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A chemical inhibitor of cell growth reduces cell size in *Bacillus subtilis*

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Abstract

Bacteria exhibit complex responses to biologically active small molecules. These responses include reductions in transcriptional and translational efficiency, alterations in metabolic flux, and in some cases, dramatic changes in growth and morphology. Here, we describe Min-1, a novel small molecule that inhibits growth of Gram-positive bacteria by targeting the cell envelope. Sub-inhibitory levels of Min-1 inhibits sporulation in *Streptomyces venezuelae* and reduces growth rate and cell length in *Bacillus subtilis*. The effect of Min-1 on *B. subtilis* cell length is significant at high growth rates sustained by nutrient rich media but drops off when growth rate is reduced during growth on less energy rich carbon sources. In each medium, Min-1 has no impact on the proportion of cells containing FtsZ-rings, suggesting that Min-1 reduces the mass at which FtsZ assembly is initiated. The effect of Min-1 on size is independent of UDP-glucose, which couples cell division to carbon availability, and the alarmone ppGpp, which reduces cell size via its impact on fatty acid synthesis. Min-1 activates the LiaRS stress response, which is sensitive to disruptions in the lipid II cycle and the cell membrane, and also compromises cell membrane integrity. Therefore, this novel synthetic molecule inhibits growth at high concentrations and induces a short-cell phenotype at sub-inhibitory concentrations that is independent of known systems that influence cell length, highlighting the complex interactions between small molecules and cell morphology.

INTRODUCTION

The impact of antibiotics on cell morphology has been an area of investigation since 1952.¹ Since then, we have learned that treating different bacteria with antibiotics of varying targets and concentrations can induce different cell morphologies. These can be used as a tool to understand the underlying molecular mechanisms that govern cell growth and development as well as aid in identifying the targets of novel antibiotics. This cytological profiling approach of characterizing the effects of antibiotics on cell morphology and subcellular organization has been harnessed to identify the target pathways for both known antibiotics

and novel natural products.^{2,3} Specifically, the nature of morphological changes induced by antimicrobial exposure are often suggestive of the compound's general macromolecular target. Activation of the SOS response by DNA damaging agents such as mitomycin C, bleomycin, and ciprofloxacin result in cell filamentation through the direct binding of FtsZ by SulA in *Escherichia coli*⁴ or YneA in *Bacillus subtilis*.⁵ Inhibition of fatty acid synthesis by cerulenin slows the rate of cell envelope synthesis and results in reduced cell size in *E. coli*, *B. subtilis*, and *Saccharomyces cerevisiae*.⁶ The small molecule A22 binds the ATP-binding pocket of MreB, an actin-like protein that serves as a treadmilling platform for peptidoglycan synthesis, resulting in significant changes in cell width in rod-shaped bacteria.^{7,8} In previous work we identified compounds, including Fil-1, Fil-2 and Fil-3, that inhibit division in several Gram-positive bacteria, resulting in cell filamentation, and strongly inhibit spore development in the actinomycete *S. coelicolor*.^{9,10} While some of these connections between antibiotic target and induced cell morphology are well-characterized, our understanding of how cells maintain their size and how it is influenced by external factors such as antibiotics is still an ongoing line of research.

Here we report another *Streptomyces* sporulation inhibitor and novel molecule, Min-1, inhibits the growth of Gram-positive bacteria, including *B. subtilis* and *S. aureus*, by targeting the cell envelope. Min-1 mediated growth inhibition is concentration-dependent and accompanied by a distinct short cell phenotype in *B. subtilis* at sub-inhibitory concentrations. Analysis of Min-1 treated cells suggests that the short cell phenotype is a result of disruption of the cell envelope and is independent of UgtP and ppGpp. The effect of Min-1 on cell size is significant in nutrient rich media supporting rapid growth, but its effect on cell size is modest in defined minimal media. Combining the short cell phenotype with the observation that Min-1 treatment does not alter the proportion of cells containing FtsZ-rings suggests that the compound reduces the mass at which FtsZ assembly is initiated. Min-1 activates the LiaRS stress response associated with disruption of the lipid II cycle, has no impact on the cell-wall damage sensing σ^W extracytoplasmic function sigma factor, increases membrane permeability, and disrupts membrane potential. These effects are related to those demonstrated for the antibacterial peptide nisin, however, unlike nisin, supplementing the media with magnesium does not block the effects of Min-1. These findings reinforce the importance of the cell envelope in determining cell size and describe the bioactivity of a novel molecule discovered by screening against *Streptomyces* sporulation.

