Supporting Information

Bleich et al. 10.1073/pnas.1414272112

SI Materials and Methods

Culture Conditions. All bioassay plates contained 20 mL, poured using a Wheaton Unispense liquid dispenser, of 0.1× 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 0.1× 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 0.1× LB, 100 mM Mops plates using 3-mm sterile glass beads. The organisms to be tested were resuspended in 1× 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 1× phosphate buffer, spun, and resuspended in 1 mL of GTE buffer [1% glucose (wt/vol) and 5 mM EDTA in 1× 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 0.1× LB agar plates on which either *B. subtilis* lawns spotted with *B. cereus* or colonies of the *B. cereus* thiocillin 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.

1. Shank EA, et al. (2011) Interspecies interactions that result in *Bacillus subtilis* forming biofilms are mediated mainly by members of its own genus. *Proc Natl Acad Sci USA* 108(48):E1236–E1243.

2. Yang JY, et al. (2012) Primer on agar-based microbial imaging mass spectrometry. *J Bacteriol* 194(22):6023–6028.

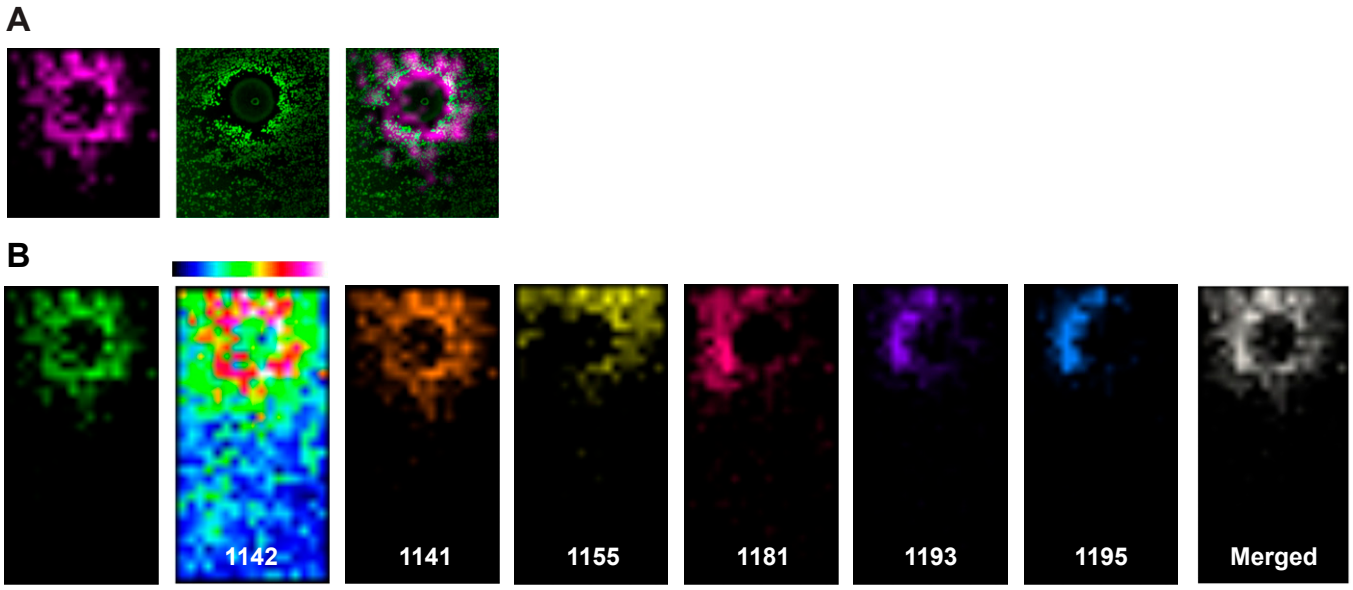

Fig. S1. Thiocillin colocalizes with *B. subtilis* fluorescence, and multiple thiocillin variants are observed in the IMS data. (*A*) The distribution of micrococcin P1 (*m/z* 1,142) as shown in Fig. 1*C* but now in magenta; the fluorescence microcolony lawn; and an overlay image showing that at the 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, $[M\text{-}H]^-$), a heat map display of the same data, micrococcin P2 (*m/z* 1,141, $[M\text{-}H]^-$), thiocillin 3 (*m/z* 1,155, $[M\text{-}H]^-$), thiocillin 1 (*m/z* 1,181, $[M\text{-}H\text{+}Na]^-$), YM-266184 (*m/z* 1,193, $[M\text{-}H\text{+}Na]^-$), thiocillin 2 (*m/z* 1,195, $[M\text{-}H\text{+}Na]^-$). The image labeled "Merged" is the merged IMS distribution of all of these thiocillin variants.

