Published in final edited form as:

ACS Chem Biol. 2018 January 19; 13(1): 235–246. doi:10.1021/acscchembio.7b00560.

Small Molecule Chelators Reveal That Iron Starvation Inhibits Late Stages of Bacterial Cytokinesis

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Abstract

Bacterial cell division requires identification of the division site, assembly of the division machinery, and constriction of the cell envelope. These processes are regulated in response to several cellular and environmental signals. Here, we use small molecule iron chelators to characterize the surprising connections between bacterial iron homeostasis and cell division. We demonstrate that iron starvation downregulates the transcription of genes encoding proteins involved in cell division, reduces protein biosynthesis, and prevents correct positioning of the division machinery at the division site. These combined events arrest the constriction of the cell during late stages of cytokinesis in a manner distinct from known mechanisms of inhibiting cell division. Overexpression of genes encoding cell division proteins or iron transporters partially suppresses the biological activity of iron chelators and restores growth and division. We propose a model demonstrating the effect of iron availability on the regulatory mechanisms coordinating division in response to the nutritional state of the cell.

Graphical abstract

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The authors declare no competing financial interest.

In bacteria, with few exceptions, cell division initiates with the polymerization of the essential cell division protein FtsZ into a ring-like structure that localizes at the division site and recruits other proteins to form a multiprotein complex called “divisome.”^{1,2} Several mechanisms regulate the spatiotemporal assembly and function of the divisome in bacteria;^{3–6} however, the molecular details underlying the coordination of these cell- cycle events are not completely understood. In addition to the systems controlling division in response to the developmental state of the cell, bacteria have evolved specific regulators that interact with components of the divisome and regulate division in response to external signals, such as nutrient availability,^{7–9} light (*e.g.*, circadian rhythms),¹⁰ and DNA damage.^{11–13} The majority of these mechanisms involve protein–protein interactions^{7,9,12–14} and target FtsZ or other components of the division machinery.^{12,13}

Metal ions such as iron, zinc, and manganese are essential in biological catalysis,¹⁵ and their absence alters bacterial cell physiology. Iron, for example, is a cofactor of many enzymes important for central metabolism, DNA biosynthesis, nitrogen fixation, photosynthesis, and respiration.^{15,16} A single bacterial cell needs $\sim 10^5$ – 10^6 iron ions to meet intracellular iron requirements;¹⁷ thus, iron-poor environments pose a major challenge to survival. To thrive in these conditions, bacteria have developed sophisticated mechanisms to harvest extracellular iron, including using small molecule siderophores.¹⁸ The classic ferric uptake regulator (Fur) and Fur-like proteins control the transcription of a large set of genes in response to iron availability.^{16,19} The role of iron in bacterial transcriptional regulation is complex, and recent genome-wide studies show that this regulatory network is still evolving.^{20,21}

Iron homeostasis plays a central role in bacterial growth and survival.^{19–21} Just as different cell stresses (*e.g.*, nutrient starvation and DNA damage) have been shown to inhibit cell division until conditions become favorable, it is plausible that iron starvation may lead to a similar phenotype. However, there is currently no evidence connecting these two processes. In this study, we investigate the consequences of iron starvation on bacterial cell division using small molecule chelators. We demonstrate that divin (**1**),²² a synthetic small molecule identified in a high-throughput screen for inhibitors of bacterial division, is a potent chelator of iron that arrests late stages of cytokinesis by blocking the physical process of constriction in dividing cells. Iron starvation induced by chemically unrelated families of iron chelators recapitulates the division defects triggered by **1**. We demonstrate that iron chelators down-regulate the transcription of genes encoding proteins involved in cell division, reduce protein biosynthesis, and affect the spatiotemporal localization of the division machinery. The combination of these cellular events arrests late stages of bacterial cell division, suggests that iron regulation and cell division are intertwined, and indicates that bacterial cytokinesis may include checkpoints akin to eukaryotic cell division.

RESULTS AND DISCUSSION

Compound 1 Arrests Cytokinesis during Late Stages of Bacterial Cell Division.

We previously demonstrated that **1** arrests late stages of bacterial cell division to create a unique phenotype not previously observed.²² However, the mechanism of action of this small molecule was not characterized in detail. *Escherichia coli* cells treated with **1** ($2\times$ minimum inhibitory concentration [MIC], 25 μM) display a distinct morphological defect