RESULTS AND DISCUSSION

Min-1 inhibits *Streptomyces* sporulation at sub-inhibitory concentrations

Min-1 is a synthetic molecule (Figure 1A) identified in a screen of 30,569 small molecules for compounds that blocked sporulation of *Streptomyces coelicolor*.⁹ It was of interest because, while the molecule blocked spore formation, it appeared to have relatively little effect on formation of vegetative substrate hyphae or sporogenic aerial hyphae.

To confirm the original screening result we tested Min-1 against the distantly related species *Streptomyces venezuelae*. Normally, *S. venezuelae* colonies go through a cycle of growth in which they first produce a beige mycelium of filamentous substrate hyphae. Later, they

produce a white layer of sporogenic aerial hyphae, which grow upwards, conferring a white, fuzzy appearance to the colony surface. When the aerial hyphae sporulate, they turn green due to the activation of the *whiE* spore pigment genes.

We spotted 1 μ L of 25 mM Min-1 on a lawn of *S. venezuelae* spores and, following growth, observed a zone of inhibition surrounded by a white halo. Further from the source of Min-1, the lawn exhibited the characteristic green pigmentation of *S. venezuelae* (Figure 1B). The white halo indicates a region where cells failed to express the *whiE* genes, a classic indication of a sporulation block in streptomycetes.¹⁰ In contrast, compounds like vancomycin, which inhibit growth but not sporulation, conferred a typical zone of inhibition with no white halo.

To confirm this phenotype, we used scanning electron microscopy (SEM) to image the surface of the bacterial lawn. Cells sampled from the green zone grew in chains of septated hyphae, indicating normal division into spores (Figure 1D). In contrast, cells in the white halo region lacked septated hyphae (Figure 1C). This confirms that Min-1 blocks sporulation in *S. venezuelae* indicating that this is a general effect of the drug against this genus. Min-1 had no other observable effects on the filamentous *Streptomyces* cells.

Min-1 inhibits growth of Gram-positive bacteria and reduces cell length of *B. subtilis*

Streptomyces cells employ molecular mechanisms for cell growth and division that are similar to those of rod shaped and coccoid bacteria though they deploy them differently. We therefore predicted that Min-1 might have antibiotic activity against other bacteria. Indeed, when we treated *B. subtilis* and *S. aureus* with Min-1 we found that it inhibited growth at a minimum inhibitory concentration (MIC) of 31 μ M when cultured in LB medium (Table 1). The MIC of Min-1 against *B. subtilis* declined with the medium-supported growth rate, decreasing 2-fold in both S7₅₀-glucose and S7₅₀-glycerol indicating greater antimicrobial potency under these slower growth conditions. The compound had no inhibitory effect against *E. coli* at concentrations up to 100 μ M though it was able to inhibit the growth of the hyperpermeable strain MC1061¹¹ at 61 μ M. This suggests that the lipopolysaccharide (lps) layer of the Gram-negative outer membrane blocks access of Min-1 to a potential target(s).

We explored the phenotypic effects of Min-1 on *B. subtilis* cultured in several different media (Figure 2A) supporting a range of doubling times. In LB-glucose, *B. subtilis* grew with a doubling time of ~21 min and reached an average length of 5.59 μ m. Addition of 3 μ M Min-1 reduced cell size to 4.78 μ m but had no effect on growth rate. Higher concentrations of the drug further decreased size and growth rate, with 12 μ M Min-1 reducing the average cell size by ~40% to 3.42 μ m and increasing the doubling time to ~29 min. While Min-1 exposure reduced cell length, we did not observe any other significant morphological changes upon Min-1 treatment (Figure 2B).

Despite having greater antibiotic potency in defined S7₅₀ media (Table 1), Min-1's effect on cell length was greatly reduced when *B. subtilis* was cultured in minimal media. Untreated cells cultured in S7₅₀-glucose¹² grew slower and were significantly smaller than cells cultured in LB-glucose, reaching an average length of 3.17 μ m. Treatment with 12 μ M Min-1 reduced cell length to 2.94 μ m. Similarly, untreated cells cultured in S7₅₀-glycerol

reached an average length of 2.8 μm , which was reduced to 2.51 μm by treatment with 12 μM Min-1. Therefore, the effect of Min-1 on cell length was influenced by growth conditions: it was greatest when cells were growing rapidly in rich, undefined medium and did not have a significant effect in defined media supporting slower growth rates.