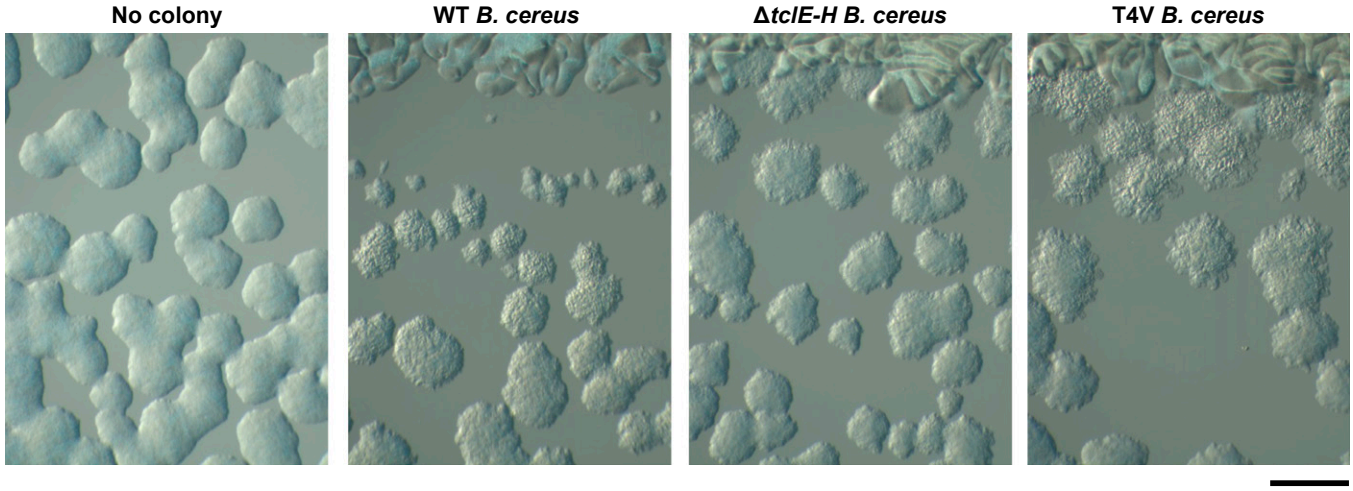

Fig. S2. *B. cereus* T4V elicits biofilm wrinkling in *B. subtilis* colonies. Images of *B. subtilis* microcolonies on 0.1× LB, 100 mM Mops agar plates grown alone or adjacent to WT *B. cereus*, Δ*tclE-H B. cereus*, or T4V *B. cereus* (colonies at top of images). The *B. subtilis* colonies grown with WT or T4V *B. cereus* are significantly more wrinkled (forming more biofilm) than those grown in the absence of *B. cereus* or with Δ*tclE-H B. cereus*. The minimal wrinkling that is observed in *B. subtilis* grown with Δ*tclE-H B. cereus* is due to the remaining *kinD*-dependent activity that this *B. cereus* colony is producing. (Scale bar: 0.5 mm.)