characterized by cytokinesis arrest after initial constriction at the division site (Figure 1a and b). Preliminary studies suggested that *divin* is effectively pumped out of *E. coli* cells by efflux machinery;²² therefore, we used a strain with a defective AcrAB-TolC efflux pump system—*E. coli* BW25113 *tolC*—for the experiments described below. Although the phenotype is apparent after 1–2 h, we chose to characterize it at later time points when it was more evident within a large population of cells. To quantify the effect of **1** on cell division of different bacteria, we used imaging flow cytometry and microscopy. Analysis of *E. coli* cells treated with **1** revealed two distinct populations of cells compared to the DMSO control (Figure 1c). A total of ~43% of the cells have an aspect ratio and area similar to those of cells treated with DMSO (Figure 1c, region I). More than 35% of the cells have an average length (*L*) of $3.5 \pm 0.7 \mu\text{m}$ (mean $\pm$ standard deviation; Figure 1c, region II and inset histogram). To avoid artifacts from cell debris or aggregates, we used a more stringent gating strategy and manually inspected the data to favor single cells. A probability density histogram of the cell population treated with **1** had a bimodal distribution (Figure 1d). The average cell length of the population treated with **1** ($L = 2.9 \pm 0.8 \mu\text{m}$, $n = 3,553$ cells analyzed) is significantly higher ($P = 0.001$) than the average length of the DMSO-treated cells ($L = 2.0 \pm 0.6 \mu\text{m}$, $n = 4,216$). We speculate that the population of shorter cells completed division at early predivisional stages during drug treatment and have a normal cell morphology; the other cell population was undergoing division prior to treatment, are susceptible to **1**, and display the cell division arrest at the constriction stage. Importantly, cells treated with **1** do not filament, which is a phenotype that commonly accompanies inhibition of cell division, *e.g.*, antibiotics that inhibit cell division by blocking DNA replication (*e.g.*, ciprofloxacin or nalidixic acid) or peptidoglycan biosynthesis (*e.g.*, cephalixin).²³

To confirm that **1**-dependent cytokinetic arrest occurs in other bacterial species, we treated wild-type *Caulobacter crescentus* with **1** ($2\times \text{MIC} = 25 \mu\text{M}$) and performed time- course imaging (Figure 1e). The average length of cells after 12 h of treatment with DMSO was $2.4 \pm 0.6 \mu\text{m}$ ($n = 961$), and the size range was $1.5\text{--}5.6 \mu\text{m}$. Fewer than 3.5% of the total cells at each time point analyzed were $>4 \mu\text{m}$ in length, which corresponds to a mean length of *C. crescentus* cells at division.²⁴ However, *C. crescentus* treated with **1** became slightly elongated at ~4 h following treatment ($L = 3.5 \pm 0.9 \mu\text{m}$, $n = 457$, $P = 0.001$ compared to the DMSO control), and 31% displayed arrested cell division. We continued to study cells at longer growth times to see what would happen to their morphology. After 6 h, >60% of the cells were two-cells-long (*i.e.*, $>4 \mu\text{m}$) and displayed the typical division arrest observed in *E. coli* cells treated with **1**. The average length of cells 12 h following treatment was $5.1 \pm 1.4 \mu\text{m}$ ($n = 624$) with a size range of $1.7\text{--}9.2 \mu\text{m}$, confirming that cells treated with **1** continue to grow slowly but do not filament, as happens with most methods of blocking cell division. Similarly, flow cytometric analysis of *C. crescentus* cells treated with **1** revealed morphological changes in the cell population when compared to DMSO-treated cells (Figure s1a). Compound **1** is also active against Gram-positive bacteria, as cultures of wild-type *Streptococcus pyogenes* treated with DMSO contained long, bead-like chains of cocci with a homogeneous cell morphology, yet treatment with **1** ($2\times \text{MIC} = 3.12 \mu\text{M}$) resulted in short chains of cells with heterogeneous morphology and size (Figure s2).

Insight into the mode of action of **1** came from chemical structure searching, structure–activity relationship (SAR) studies, and whole genome sequencing of spontaneous mutants. A number of cell-permeable anticancer agents contain an arylhydrazone moiety that is reported to chelate iron.^{25–27} Our previous SAR studies of **1** demonstrated that the 2-hydroxynaphthalenyl hydrazide portion of the molecule is essential for its biological activity (Figure 1a, region highlighted in red).²⁸ Whole genome sequencing of *C. crescentus* spontaneous mutants resistant to 1× MIC of **1** revealed mutations mapping to the gene encoding the putative TonB- dependent hemin receptor (CC_2194), which is an outer membrane protein involved in iron uptake.²⁹

These observations led us to hypothesize that the antibacterial activity of **1** and its role in inhibiting cytokinesis arises from its function as an iron chelator. This hypothesis leads to several testable predictions; the following sections test these predictions, describe data supporting the hypothesis, and include a model explaining how **1** and other iron chelators arrest bacterial cytokinesis.

Iron, Cobalt, and Copper Antagonize the Biological Activity of **1**.

To verify the antagonistic effect of metal ions on the biological activity of **1**, we compared the growth of *E. coli* cells in medium containing **1** and various concentrations of metals. Compound **1** completely inhibited bacterial growth in all conditions tested, except when cobalt (100 μM), copper (100 μM), or iron (10 μM and 100 μM) was supplemented in the medium (Figure 2a). Cells treated with a mixture of **1** and 100 μM of CoCl_2 , FeCl_3 , or FeSO_4 grew similarly to cells treated with DMSO. The addition of 100 μM of CuSO_4 partially antagonized the biological activity of **1**, as the final absorbance of the culture reached ~40% of the absorbance measured for cells treated with DMSO.