While a reduction in cell size is accompanied by slower growth rate under nutrient-limiting conditions, as we observed in the untreated cultures, the two factors are not strictly linked. Alterations in temperature,¹³ transcription inhibition,⁶ and thymine limitation¹⁴ can result in changes to either growth rate or cell size without impacting the other, demonstrating that the cell cycle can be decoupled from growth. Also, while various molecules and mechanisms induce cell filamentation (ciprofloxacin, ampicillin) or rounding (A22, mecillinam), small molecule induced cell shortening is a less frequently observed and understood phenotype.¹⁵ Disruption of some cellular mechanisms, specifically UgtP¹⁶ and fatty acid synthesis,⁶ have resulted in short cells and cytological profiling studies have shown that small molecule membrane disruption at high concentrations can lead to short cells.¹⁵ We were therefore interested in further understanding the bioactivity of this molecule and how it disrupts cell growth and the cell cycle.

Structural analogs of Min-1 have altered effects on growth rate and cell length

To identify chemical features responsible for growth inhibition and the short cell phenotype we compared the activities of Min-1 with 13 structural analogues. The results indicate that minor changes in structure have profound effects on the molecule's activity (Figure 3). For example, when compared with Min-1, the analogues Min-1-5, Min-1-8, Min-1-9, Min-1-10, Min-1-11, Min-1-12 and Min-1-14 had greatly reduced potency (MIC greater than 100 μM) or in some cases no ability to block growth (MIC greater than 250 μM). Min-1-3, Min-1-13, Min-1-15 were active but exhibited higher MICs than the parent molecule, indicating lower potency. The potency of Min-1-6 and Min-1-9 were similar to Min-1's in this assay and Min-1-7 exhibited improved potency with an MIC of 10 μM . We note that a dynamic light scattering experiment indicated that, unlike the rest of the compounds, M1-3, M1-4, M1-7 and M1-15 were prone to aggregation (not shown).

Changes to Min-1 structure also altered the effects of the molecules on cell length in *B. subtilis* cultured in LB-glucose (Figure 4). Testing the analogs against *B. subtilis* at 6 μM , we observed that the more active molecules also shortened cells to a greater degree (Figure 4A). Indeed, we observed a striking correlation between the reduction in growth rate and the short cell phenotype (Figure 4B). This suggests that the effect of Min-1 on cell size is directly coupled to its antimicrobial potency when cultured in nutrient rich media.

Min-1 disrupts coordination between growth and FtsZ ring assembly

Polymerization of the tubulin homologue FtsZ into a ring-like structure establishes the location of the bacterial division site. The FtsZ ring serves as a framework for assembly of the division machinery and is thus an excellent marker for cell cycle progression. The proportion of cells with FtsZ rings localized at midcell in a population reflects the relative time at which rings first form following the previous cell division. This frequency remains constant if the time at which rings first form relative to the time from cell birth to division is

unchanged. In *B. subtilis*, the percentage of cells with FtsZ rings is high (~90%) in long, fast growing bacteria cultured in nutrient rich medium, consistent with their rapid mass doubling time. In contrast, the percentage of cells with FtsZ rings is lower in short, slow growing bacteria cultured in nutrient poor medium, consistent with their longer cell cycle time.¹⁷

To assess the influence of Min-1 on the cell cycle, we first determined the effect of Min-1 on the fraction of cells with FtsZ rings during growth in LB-glucose, a nutrient rich medium. Consistent with previous findings,¹⁷ an average of 95.4% of cells grown in LB-glucose contained a visible FtsZ-ring. This number was reduced to 79.1% and 63.7% in S7₅₀-glucose and S7₅₀-glycerol respectively, demonstrating that shorter and slower growing cells cultured in minimal media and different carbon sources display a delay in FtsZ ring formation.