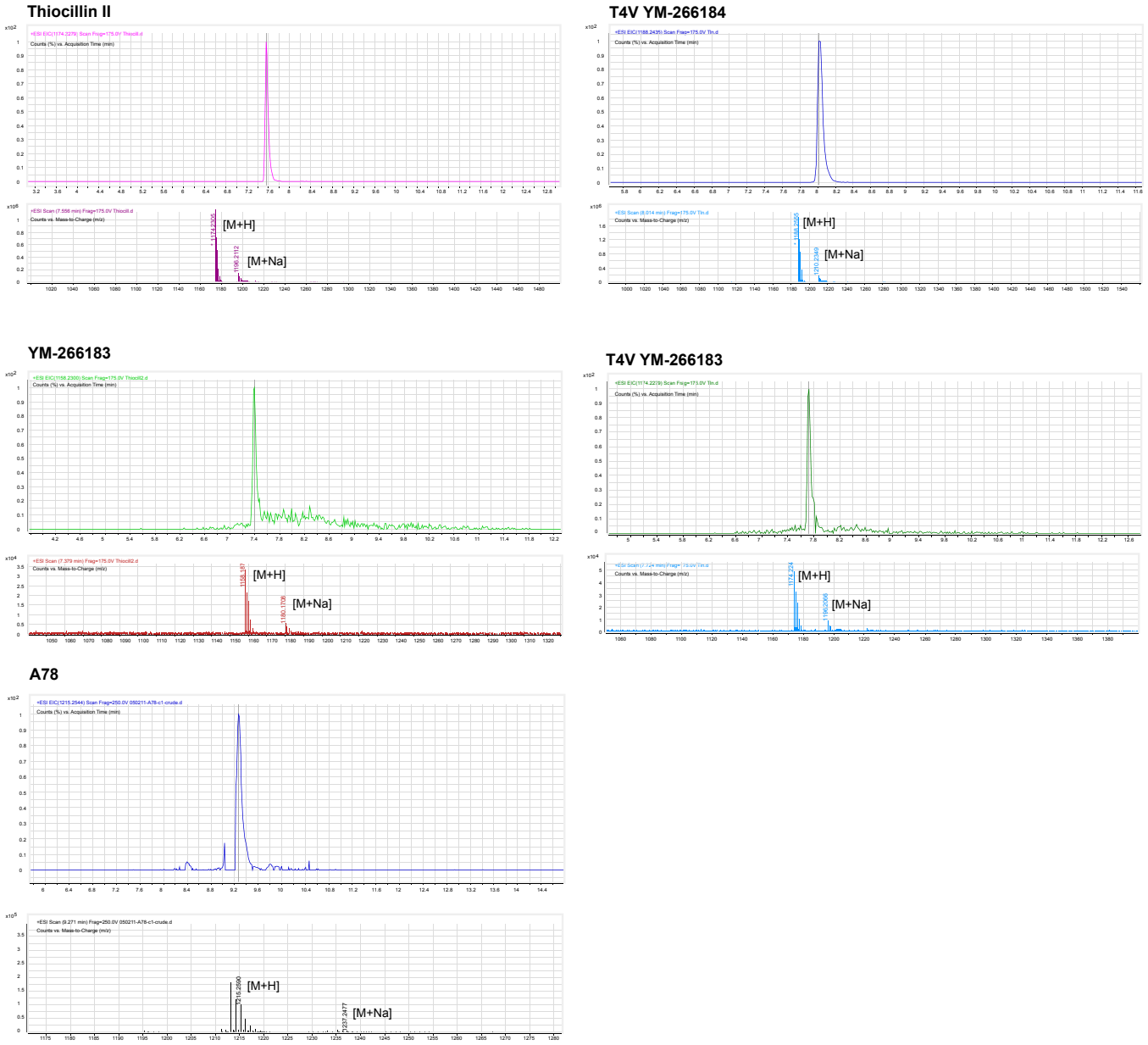

Fig. S3. LC-MS traces of purified thiocillin variants. Extracted ion chromatograms of [M+H] and [M+Na] for purified thiocillin variants are shown. For each compound, *Upper* shows counts (%) vs. acquisition time (min), and *Lower* shows counts vs. mass-to-charge. All observed masses fall within 10 ppm 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).