We further measured the antagonist effect of CoCl_2 , CuSO_4 , FeCl_3 , and FeSO_4 on **1** by assessing the growth of *E. coli* in medium containing various concentrations of **1** (Figure 2a, inset heat map). In the absence of metal ions, cell growth inhibition required 12.5 μM of **1**. An increase in the concentration of metals was necessary to rescue growth of cells in increasing concentrations of **1**. A concentration of iron of 50 μM and higher was sufficient to support bacterial growth in the presence of 100 μM of **1** (8× MIC). We found similar results for cultures of *C. crescentus* (Figure s1b).

We confirmed the antagonistic effect of these specific metals in growth curve assays. As expected, only CoCl_2 , CuSO_4 , FeCl_3 , and FeSO_4 antagonized the inhibitory effect of **1** and enabled *E. coli* cells to grow in medium containing **1** when the metal ions were added immediately following drug treatment (Figure s3a). Similarly, bacterial growth and division was rescued when cobalt, copper, or iron was added after cells had been treated with **1** for 16 h (Figure s3b and c). These results suggest that **1** chelates cobalt, copper, and iron ions preferentially.

Compound **1** binds Fe^{3+} and Triggers the Iron Starvation Response in *E. coli*.

We used molecular modeling to predict the structure and binding affinities of different metal–**1** complexes, as the solubility limits of **1** made it difficult to measure binding constants.

Our model reveals two **1** molecules complexed to Fe³⁺ through an octahedral geometry arising from three coordination sites on each ligand (Figure 2b). The coordination sites map to the 2-hydroxynaphthalenyl hydrazide region of **1**, which is in agreement with our previous SAR studies.²⁸ The predicted binding affinity of **1** for Fe³⁺ ($G = -35.7$ kcal mol⁻¹) is preferred over binding to Fe²⁺ (-3.8 kcal mol⁻¹), Co²⁺ (-5.9 kcal mol⁻¹; octahedral coordination sphere), and Cu²⁺ (-9.5 kcal mol⁻¹; square planar configuration). As a point of comparison, we modeled the complexes of these metals bound to the chelator 2,2'-bipyridine (bipy, **2**), which predicted three **2** molecules complexed to Fe²⁺, Fe³⁺, Co²⁺, or Cu²⁺ in a conserved octahedral coordination sphere (Figure 2b). The models reveal that **1** is more selective for binding to Fe³⁺ than to other metal ions (compared to **2**), making it a potentially more useful probe for studying iron starvation in bacteria. Details of binding calculation and justification for this technique are included in the Supporting Information.

The ferric uptake regulator (Fur) binds Fe²⁺ and controls iron homeostasis in *E. coli* and many other Gram-negative bacteria.¹⁶ In *E. coli*, Fur regulates the iron-dependent expression of more than 90 genes.^{16,19–21} The classical view of Fur regulation in *E. coli* involves the binding of Fur-Fe²⁺ (holo-Fur) to the promoter region of Fur-regulated genes as a repressor. Iron-depleted conditions release repression by Fur and trigger the expression of genes involved in iron uptake. For example, the outer membrane ferric enterobactin receptor FepA is involved in the active transport of iron bound to enterobactin from the extracellular space into the periplasm and is transcriptionally repressed by Fur-Fe²⁺.

To determine whether bacterial cells treated with **1** display the phenotypic hallmarks of the iron starvation response, we constructed iron-responsive biosensors using GFP-promoter fusions and measured time-dependent GFP levels after treatment with **1**. *E. coli* cells harboring a plasmid in which the promoter region of the gene *fepA* regulates the expression of GFP (pUA66-*P_{fepA}*-*gfpmut2*) showed an increase (~105% increase compared to the DMSO control) in GFP expression when treated with **1** for ~6 h (Figure 2c and d). Similarly, we observed a ~72% increase in GFP expression following treatment with the intracellular iron chelator **2**. Addition of 100 μ M of FeSO₄ caused a ~54% reduction in GFP expression compared to the control. The repression observed for cells harboring pUA66-*P_{fepA}*-*gfpmut2* in medium supplemented with FeSO₄ was lost in *E. coli fur* cells (Figure 2c and d), demonstrating that this system is Fur-regulated. We observed similar results for cultures harboring a plasmid (pUA66-*P_{yncE}*-*gfpmut2*) in which the promoter region of the Fur-regulated gene *yncE* controls GFP expression (Figure s4). In both examples, *E. coli tolC fur* mutants produce more GFP in medium supplemented with DMSO or FeSO₄ when compared to *E. coli tolC*.

Other Iron Chelators Arrest Bacterial Cell Division Similarly to **1**.

We expected that cells treated with inhibitory concentrations of other iron chelators that are structurally unrelated to **1** would also arrest cell division. We found this to be the case for **2** (Figures 1b–e and s3d), a range of other small molecule chelators (Figures s5 and s6 and Supporting Information), and iron-depleted growth medium (Figure s7). The antagonistic effect of metals was not observed for other common antibiotics that target protein or peptidoglycan biosynthesis (Figure s8).