Based on the reduction in growth rate and cell size induced by Min-1, we expected Min-1 to also reduce the proportion of cells containing FtsZ rings relative to the untreated population. Strikingly, cells cultured with up to 12 μ M Min-1 exhibited no significant change in the proportion of cells with FtsZ-rings in all three media (Figure 5A). In *B. subtilis* cultures treated with 12 μ M Min-1 we observed an average of 91.8% of cells cultured in LB-glucose, 83.9% in S7₅₀-glucose, and 71.7% in S7₅₀-glycerol contained FtsZ-rings.

Changes in the length-to-ring ratio of a population of cells can help to identify instances in which a treatment disrupts coordination between growth and the cell cycle. Quantifying the total length of cells in the population and the number of FtsZ-rings present when cultured in LB-glucose revealed an average cell length-to-ring ratio of 7.3 μ m/FtsZ rings (Figure 5B). Addition of up to 12 μ M Min-1 reduced this ratio to 4.7. The degree to which Min-1 affected the length-to-ring ratio varied with the growth medium, decreasing from 4.8 in untreated culture to 4.2 with 12 μ M Min-1 in cells cultured in S7₅₀-glucose, and from 5.5 to 4.4 in cells cultured in S7₅₀-glycerol. Together these data suggest that Min-1 reduces the mass at which FtsZ assembly is initiated, disrupting the coordination between growth and FtsZ-ring assembly, and that this effect is most significant when cells are grown in nutrient rich media.

To evaluate possible mechanisms through which Min-1 could impact cell length, we first investigated the UDP-glucose dependent division-inhibitor and glycosyltransferase UgtP.¹⁶ UgtP coordinates carbon availability with cell size in *B. subtilis* via changes in FtsZ assembly dynamics. During growth in carbon rich medium, high concentrations of its substrate UDP-glucose stimulates interaction between UgtP and FtsZ, delaying assembly of the division machinery and linking nutrient availability to cell length. Because UgtP impacts the cell cycle, defects in the three genes in the UDP-glucose biosynthesis pathway, *ugtP*, *gtaB* and *pgcA* reduce cell length under nutrient rich conditions but do not substantially impact growth rate.

To evaluate whether Min-1 acts through UgtP to induce the short cell phenotype, we compared the effect of Min-1 on wild type cells with its effects on strains bearing *ugtP::cat*, *gtaB::spc*, and *pgcA::Tn10* mutations. As expected, the lengths of the untreated mutants were ~20% shorter than wild-type (Figure 5C). Application of 6 μ M Min-1 to each mutant strain cultured in LB-glucose reduced cell length by an additional 10–15%, an effect

equivalent to that on wild type cells. Furthermore, the MIC of Min-1 against the mutants was 31 μ M, identical to that in the wild type. This demonstrates that Min-1 action is independent of the UgtP pathway.

ppGpp is a pleiotropic inhibitor of macromolecular synthesis in bacteria that reduces cell size, largely due to its impact on lipid synthesis.⁶ To determine whether the cell length effect of Min-1 is mediated through ppGpp, we tested Min-1 against a ppGpp-deficient strain of *B. subtilis* (JH642 *yjbM ywaC::kan relA::mIs*).¹⁸ If the cell shortening and growth inhibition phenotypes were mediated through Min-1 induction of ppGpp we would expect the ppGpp-deficient strain to be resistant to the Min-1 induced shortening and growth inhibition. Consistent with Min-1 impacting growth independently of ppGpp, the MIC of Min-1 for the ppGpp-deficient strain is the same as wild-type *B. subtilis* (31 μ M). We also found that treatment with 6 μ M of Min-1 reduced the length of ppGpp-deficient *B. subtilis* by more than 20%, the same magnitude as observed following treatment of the wild-type strain (Figure 5D). This demonstrates that Min-1 acts independently of ppGpp.

Min-1 targets the cell envelope

The cell envelope plays a vital role in determining and maintaining cell shape. To assess the possible impact of Min-1 on cell wall synthesis, we used two *B. subtilis* reporter strains, P_{liaR} -*lacZ*¹⁹ and P_{σ^W} -*lacZ*.²⁰ These reporters are activated upon inhibition of different components of the cell wall synthesis machinery. P_{liaR} is the promoter of the LiaRS two-component system that is strongly activated by direct interference with the lipid II cycle or by daptomycin, and, to a lesser extent, certain organic solvents and alkaline shock.^{21,22} Lipid II transports precursors for the biosynthesis of peptidoglycan across the bacterial membrane and this pathway is the target of many different antibiotics, including bacitracin,²³ nisin,²⁴ teixobactin²⁵ and malacidin A.²⁶ σ^W is an extracytoplasmic function (ECF) sigma factor that activates a cell wall stress response to antibiotics that interfere with the assembly of peptidoglycan, including vancomycin and the β -lactams, but less so for those that directly interfere with the lipid II cycle.^{27,28}