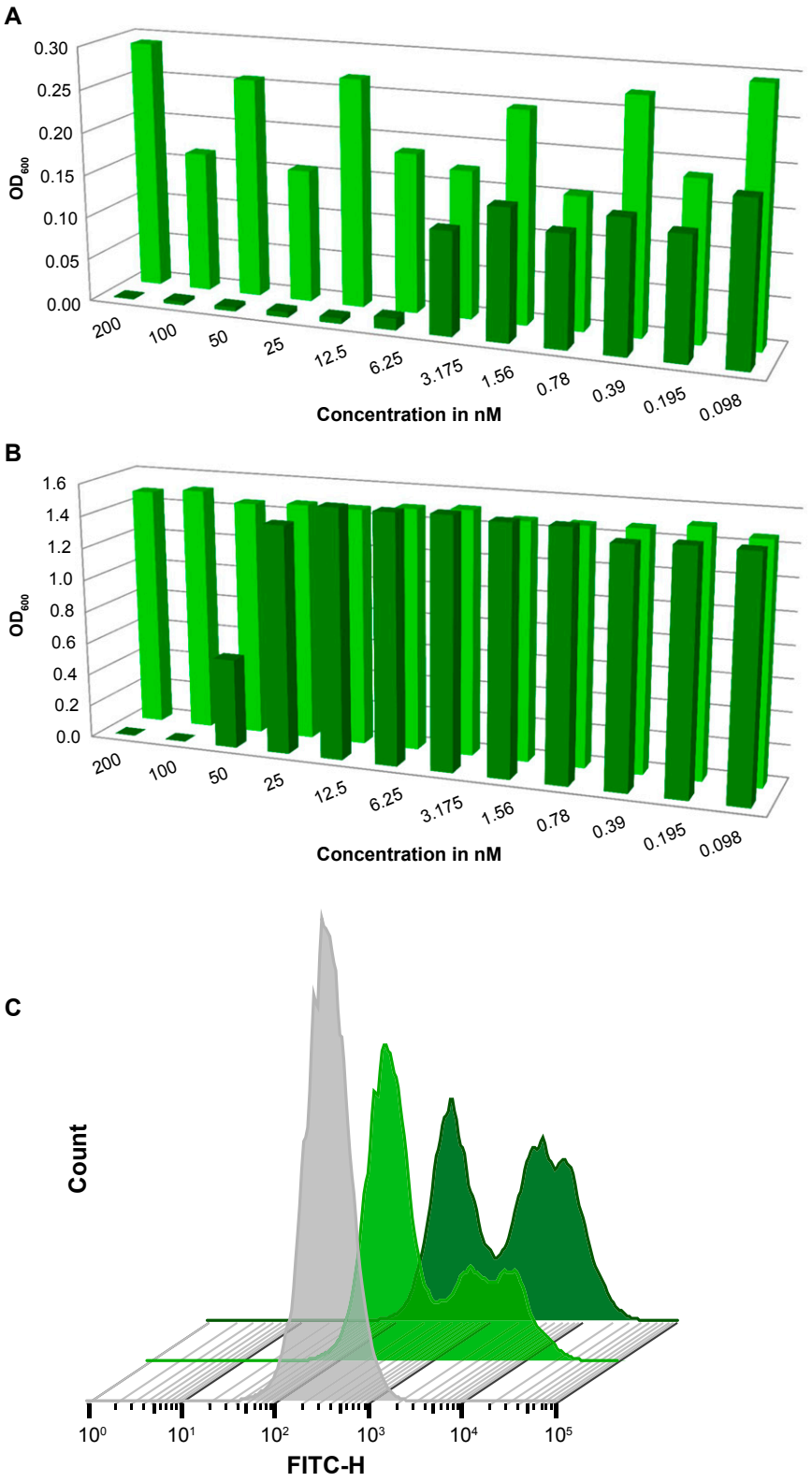

Fig. S4. Thiocillin minimum inhibitory concentration determinations and matrix gene expression during growth inhibition. (*A* and *B*) Absorbance readings (OD_{600}) after either 2.5 h (*A*) or overnight (*B*) growth of *B. subtilis* cells in the presence of the indicated concentrations of YM-266183. (*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.