Iron Deprivation Affects General Biosynthesis of Macromolecules.

Bacteria have many iron-containing proteins that biosynthesize essential macromolecule precursors, including deoxyribonucleotides for DNA biosynthesis and isoprenoids involved in the biosynthesis of cell wall carbohydrate polymers (*e.g.*, peptidoglycan and teichoic acids). To investigate the consequences of iron starvation on the biosynthesis of macromolecules and establish a connection to arrested cell division, we measured the incorporation of radioactive precursors of peptidoglycan, DNA, RNA, and protein in *E. coli* following treatment with DMSO, **1**, or **2**. Both **1** and **2** blocked incorporation of all types of precursors within 1 h after treatment (Figure 3a), confirming the quick and pleiotropic effect of iron starvation on major biosynthetic processes. A large decrease (~90%) in incorporation of radiolabeled L-leucine following treatment with **1** suggests that protein biosynthesis is extensively inhibited. Compound **1** reduced peptidoglycan and RNA biosynthesis by ~40%, and DNA biosynthesis by ~15%; we also observed reduction of these processes using sub-MIC concentrations of **1** (Figure s9). Similarly, treatment with **2** reduced incorporation of all four families of precursors (Figure s9). To confirm a similar response in Gram-positive bacteria, we studied the biosynthesis of macromolecules in *B. subtilis* and found a ~35–50% reduction in precursor incorporation for all four processes following treatment with **1** (Figure s9).

We measured the ATP levels of *E. coli* cells to determine their metabolic activity and found a difference in ATP levels following 1 h of treatment with **1** ($P = 0.04$) or **2** ($P = 0.03$; Figure 3b); the small change observed (an ~5% reduction in the ATP levels in cells treated with either chelator) is unlikely to account for the division defect or the decrease in the incorporation of the four precursors that we observed. As a point of comparison, treatment of *E. coli* cells with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP)—a compound that disrupts transmembrane potential and reduces oxidative phosphorylation—caused an ~43% reduction in cellular ATP levels.

Iron Deprivation Inhibits Divisome Assembly and Reduces Peptidoglycan Synthesis at the Division Site.

The assembly of the bacterial divisome occurs in a sequential series of defined steps that requires multiple essential cytoplasmic, membrane, and periplasmic proteins.^{30,31} We hypothesized that the inhibition of cell division by **1** may arise from the mislocalization of the divisome or several of its key components. To study the effects of iron starvation on divisome assembly, we observed the spatiotemporal localization of the division machinery in *C. crescentus* cells treated with DMSO, **1**, or **2** for 6 h. Although we observe division inhibition as early as 1 h after treatment with a compound, we used 6 h for this experiment, as it corresponds to the approximate time required to observe division arrest in >60% of the cell population (Figure 1e). We used functional, fluorescently labeled versions of cell division proteins as a proxy for divisome localization and a selection of proteins (ZapA, FzIA, FtsE, MurG, FtsN, FtsK, FtsI, FtsW) that are recruited to the incipient division site at different stages in the process.³¹ Fluorescent fusions were located at the original loci on the chromosome under regulation of their native promoter.

Cells treated with DMSO displayed the characteristic localization pattern observed in other studies³¹ in which fluorescent foci accumulated at the incipient division site prior to cell septation (Figures 4 and s10). With one exception (FtsE), division protein localization was significantly perturbed in cells treated with **1** or **2**; in some cases (*e.g.*, FzlA, MurG, and FtsW), the fluorescent signal became diffuse over time. After treating cells with the iron chelators, ZapA, FtsN, FtsK, and FtsI were enriched at the division site; however, there was a significant reduction in the fluorescent signal (Figure 4). These results demonstrate that iron chelators affect the assembly and localization of the divisome, providing an explanation for arrested cytokinesis.

We also analyzed the synthesis and remodeling of septal peptidoglycan following treatment with **1** and **2** to determine whether it was inhibited and therefore the responsible factor for arresting cytokinesis. We studied synthesis of nascent peptidoglycan by *in vivo* pulse labeling using 7-hydroxycoumarin-3-carboxylic acid-3-amino-D-alanine (HADA).³² *C. crescentus* cells treated with DMSO for 6 or 16 h displayed intense synthesis/remodeling of peptidoglycan at midcell in dividing cells (Figure 5). HADA-labeling of peptidoglycan at the division site was reduced in cells treated with **1** or **2** for 6 h and became diffuse after 16 h (Figure 5). The results indicate that peptidoglycan synthesis/remodeling is inhibited at the site of cell division.

Iron Deprivation Downregulates the Expression of Cell Division Proteins.