When we applied Min-1 to the P_{liaR} -*lacZ* reporter strain we observed a clear blue halo around the zone of inhibition (Figure 6A), indicating activation of the reporter. In contrast, when we applied Min-1 to a P_{σ^W} -*lacZ* *B. subtilis* reporter strain we did not observe any reporter activation (Figure 6A). This suggests that Min-1 targets the cell envelope at the membrane, either through the lipid II cycle or by directly disrupting membrane integrity.

Interestingly, this activity profile resembles that of nisin, an antimicrobial peptide that elicits a positive response from P_{liaR} -*lacZ*²¹ but not P_{σ^W} -*lacZ*.²⁷ It has also been reported that sub-MIC concentrations of nisin reduce cell length in *B. subtilis*.²⁹ Nisin action involves direct binding of lipid II with the concomitant inhibition of peptidoglycan synthesis; moreover, nisin also forms depolarizing pores across the cell membrane. This membrane activity is considered to be its principle mechanism of antimicrobial action.²⁴

To determine whether Min-1 has an effect on membrane potential and permeability, we employed the DNA-binding fluorescent dyes, TO-PRO-3 iodide and DiOC₂(3). TO-PRO-3 iodide strongly fluoresces when bound to DNA, however, it is unable to cross intact bacterial

membranes and can therefore be used to monitor membrane integrity.^{30,31} DiOC₂(3) is a cell permeant dye that, in response to a bacterial membrane potential, quenches its fluorescence output. Disruption of the membrane potential reduces this quenching and significantly increases the fluorescence emission intensity.³² These dyes can be combined into a single assay to distinguish disruption of membrane permeability from depolarization.³¹ Consistent with disruption of both the cell membrane integrity and depolarization, Min-1 cells exhibited a significant increase in TO-PRO-3 iodide and DiOC₂(3) fluorescence beginning at the sub-inhibitory concentration of 10 μ M and increasing through the MIC (Figure 6B).

Finally, to determine whether protecting membrane integrity compromises the antibacterial effect of Min-1, we performed a checkerboard assay in which varying concentrations of Min-1 were incubated with cells in the presence of varying concentrations of supplemental Mg²⁺. Supplemental magnesium stabilizes the bacterial membrane, as demonstrated by its ability to stabilize spheroplasts^{33,34} and ability to mitigate osmotic shock.³⁵ It has also been used to suppress activation of the *ywaC* cell-wall stress response when treated with various antibiotics that target the cell envelope.³⁶ We observed no effect of added Mg²⁺ on the MIC of Min-1, indicating that the action of the compound is more complex than simple disruption of the membrane. In contrast, the addition of supplemental Mg²⁺ had a strong protective effect against nisin: the MIC was doubled in the presence of 5 mM Mg²⁺ and increased more than 8-fold by 40 mM Mg²⁺ (Figure 7B). In summary, these data suggest that Min-1 compromises the lipid II cycle, thereby activating LiaRS. This could involve a direct interaction with lipid II or one of the enzymes that binds to it. Alternatively, Min-1 might interact with the membrane, compromising lipid II indirectly.

Min-1 is a novel inhibitor of bacterial growth.

We have demonstrated that Min-1, discovered initially for its capacity to inhibit sporulation in streptomycetes, also has antibacterial activity against *B. subtilis* and *S. aureus*, conferring a distinctive short cell phenotype at sub-MIC concentrations on the latter. The reduction in *B. subtilis* cell size is accompanied by an increase in mass doubling time but does not change the proportion of cells exhibiting FtsZ-rings. Therefore, Min-1's effect does not involve compromising cell cycle progression while reducing cell length. Instead, we propose that the reduced cell size at sub-MIC concentrations is the result of FtsZ-rings forming at reduced cell mass relative to an untreated population. This effect is independent of two known cell size regulators that result in short cell phenotypes: the UgtP pathway and ppGpp. Rather, the mechanism by which this occurs involves depolarization of the membrane and a defect in the lipid II cycle, resulting in the activation of the LiaRS stress response.

