and 50 μL of this dilution was spread on 20 mL of 0.1x LB, 100 mM Mops plates using 3-mm sterile glass beads. The organisms to be tested were resuspended in 1x LB broth to an OD_{600} of 0.5, and 3 μL of the cell suspension was spotted onto a dried plate freshly inoculated with a *B. subtilis* reporter microcolony lawn. Plates were grown at 24 °C. After 24–26 h of growth, plates were scanned for fluorescence by using a Typhoon fluorescence imager (488 nm excitation, 526 nm emission, 500 PMT, 100-μm resolution, 3-mm scan height).

Fluorescence Quantification. Typhoon data files (.gel) were loaded into Metamorph (Version 7.1), and brightness and contrast were linearly adjusted. Quantification was performed essentially as described in ref. 1, except that thresholding was not used. In brief, four concentric regions of interest were defined around each colony spot, with the difference between the outer two representing the background fluorescence of the plate, and the difference between the inner two representing the fluorescence intensity in the area of matrix-induction. After background subtraction, the average integrated intensity per area values were normalized by the background values to account for interplate variability. Values from each replicate assay were scaled relative to the maximum measured intensity for WT *B. cereus* for that assay to reduce noise and allow comparisons between biological replicates. Values for at least three independent experiments were averaged. Error bars are the SD.

Flow Cytometry. *B. subtilis* cultures were initiated by diluting a midlog culture to OD_{600} = 0.004 in 1 mL of LB in 24-well plates (Falcon). A total of 5 μL of DMSO or thiocillin (final = 12.5 nM) was added, and the plates were covered with Aeroseals and grown at 37 °C with shaking at 200 rpm. OD_{600} measurements were taken by using a SpectraMax or Tecan GENios plate reader. Cells for flow cytometry were prepared by harvesting 1 mL of culture, spinning it 2 min at 16,000 × g, and removing the supernatant. Cells were resuspended in 200 μL of 4% (wt/vol) paraformaldehyde and incubated for 7 min before being spun 2 min at 16,000 × g. The cells were washed with 1 mL of 1x phosphate buffer, spun, and resuspended in 1 mL of GTE buffer [1% glucose (wt/vol) and 5 mM EDTA in 1x phosphate buffer, pH 7.4]. Cells were sonicated for 12 pulses lasting 1 s each with a 1-s pause in between, put on ice for 30 s, and then sonicated for another set of 12 pulses, each lasting 1 s. Cells were stored at 4 °C until being filtered through a 38-μm nylon mesh, and their YFP fluorescence was measured using an LSR II Flow Cytometer (BD Biosciences).

IMS. IMS was performed as described in ref. 2. Pieces of 10-mL 0.1x LB agar plates on which either *B. subtilis* lawns spotted with *B. cereus* or colonies of the *B. cereus* thiocticillin mutants had been grown were excised from the plate and placed onto Bruker MSP 96 target ground steel target plates (Bruker part no. 224990) and covered with Universal MALDI matrix (Sigma, Fluka 50149) using a 53-μm stainless steel sieve (Hogentogler & Co, part 1312). The sample was dried onto the target plate overnight at 37 °C. Excess matrix was removed, and a peptide calibration standard was spotted onto the plate (Bruker part no. 206195, Pepmix4). IMS data were collected across the samples by using a Bruker Microflex LRF MALDI-TOF mass spectrometer and FlexControl and FlexImaging software. Data were collected in both linear-positive and linear-negative mode for both samples, with 80 shots averaged from each pixel of 400–800 μm across an m/z of 0–5,000 Da. The data were then examined manually in 0.5-Da increments for mass signals of interest, which were false-colored for display.


Fig. S1. Thiocillin colocalizes with *B. subtilis* fluorescence, and multiple thiocillin variants are observed in the IMS data. (A) The distribution of micrococcin P1 (m/z 1,142) as shown in Fig. 1C but now in magenta; the fluorescence microcolony lawn; and an overlay image showing that at the highest concentrations of thiocillin (closest to the *B. cereus* colony), there is growth inhibition of *B. subtilis*, but further from *B. cereus* the fluorescence and IMS data are colocalized. (B) The distributions of various structural variants of thiocillin observed in the IMS data that correspond to known masses of the thiocillins. From left: micrococcin P1 (m/z 1,142, [M-H]−), a heat map display of the same data, micrococcin P2 (m/z 1,141, [M-H]−), thiocillin 3 (m/z 1,155, [M-H]−), thiocillin 1 (m/z 1,181, [M-H+Na]−), YM-266184 (m/z 1,193, [M-H+Na]−), thiocillin 2 (m/z 1,195, [M-H+Na]−). The image labeled "Merged" is the merged IMS distribution of all of these thiocillin variants.