We performed a small target- multicopy suppression screen to identify potential molecular targets of **1** and understand the downstream consequences of iron deprivation on cell division. In this approach, the copy number of a protein target is increased, exceeds the toxicity of a compound, and suppresses growth inhibition. We selected 79 genes from a library of *E. coli* clones harboring a complete set of operational reading frames on a multicopy plasmid vector.³³ A total of 24 of the selected genes encode proteins involved in cell division, cell-cycle regulation, or envelope biogenesis, and the remaining 55 genes encode proteins involved in iron metabolism (*e.g.*, transcription factors and iron transporters) or iron-containing proteins. Overexpression of some genes from each group partially suppressed the biological activity of **1** and enabled cell growth and division (Figure 6a, right panel). For example, overexpression of genes encoding “late” cell division proteins (*e.g.*, FtsK, FtsQ, FtsW, FtsN) partially antagonized the inhibitory effect of **1**. Of the genes involved in iron metabolism, overexpression of *fecA* (gene encoding the outer membrane component of the ferric dicitrate uptake system), *fecC* (gene encoding a permease component of the ferric dicitrate uptake system), and *fhuA* (gene encoding the outer membrane protein receptor for the ferric hydroxamate uptake system) supported more bacterial growth when compared to the empty vector control (>10% growth compared to the empty vector control). Overexpression of some of the selected genes seems to be toxic in the conditions tested.

Some iron chelators of the pyridoxal isonicotinoyl hydrazone class downregulate the expression of proteins that control cell-cycle progression in eukaryotes.²⁷ Because the antagonistic effect of **1** can be partially suppressed by individual over-expression of genes encoding some of the proteins involved in cell division, we hypothesized that **1** could

similarly affect expression of a set of bacterial genes encoding proteins necessary for cell division. We used quantitative reverse transcription PCR (qRT-PCR) to assess changes in transcription of *ftsZ*, *ftsQ*, and *ftsW* in *E. coli* cells grown under iron-replete or iron-depleted conditions. We observed a small but significant ($P = 0.05$) decrease in transcription of *ftsZ* (~2.5-fold), *ftsQ* (~2-fold decrease), and *ftsW* (~2-fold decrease) in cells treated with **1** or **2** compared to cells grown in iron-replete conditions (Figure 6b). For further comparison, we measured the transcription of the iron- deprivation-induced genes *entC* and *fepA*.^{16,34} Both genes were highly transcribed (~20-fold increase) in cells treated with **1** or **2**, suggesting that the decrease in RNA levels observed for the genes encoding cell division proteins is not the consequence of a general impact of transcription by sequestration of iron.

DISCUSSION

Bacterial cells adapt to environmental changes rapidly and have evolved multiple mechanisms to regulate cell division in response to environmental cues.³⁵ Using the small molecule iron chelator **1**—and other structurally unrelated chelators—we demonstrate that perturbations in iron homeostasis directly affect the cell division regulatory network. Surprisingly, the outcome does not arise through a scenario in which one might anticipate cell metabolism grinding to a halt in the absence of iron; the result is much more nuanced and interesting. We found that depletion of iron arrests a very late stage in bacterial cell division by affecting the structure and localization of a functional divisome.

Compound **1** is selective for Co^{2+} , Cu^{2+} , and $\text{Fe}^{2+}/\text{Fe}^{3+}$ over abundant cellular alkali (Na^+ and K^+), alkaline earth (Mg^{2+} and Ca^{2+}), and other biologically relevant metals (Mn^{2+} , Ni^{2+} , and Zn^{2+}). This observation corroborates previous findings that anticancer cells containing an arylhydrazine moiety have a high affinity for Fe^{3+} in addition to a range of other metals.³⁶ Unable to solve a crystal structure of the **1**- Fe^{3+} complex, we used molecular modeling to predict a 2:1 ligand/iron (Fe^{2+} or Fe^{3+}) complex. In addition to chelating extracellular Fe^{3+} , evidence from other studies with salicylaldehyde isonicotinoyl hydrazone (SIH), a structural analog of **1**, supports internalization of **1** into cells and chelation of intracellular iron.³⁷

Iron homeostasis in bacteria is maintained through a balance of iron acquisition, storage, and incorporation into proteins as cofactors. These iron-containing proteins participate in a variety of metabolic and developmental processes.^{15,16} The transcription factor Fur regulates iron-dependent expression of numerous genes to maintain a precise concentration of the cytoplasmic levels of iron and prevent iron-induced toxicity. A recent reconstruction of the Fur regulatory network in *E. coli* shows that Fur may coordinate cellular processes beyond its canonical role in iron metabolism.²⁰ We envision a model in which **1** and other iron chelators (*e.g.*, **2**) deplete the intracellular iron pool, downregulate genes encoding proteins involved in cell division, block biosynthesis of the corresponding proteins, prevent assembly/localization of the complete divisome, and inhibit late stages of cell division (Figure 7). Our model posits a direct effect of iron starvation on the division machinery as opposed to indirect cellular effects propagated through general metabolic constraints and energy depletion. As divisome assembly is a quasi-stepwise process and is dependent on the concentration and position of proteins, in principle, a reduction in the concentration of a single family of division proteins is sufficient to alter the position of other proteins or the

divisome. However, our model does not exclude the possibility that the division arrest results from a combination of additional effects of iron starvation on other cellular processes.