The morphological impact of numerous antibiotics have been described previously and, to our knowledge, the spectrum of effects exhibited by Min-1 is not common. For example, of 43 known antibiotics that have undergone cytological profiling, 11 resulted in a reduction in cell length.¹⁵ These include ionophores or other molecules that destabilize the bacterial membrane, such as nisin, CCCP, nigericin, and spirohexenolide A, or target fatty acid synthesis, such as cerulenin or triclosan. Therefore, either direct or indirect disruption of the membrane inhibits membrane expansion and results in reductions in cell length. This could be caused by reduction in nutrient uptake or disruption of ATP synthesis via membrane

depolarization. However, Min-1 also induces the LiaRS stress response, which is also activated by the lipid II targeting nisin, bacitracin, and vancomycin, and the membrane disrupting daptomycin, but not the membrane disrupting molecules peptides polymyxin B or friulimicin B.³⁷ This level of specificity suggests that the action of Min-1 is more complex than simple membrane disruption.

METHODS

Bacterial Strains, Reagents, and General Methods

The following strains were used for MIC determination of Min-1 and its analogs, *B. subtilis* JH642, *S. aureus* ATCC 29213, *S. aureus* ATCC BAA-44, *S. aureus* ATCC BAA-41, *E. faecalis* ATCC29212, *E. coli* K-12 and *E. coli* MC1061. Microscopy was performed using *B. subtilis* JH642. *Streptomyces* sporulation assay was performed using *S. venezuelae*. The *B. subtilis* reporter strains used are as follows: *B. subtilis* HB0050 (σ^W)²³ and *B. subtilis* BFS2470 (*liaI*).²⁴ The UgtP pathway mutants strains used were AHL35 (*gtaB::spc*), PL1310 (*pgcA::Tn10 cat*) and BW421 (*ugtP::cat*).² Growth media was liquid or agar Lysogeny Broth (LB) for all strains with the exception of *S. venezuelae*, which was grown on MYM agar at 30°C. For cell size assays, LB broth was supplemented with 0.2% glucose and S7₅₀ minimal media¹⁸ was supplemented with either 1% glucose or glycerol, and due to the JH642 auxotrophies, 40 µg/ml Trp and Phe. All chemicals were purchased from Sigma Aldrich. Antibiotic selection was performed using the following concentrations, erythromycin (1 µg mL⁻¹), neomycin (10 µg mL⁻¹), lincomycin (25 µg mL⁻¹), chloramphenicol (25 µg mL⁻¹) and spectinomycin (50 µg mL⁻¹).

Streptomyces development inhibition assay and Scanning Electron Microscopy

S. venezuelae spore stock was diluted in sterile saline, spread on MYM agar and allowed to dry. 1 µL of Min-1 was then spotted into the agar and allowed to dry before incubating at 30 °C for 48 hours. For other antibiotics, 10 µL was added to a drug disk prior to being placed on the agar plate. In order to prepare the samples for SEM, the agar was cut at the edge of the zone of inhibition and fixed with 2% glutaraldehyde in 0.1% sodium cacodylate buffer followed by sputter coating with gold. Images were obtained using an FEI XL30 ESEM.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) defines the lowest concentration of a compound required to inhibit the growth. A single colony was grown in 5 mL of liquid growth media. Following overnight growth, the culture was diluted 100-fold into fresh LB, S7₅₀-glucose or S7₅₀-glycerol medium and incubated at 37 °C until the cells reached mid-log phase (OD₆₀₀ of 0.3 to 0.5). The cells were diluted 10,000-fold into fresh medium and the MIC determined for each of the strains by adding 198 µL of the diluted cells to wells of a clear 96-well plate containing the compound at 2-fold dilutions. The plates were incubated at 37 °C overnight and the MIC determined as the lowest concentrations that inhibited growth as measured by OD₆₀₀ in a BioTek Eon plate reader.

***B. subtilis* reporter assays**

Screening for activation of P_{lial} and $P_{\sigma W}$ was performed by disc diffusion. A single colony of the reporter strain was incubated in growth media overnight at 37 °C with the corresponding antibiotics. The overnight culture was diluted 20-fold in liquid LB and 500 μ L of the diluted culture was added to an LB agar plate containing 500 μ L of a 2% X-Gal solution. The antibiotics were either spotted directly to the LB agar plate or absorbed into a drug disk that was placed on the agar prior to incubating overnight at 37 °C. After incubation, the plates were scored for appearance of the blue rings at or near the edge of the zone of inhibition.