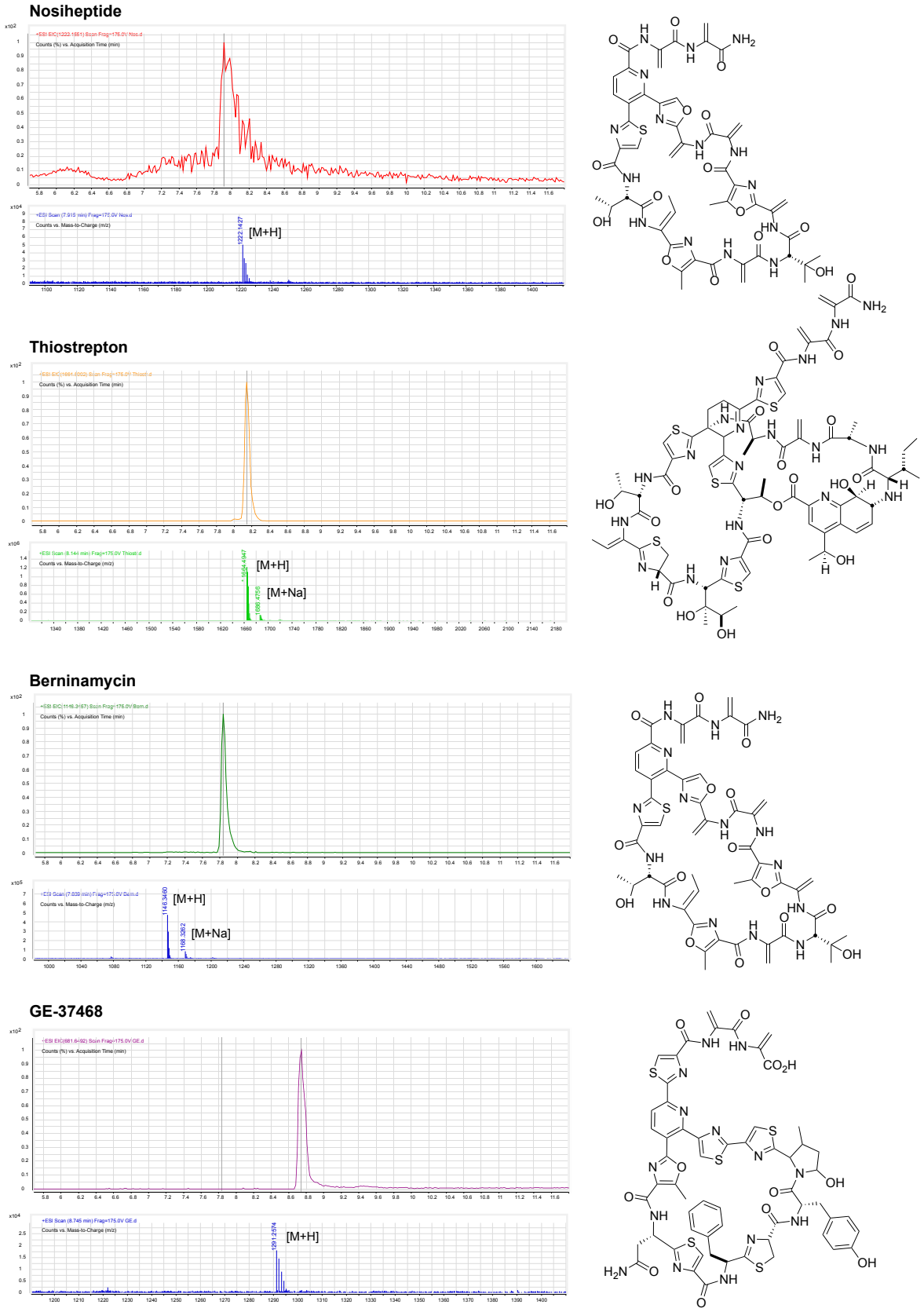

Fig. S5. LC-MS traces of purified thiopeptides and their chemical structures. Extracted ion chromatograms of [M+H] and [M+Na] for the purified thiopeptides. All observed masses fall within 10 ppm of their expected masses: nosiheptide (expected mass 1,222.1551, observed mass 1,222.1427), thiostrepton (expected mass 1,664.4903, observed mass 1,664.4947), berninamycin (expected mass 1,146.3483, observed mass 1,146.3460), and GE-37468 (expected mass 1,291.2460, observed mass 1,291.2574).