The direct effect of iron depletion on key regulators of the eukaryotic cell cycle involves multiple molecular targets, including ribonucleotide reductase, cyclins, and cyclin-dependent kinases (cdks).^{27,38} Iron depletion in eukaryotic cells caused by chemically diverse iron chelators—including a compound (referred to as 311) containing an arylhydrazine—decreases the protein level of cyclins and cdks.^{27,39,40} Treating eukaryotic cells with a complex consisting of compound 311 bound to iron prevented the negative effect of this small molecule on cyclin expression, supporting the connection between iron availability and expression of cell-cycle regulators.²⁷ Compounds 1 and 2 downregulate the transcription of cell division genes, while upregulating the transcription of genes involved in iron acquisition (Figure 6b), and reduce protein biosynthesis by ~90% in *E. coli* (Figure 3a), suggesting an analogous effect—to eukaryotes—of iron chelators on bacterial gene expression. Importantly, *E. coli* cells treated with protein biosynthesis inhibitors (*e.g.*, chloramphenicol, tetracycline, kanamycin)²³ do not display the characteristic division defect observed in cells treated with iron chelators, suggesting that division arrest promoted by iron chelators does not arise from a general collapse in protein biosynthesis. Instead, our results support the presence of an iron-dependent checkpoint on bacterial division.

Cellular stress has an important role in regulating the bacterial cell cycle by influencing the assembly and activity of the divisome.^{35,41} Some types of metabolic stress, such as carbon starvation and prolonged cultivation in the stationary phase, have been linked to a decrease in the level of FtsZ and consequent cell filamentation in *C. crescentus*.^{42,43} Similarly, controlled inactivation of the master cell-cycle regulator CtrA in *C. crescentus* during NaCl and ethanol stress conditions results in downregulation of the cell division genes *ftsA*, *ftsQ*, and *ftsW*, blocking cell division and causing cell filamentation.⁴¹ We did not observe filamentation in cells treated with **1** or **2** for up to 12–16 h (Figure 1d and e), suggesting that the decrease in expression of cell division genes may be insufficient to trigger molecular events that activate filamentation or may avoid filamentation by design, in which division is inhibited and cell growth is reduced. Recent transcriptional network analysis in cyanobacteria indicates that the late division protein *ftsW* is downregulated under iron starvation conditions.⁴⁴

E. coli or *C. crescentus* cells incubated in culture media supplemented with iron grow in the presence of concentrations of **1** or **2** that would be inhibitory otherwise (Figures 2a, s1b, s3a, and s3b). We performed time-lapse experiments to explore how an increase in iron availability following long-term exposure to **1** or **2** affects division in arrested cells. Surprisingly, we found a subpopulation of cells that is unable to resume growth and divide even after 2 h of recovery in iron-replete conditions (Figure s11). Interestingly, a subpopulation of cells starts dividing at a new site in the cell quartile and never complete constriction at the primary, arrested division site at midcell. These results suggest that the cellular damage caused by long-term iron deprivation may alter the division machinery and prevent its reactivation.

The antibacterial potential of small molecule iron chelators has been long acknowledged;^{45,46} however, clinical applications of metal chelators are limited to the treatment of diseases that require removing metals from the body (*e.g.*, iron overload and heavy metal toxicity).⁴⁷ We have previously demonstrated the antibacterial activity of **1** and its chemical analogs against various clinical strains of pathogenic bacteria, including *Acinetobacter baumannii*, *Enterobacter aerogenes*, *Salmonella enterica enterica* serovar Typhimurium, *Shigella boydii*, and *Vibrio cholerae*.^{22,28} Iron is essential in a variety of biological processes in bacteria, and consequently the activity of **1** and other antimicrobial iron chelators arises from affecting multiple downstream targets. The ability of iron chelators to interfere with the activity of multiple cellular targets can reduce the frequency at which bacteria develop resistance; this point is supported by our challenge isolating **1**-resistant strains of *E. coli* and *C. crescentus* through a variety of different approaches (*e.g.*, through multiple passaging experiments). Recent studies have proposed the therapeutic use of metal chelators in combination with other drugs as an adjuvant to overcome antibiotic resistance.^{46,48}

CONCLUSIONS

We demonstrate that **1** and other structurally unrelated iron chelators perturb bacterial iron homeostasis and arrest late stages of cell division by preventing the formation of a functional divisome. The observation that cells that are able to engage in division become arrested at a specific point in the process (*i.e.*, during late stages of cytokinesis) suggests the existence of iron-dependent cellular checkpoints in bacterial cell division. These studies provide insight into mechanisms by which iron homeostasis and cell division are coordinated in bacteria and will guide future experiments testing whether disruption of iron homeostasis may be a viable target for development of new families of antibacterial agents.

METHODS

Bacterial Strains and Growth Conditions.