Membrane potential and permeability disruption

An overnight culture of *B. subtilis* JH642 was sub-cultured and grown to a mid-log growth phase ($OD_{600} = 0.4$) in liquid LB media at 37 °C on a rotating incubator set to 300 rpm. Cells were pelleted at 3000g for 10 minutes, the supernatant decanted, and washed twice in PBS+ (PBS supplemented with 10 mM glucose and 0.5 mM $MgCl_2$). The pellet was then resuspended in PBS+ and diluted to an OD_{600} of 0.1. Concurrently, a blank PBS+ sample was also prepared. Dyes were aliquoted into both samples to a final concentration of 625 nM TO-PRO-3 Iodide ($\lambda_{Ex} = 600$ nm, $\lambda_{Em} = 650$ nm) and 10 μ M DiOC₂(3) ($\lambda_{Ex} = 450$ nm, $\lambda_{Em} = 510$ nm). Samples were allowed to incubate in darkness at room temperature for 5 minutes, then subject to compound treatment. Fluorescence was read immediately following treatment using a BioTek Synergy H1 plate reader.

Cell length measurements and FtsZ immunofluorescence labeling

B. subtilis strains were cultured on a roller drum at 37 °C from a single colony in LB + 0.2% glucose to $OD_{600} = 0.2$, then diluted 1:100 in LB-glucose with sub-inhibitory concentrations of Min-1 and grown to early exponential phase ($OD_{600} = 0.15$ – 0.3). For samples cultured in minimal media, cells were first cultured from a single colony in S7₅₀-glucose or S7₅₀-glycerol at 37 °C overnight. The overnight cultures were diluted into minimal medium to an $OD_{600} = 0.005$, then cultured to $OD_{600} = 0.2$ before being diluted 1:100 with, and without, Min-1 as indicated for the LB-glucose cultures. Early exponential phase samples were fixed with paraformaldehyde/glutaraldehyde (2.6%/0.008%) for 15 min at room temperature followed by a 30-min incubation on ice. Fixed cells were then adhered to an 18 well slide with 1% poly-L-lysine. Adherent cells were briefly incubated with 1 mg mL⁻¹ lysozyme, then washed with PBS, followed by incubation with wheat germ agglutinin-tetramethylrhodamine to label the cell wall. FtsZ rings were labeled by immunofluorescence, using affinity-purified polyclonal rabbit anti-FtsZ antibody¹⁸ followed by a goat anti-rabbit secondary antibody conjugated to Alexa-488. Images were acquired with an Olympus BX51 microscope with an OrcaERG camera and captured using Nikon Elements. Cell lengths were determined by manually measuring the distance between adjacent septa using Nikon Elements analysis software. Cells were scored as positive for FtsZ-ring formation if they contained a fluorescent band across the width of the cell, two fluorescent foci across from one another at midcell, or a single focus visible at the midpoint of an invaginating septum.

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Figure 1.

Effect of Min-1 on *S. venezuelae* development. a) Chemical structure of Min-1. b) Spot diffusion assay of *S. venezuelae* lawns treated with vancomycin and Min-1. c) SEM image of the *S. venezuelae* lawn in the Min-1 sub-MIC region. d) SEM image of untreated *S. venezuelae* lawn with arrows indicating septation of the hyphae.

Figure 2. Min-1 inhibits growth of Gram-positive organisms and reduces cell length in *B. subtilis*. a) Impact of increasing concentrations of Min-1 on *B. subtilis* cell length cultured in LB-glucose, S7₅₀-glucose, and S7₅₀-glycerol. Larger and darker points represent higher Min-1 concentrations, from 0 μM to 12 μM. Error bars represent the standard error of the mean from three biological replicates with at least 100 cell lengths measured per replicate. b) Microscopy images of untreated (left) and Min-1 treated at 6 μM (right) *B. subtilis* cultured in LB-glucose.