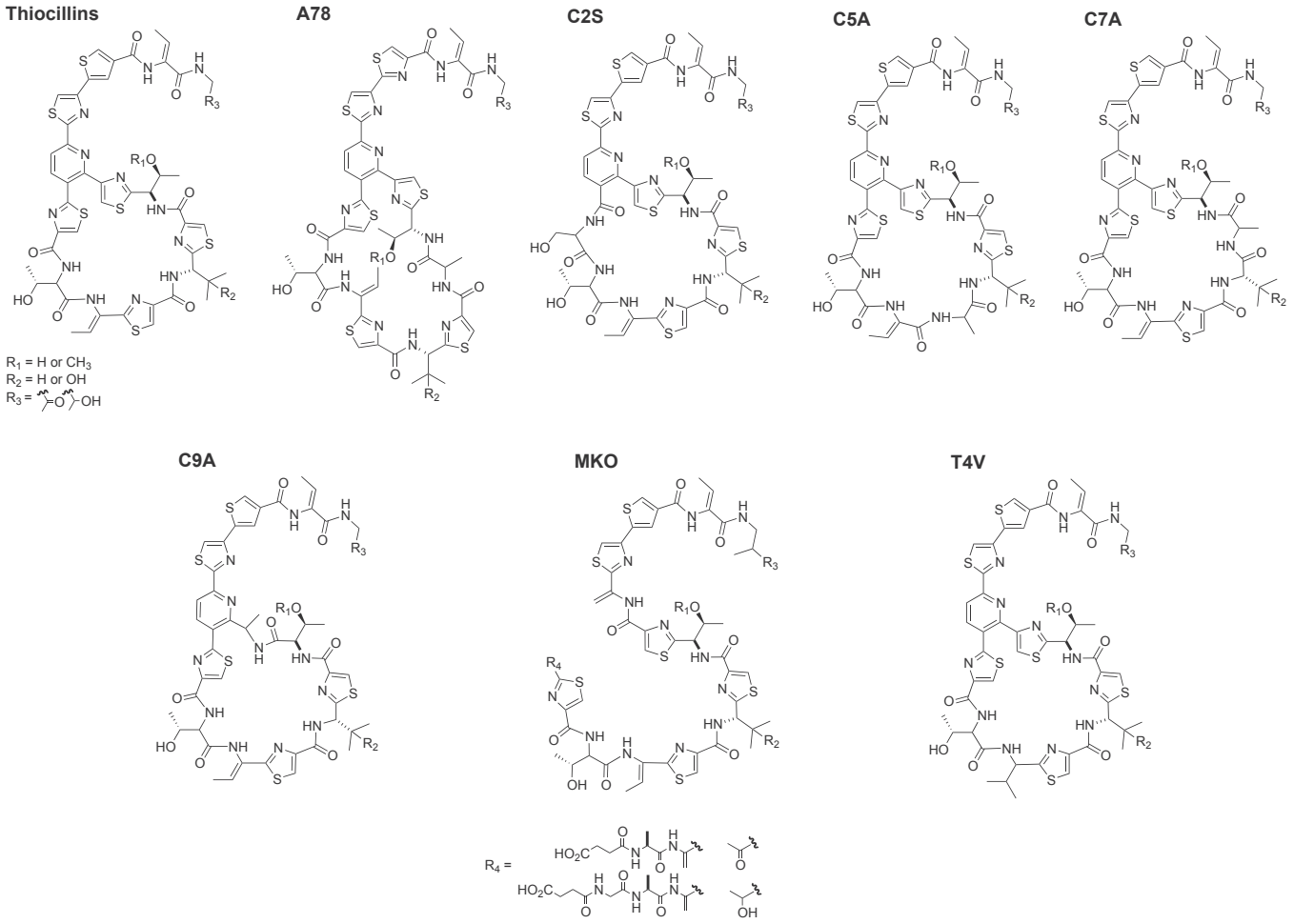

Fig. S6. Structures of thiocillin mutants.