Fig. S2. *B. cereus* T4V elicits biofilm wrinkling in *B. subtilis* colonies. Images of *B. subtilis* microcolonies on 0.1× LB, 100 mM Mops agar plates grown alone or adjacent to WT *B. cereus*, ΔtclE-H *B. cereus*, or T4V *B. cereus* (colonies at top of images). The *B. subtilis* colonies grown with WT or T4V *B. cereus* are significantly more wrinkled (forming more biofilm) than those grown in the absence of *B. cereus* or with ΔtclE-H *B. cereus*. The minimal wrinkling that is observed in *B. subtilis* grown with ΔtclE-H *B. cereus* is due to the remaining *kind*-dependent activity that this *B. cereus* colony is producing. (Scale bar: 0.5 mm.)
Fig. S3. LC-MS traces of purified thiocillin variants. Extracted ion chromatograms of [M+H] and [M+Na] for purified thiocillin variants are shown. For each compound, Upper shows counts (%) vs. acquisition time (min), and Lower shows counts vs. mass-to-charge. All observed masses fall within 10 ppm of their expected masses: thiocillin II (expected mass 1,174.2279, observed mass 1,174.2305), YM-266183 (expected mass 1,158.1966, observed mass 1,158.1877), T4V YM-266184 (expected mass 1,188.2435, observed mass 1,188.2555), T4V YM-266183 (expected mass 1,174.2279, observed mass 1,174.2247), and A78 thiocillin (expected mass 1,215.2544, observed mass 1,215.2590).
Fig. S4. Thioctxillin minimum inhibitory concentration determinations and matrix gene expression during growth inhibition. (A and B) Absorbance readings (OD$_{600}$) after either 2.5 h (A) or overnight (B) growth of *B. subtilis* cells in the presence of the indicated concentrations of YM-266183. (C) Flow-cytometry data from 4-h time point shows that, even during growth inhibition, a larger percentage of cells are expressing matrix genes when treated with YM-266183 (gray, front, WT cells with DMSO; light green, middle, *P$_{tapA}$–yfp* cells with DMSO; dark green, back, *P$_{tapA}$–yfp* cells with YM-266183). A total of 30,000 cells were quantified for each sample.
Fig. S5. LC-MS traces of purified thiopeptides and their chemical structures. Extracted ion chromatograms of [M+H] for the purified thiopeptides. All observed masses fall within 10 ppm of their expected masses: nosiheptide (expected mass 1,222.1551, observed mass 1,222.1427), thiostrepton (expected mass 1,291.2574, observed mass 1,291.2574), berninamycin (expected mass 1,146.3483, observed mass 1,146.3460), and GE-37468 (expected mass 1,291.2460, observed mass 1,291.2574).
Fig. S6. Structures of thiocillin mutants.
Fig. S7. Quantification of fluorescence induction due to thiocillin and other Bacilli. (A) Minor thiocillin variants have different abilities to induce $P_{tapA-yfp}$ gene expression and do so in a dose-dependent manner. Quantification of the fluorescence intensity caused by 450 ng (or the indicated dilutions) of thiocillin 2 or YM-266183 spotted onto a lawn of WT $B. subtilis$ $P_{tapA-yfp}$ ($n=3$). *$P<0.1$; ***$P<0.005$, ****$P<0.0001$. (B) Quantification of fluorescence induction by other Bacilli spp. We quantified the ability of these $Bacillus$ strains (from ref. 1) to induce fluorescence in the $P_{tapA-yfp}$ reporter in both WT and kinD $B. subtilis$ microcolony lawns. $B. atrophaeus$, $B. vallismortis$, and $B. mojavensis$ induction is not significantly different between the WT and kinD $B. subtilis$ lawns, indicating that this matrix induction is kinD-independent. **$P<0.05$; ****$P<0.0001$.

**B. subtilis WT**

![B. subtilis WT](image)

**B. subtilis kinD**

![B. subtilis kinD](image)

**Table S1. Strains used in this work**

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