Unless stated otherwise, *E. coli* strains were routinely grown aerobically in M8 minimal medium (1× M8 salts [8.5 mM NaCl, 22 mM KH₂PO₄, and 48 mM Na₂HPO₄·7H₂O], supplemented with 2 mM MgSO₄, 0.4% [w/v] glucose, and 0.5% [w/v] casamino acids) or in M8 minimal medium containing 1.5% (w/v) agar at 37 °C. *C. crescentus* strains were routinely grown aerobically in peptone-yeast extract (PYE) broth (0.2% [w/v] peptone, 0.1% [w/v] yeast extract, 0.8 mM MgSO₄, and 0.5 mM CaCl₂) or in PYE broth containing 1.5% (w/v) agar at 30 °C. *B. subtilis* 168 was routinely grown aerobically in Belitsky minimal medium (BMM; 8 mM MgSO₄, 27 mM KCl, 7 mM sodium citrate, 50 mM Tris-HCl (pH 7.5), 2 mM CaCl₂, 0.6 mM KH₂PO₄, 15 mM (NH₄)₂SO₄, 10 μM MnSO₄, 1 μM FeSO₄, 0.5% [w/v] glucose, 250 μM thymine, and 0.025% [w/v] of each of the 20 standard L-amino acids) at 37 °C. *S. pyogenes* ATCC 12344 was routinely grown aerobically in brain–heart infusion (BHI) broth (BD Difco) at 37 °C. We added ampicillin (50 μg mL⁻¹), chloramphenicol (25 μg mL⁻¹), kanamycin (30 μg mL⁻¹ for *E. coli*; 5 μg mL⁻¹ for *C. crescentus*), spectinomycin (25 μg mL⁻¹), or streptomycin (2.5 μg mL⁻¹) to the liquid growth medium or agar as needed. For some experiments, culture medium was treated with the transition-metal-chelating resin Chelex 100 (BioRad). Briefly, culture medium

containing 5% (w/v) Chelex 100 was incubated in a rotating platform for 1 h, centrifuged for 10 min, and filtered through a 0.20 μm filter.

Metal Ion Screen.

Cultures of *E. coli* BW25113 *tolC* were grown overnight in M8 minimal medium for 12 h at 37 °C with agitation (200 rpm), diluted (1:100) in fresh medium, incubated at 37 °C with agitation (200 rpm), and calibrated to an absorbance of 1.0 ($\lambda = 600$ nm). Cultures of *C. crescentus* CB15N were grown in PYE broth for 24 h at 30 °C with agitation (200 rpm) and calibrated to an absorbance of 1.0 ($\lambda = 600$ nm). Calibrated cultures were diluted (1:10,000) in fresh media and used as inoculum in the metal screen and growth curve experiments. To identify metals that antagonize the inhibitory properties of **1**, we inoculated a suspension of *E. coli* cells in 200 μL of M8 minimal medium containing 25 μM ($2\times$ MIC) of **1** and various concentrations of metals. We measured the initial absorbance ($\lambda = 600$ nm) of the *E. coli* cell suspensions at 25 °C in a Tecan Infinite M1000 microplate reader (Tecan). The 96-well plate was incubated at 37 °C with agitation (200 rpm) and the final absorbance ($\lambda = 600$ nm) was measured after 16 h. Control experiments to verify culture viability and the inhibitory effects of **1** were performed in parallel. We calculated bacterial growth in each condition as a percentage relative to the growth of cells in a medium containing 100 μM of the tested metal (higher concentration tested) without **1**. To study the antagonistic relationship between the metals of interest and **1**, we inoculated a cell suspension of *E. coli* or *C. crescentus* cells in 200 μL of M8 minimal medium or PYE broth, respectively, containing various concentrations of **1** and the following metals: $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$, $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$, or $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$. We measured the initial absorbance ($\lambda = 600$ nm) of the cell suspensions at 25 °C, incubated the 96-well plate with agitation (200 rpm), and measured the final absorbance ($\lambda = 600$ nm) after 16 h (at 37 °C for *E. coli*) or 24 h (at 30 °C for *C. crescentus*). We performed control experiments to verify culture viability and metal toxicity.

Microscopy and Image Analysis.

To visualize the phenotypic alterations in bacterial cells treated with inhibitory concentrations of the tested compounds, cultures of *E. coli* BW25113 *tolC* or *C. crescentus* CB15N were grown overnight in M8 minimal medium or PYE broth, respectively, for 12 h at 37 °C with agitation (200 rpm), diluted (1:100) in fresh M8 minimal medium, and incubated at 37 °C with agitation (200 rpm) to an absorbance of 0.1 ($\lambda = 600$ nm). *S. pyogenes* cultures were grown overnight for 12 h at 37 °C in BHI broth and calibrated to an absorbance of 0.1 ($\lambda = 600$ nm). We treated the cultures with DMSO or the test compounds, incubated the cultures at adequate conditions for different amounts of time, and imaged the cells as described below.