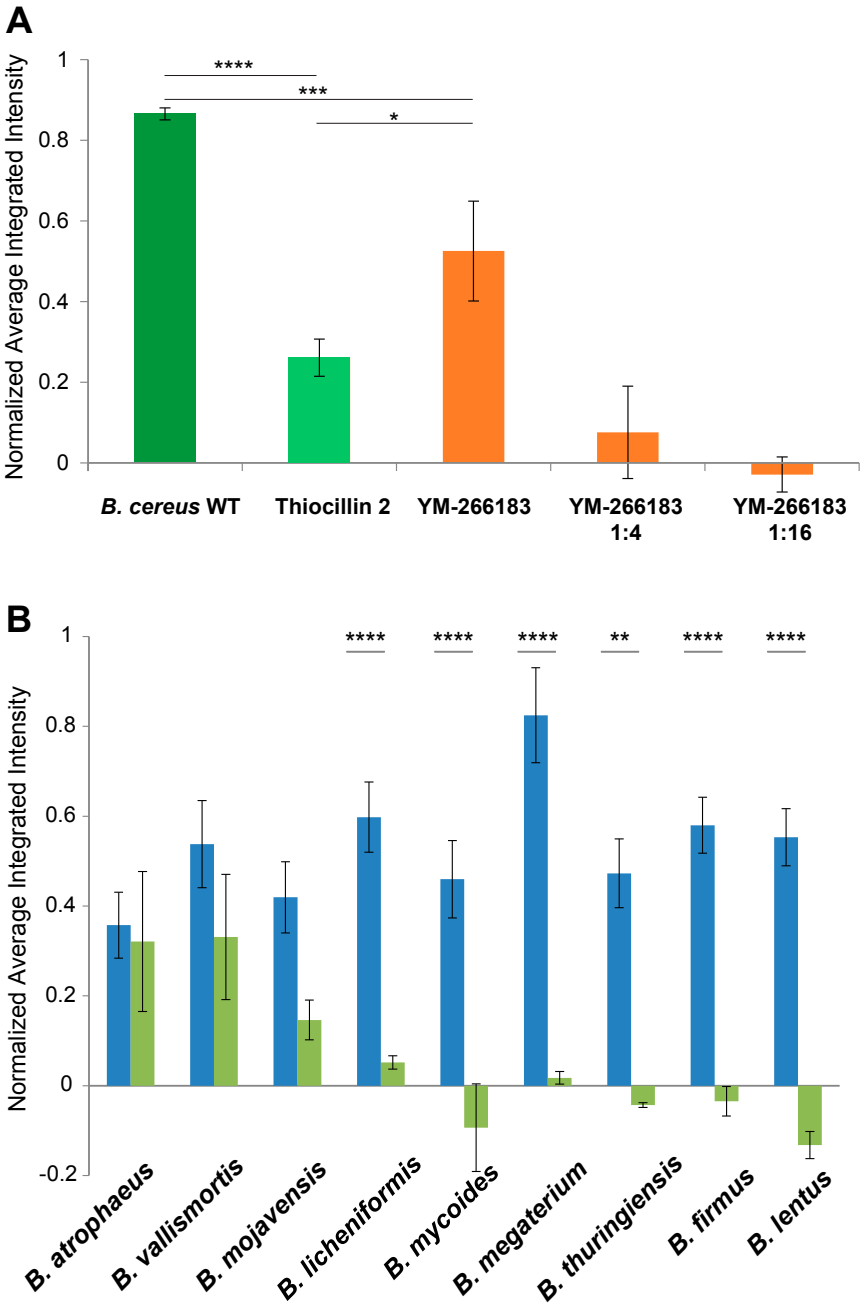

Fig. S7. Quantification of fluorescence induction due to thiocillin and other *Bacilli*. (*A*) Minor thiocillin variants have different abilities to induce P_{tapA}–*yfp* gene expression and do so in a dose-dependent manner. Quantification of the fluorescence intensity caused by 450 ng (or the indicated 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. **$P < 0.05$; ****$P < 0.0001$.

1. Shank EA, et al. (2011) Interspecies interactions that result in *Bacillus subtilis* forming biofilms are mediated mainly by members of its own genus. *Proc Natl Acad Sci USA* 108(48):E1236–E1243.