	Structure						MIC (μM)	
	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	<i>B. subtilis</i>	<i>S. aureus</i>
Min-1	-OH	-H	-H	-H	-H	-CF ₃	31 $\pm$ 1	30 $\pm$ 3
M1-3	-OH	-CH ₃	-H	-H	-H	-CF ₃	74 $\pm$ 14	59 $\pm$ 3
M1-4	-OH	-OH	-H	-H	-H	-CF ₃	44 $\pm$ 16	34 $\pm$ 13
M1-5	-OH	-H	-H	-H	-F	-CH ₃	126 $\pm$ 1	125 $\pm$ 0
M1-6	-OH	-H	-H	-H	-F	-CF ₃	28 $\pm$ 5	31 $\pm$ 0
M1-7	-OH	-H	-H	-Ph	-H	-CH ₃	10 $\pm$ 5	11 $\pm$ 4
M1-8	-OCH ₃	-H	-H	-H	-Ph	-CH ₃	125 $\pm$ 0	>250
M1-9	-OCH ₃	-H	-H	-H	<i>i</i> Pr	-CH ₃	31 $\pm$ 0	>250
M1-10	-OCH ₃	-H	-F	-H	-H	-CH ₃	125 $\pm$ 0	>250
M1-11	-OC ₂ H ₅	-H	-H	-H	-F	-CH ₃	>250	>250
M1-12	-OCH ₃	-H	-H	-H	-H	-CH ₃	94 $\pm$ 36	>250
M1-13	-OCH ₃	-H	-H	-H	-F	-CH ₃	63 $\pm$ 0	94 $\pm$ 31
M1-14	-OH	-H	-H	-H	-Ph	-H	>250	>250
M1-15	-OCH ₃	-H	-H	-H	-Cl	-CH ₃	42 $\pm$ 18	42 $\pm$ 15

Ph = phenol *i*Pr = isopropanol

Figure 3.
Structure and activity of Min-1 analogs against *B. subtilis* and *S. aureus*

Figure 4.

a) Min-1 analogs demonstrate a link between cell length phenotype and inhibitory activity when cultured in LB-glucose, with the more active analogs inducing a shorter cell phenotype. b) The Min-1 analogs that reduce growth rate, as shown by cell doublings per hour, also have a greater impact on cell length. Error bars for each panel represent the standard error of the mean from three biological replicates with at least 100 cell lengths measured per replicate.

Figure 5.

Min-1 disrupts coordination between growth and FtsZ ring assembly independent of known cell size mechanisms. a) Proportion of treated *B. subtilis* cells with FtsZ rings is reduced with decreasing nutrient availability, but is not impacted over changing growth rates induced by increasing Min-1 concentrations. Min-1 treatment concentrations range from 0 μ M (light, small) to a maximum of 12 μ M (dark, large). b) The cell length to Z-ring ratio decreases as the growth rate slows at higher Min-1 concentrations. c) Cell length of knock-out mutants in the *B. subtilis* UgtP pathway, including *ugtP*, *gtaB*, and *pgcA* treated with 6 μ M Min-1. d) Impact of 6 μ M Min-1 on the cell length of a ppGpp null *B. subtilis* strain. Error bars for each panel represent the standard error of the mean from at least three biological replicates with at least 100 cells measured per replicate.

Figure 6.

Min-1 targets the cell envelope. a) Impact of Min-1 and control antibiotics on *B. subtilis* reporter strains for cell wall assembly ($P_{liaI} - lacZ$ and $P_{\sigma W} - lacZ$). b) TO-PRO-3 iodide fluorescence (red ● - left axis), DiOC₂(3) fluorescence (green ▲ - left axis) and *B. subtilis* optical density at 600 nm (■ - right axis) with increasing Min-1 treatment concentration. Error bars represent the standard error of the mean from three replicates.

Figure 7. Supplemental magnesium does not impact growth inhibition of Min-1. Checkerboards of (a) Min-1 and supplemental magnesium and (b) nisin and supplemental magnesium against *B. subtilis* grown in LB.

Table 1.

Min-1 inhibits growth of Gram-positive organisms.

Strain	Min-1 MIC (μ M)
<i>B. subtilis</i> JH642 (LB)	31
<i>B. subtilis</i> JH642 (S7 ₅₀ -glucose)	16
<i>B. subtilis</i> JH642 (S7 ₅₀ -glycerol)	16
<i>S. aureus</i> ATCC 29213	30
<i>S. aureus</i> ATCC BAA-44	22
<i>S. aureus</i> ATCC BAA-41	33
<i>E. coli</i> K-12	>250
<i>E. coli</i> MC1061	61