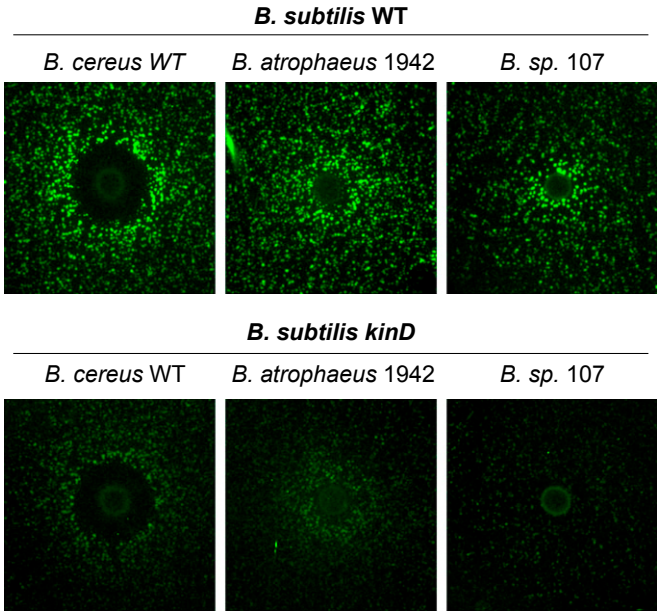

Fig. S8. Representative examples of the fluorescence of *B. subtilis* P_{tapA}–*yfp* microcolonies in response to other cryptic-thiopeptide–producing *Bacillus* species.

Table S1. Strains used in this work

ES strain name	Pseudonym	Other names	Genotype	Reference
ES3	*B. subtilis* WT	NCIB3610	WT	
ES136	*B. subtilis* P_{tapA}–*yfp*		3610 *amyE*::P_{tapA}–*yfp* (spec)	1
ES302	*B. subtilis* P_{tapA}–*yfp*, *kinD*		3610 *amyE*::P_{tapA}–*yfp* (spec), *kinD*::tet	1
ES348	*B. cereus* 14579	ATCC 14579	ATCC 14579 WT (Type Strain)	
ES349	*B. cereus* Δ*tclE-H*		Δ*tclE-H*	2
ES527	*B. cereus* T4V		*tclE-H*::*tclE*(T4V)	2
ES528	*B. cereus* A78		*tclE-H*::*tclE*(A78)	This work
ES529	*B. cereus* C2S		*tclE-H*::*tclE*(C2S)	3
ES530	*B. cereus* C5A		*tclE-H*::*tclE*(C5A)	3
ES531	*B. cereus* C7A		*tclE-H*::*tclE*(C7A)	3
ES526	*B. cereus* C9A		*tclE-H*::*tclE*(C9A)	3
ES532	*B. cereus* MKO		Δ*tclM*	4
ES533	*B. atrophaeus* 1942	BGSC11A3	WT	5
ES534	*B. sp.* 107	*Bacillus* sp. 278922_107	WT	

1. Shank EA, et al. (2011) Interspecies interactions that result in *Bacillus subtilis* forming biofilms are mediated mainly by members of its own genus. *Proc Natl Acad Sci USA* 108(48):E1236–E1243.
2. Yang JY, et al. (2012) Primer on agar-based microbial imaging mass spectrometry. *J Bacteriol* 194(22):6023–6028.
3. Acker MG, Bowers AA, Walsh CT (2009) Generation of thiocillin variants by prepeptide gene replacement and in vivo processing by *Bacillus cereus*. *J Am Chem Soc* 131(48):17563–17565.
4. Bowers AA, Acker MG, Koglin A, Walsh CT (2010) Manipulation of thiocillin variants by prepeptide gene replacement: structure, conformation, and activity of heterocycle substitution mutants. *J Am Chem Soc* 132(21):7519–7527.
5. Bowers AA, Walsh CT, Acker MG (2010) Genetic interception and structural characterization of thiopeptide cyclization precursors from *Bacillus cereus*. *J Am Chem Soc* 132(35):12182–12184.