To study the spatiotemporal localization of cell division proteins, we used *C. crescentus* strains containing functional, fluorescently labeled versions of cell division proteins (Table S1). Cells were grown overnight in PYE broth, diluted (1:10) in fresh PYE broth, and incubated at 30 °C with agitation (200 rpm) to an absorbance of 0.1 ($\lambda = 600$ nm). We treated 2 mL aliquots of the culture with DMSO and $2\times$ MIC of **1** (25 μM) or **2** (400 μM) and incubated the cultures for 6 or 16 h at 30 °C with agitation (200 rpm).

To image cells, we added 4- μ L aliquots of the culture on 2% (w/v) agarose pads prepared in 1 $\times$ PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4), for *E. coli* and *S. pyogenes*, or 1 $\times$ M2 salts (6.1 mM Na₂HPO₄, 3.9 mM KH₂PO₄, 9.3 mM NH₄Cl, pH 7.0), for *C. crescentus*, and imaged the cells at 25 °C on an inverted Nikon Eclipse Ti microscope equipped with a Photometrics CoolSNAP HQ2 charge-coupled-device (CCD) camera (Photometrics). Images were acquired using a 100 $\times$ objective (Nikon Plan Apo 100 $\times$ /1.40 oil Ph3 DM) and the Nikon Instruments Software (NIS)-Elements Advanced Research (AR) microscope imaging software program (Version 4.000.07; Nikon Inc.). Data were collected on the CCD camera using an exposure time of 50 ms; for fluorescent images, we used an exposure time of 50 ms or 2 s. To visualize data, we overlaid phase contrast images and the corresponding fluorescence images and false-colored them using ImageJ (version 1.48); no further adjustments or other image processing was performed. To quantify cell length and study the localization of the labeled proteins, we created cell length profiles and fluorescence intensity profiles of cells using Oufiti.⁴⁹ We verified automated cell detection by manual inspection.

Statistical Analysis.

Statistical analyses were performed using the computing environment R (version 3.2.0),⁵⁰ and *P* values <0.05 were considered significant. Refer to the figure legends for details about specific analysis performed for each experiment.

Other details about the materials and procedures used in this study can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We acknowledge materials from E. Goley and M. Thanbichler. This research was supported by the National Institutes of Health (1DP2OD008735, D.W.; R01-GM106443, Q.C.), National Science Foundation (DMR-1121288, D.W.), and the Human Frontier Science Program (RGY0076/2013, D.W.). The flow cytometry instrumentation facility is supported by the University of Wisconsin Carbone Cancer Center Support (Grant P30 CA014520). T.S. thanks the Louis and Elsa Thomsen Wisconsin Distinguished Graduate Fellowship Award.

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Figure 1.

Compounds **1** and **2** inhibit late stages of bacterial cytokinesis. (a) Chemical structure of **1** and **2**. Regions highlighted in red are essential for the biological activity of **1**.²⁸ (b) Microscopy images of *E. coli* BW25113 $\Delta tolC$ treated DMSO, 2 $\times$ MIC of **1** or **2** for 16 h. (c) Scatter plots displaying the area and aspect ratio of *E. coli* BW25113 $\Delta tolC$ treated with DMSO, 2 $\times$ MIC of **1** or **2** for 16 h. Inset: A probability density histogram of the cell length distribution of the population gated in region II. (d) Probability density histograms of the cell length distributions of *E. coli* BW25113 $\Delta tolC$ treated with DMSO, 2 $\times$ MIC of **1** or **2**

for 16 h. Red dashed line represents the kernel density estimation (KDE) of the cell length distribution for the DMSO-treated population. In (c) and (d), shaded blue area overlaying the histogram corresponds to the KDE of cell length distribution. Average cell length (L) is given with standard deviations for a total of n cells. Statistical significance for multiple comparisons was determined using a Kruskal–Wallis test with Dunn’s posthoc test. (e) Time-course experiments showing the morphological changes and the KDE of cell length distribution of *C. crescentus* CB15N treated with DMSO, 2× MIC of **1** or **2**. Average cell length (L) is given with standard deviations for a total of n cells.

Figure 2.

Compound **1** binds iron with high affinity and triggers the iron-starvation response in bacteria. (a) Growth of *E. coli* BW25113 *tolC* in the presence of $2 \times \text{MIC}$ (25 μ M) of **1** and various metals. Bars show the percentage of growth compared to cells treated with DMSO and the highest concentration of the respective metal. Inset: Checkerboard growth assays of the effect of cobalt, copper, and iron in combination with **1** on the growth of *E. coli* BW25113 *tolC*. Dark colored squares to white squares represent full growth and complete growth inhibition, respectively. Analyses represent the combination of two independent experiments. (b) Optimized structures and calculated binding affinity of **1** and **2** complexed with copper, cobalt, or iron. (c) Ratio of GFP fluorescence intensity and absorbance of *E. coli* BW25113 *tolC* or *E. coli* BW25113 *tolC fur* harboring the plasmid pUA66-P_{fepA}-gfpmut2 following culturing in M8 minimum medium supplemented with DMSO, **1** (6.25 μ M), **2** (100 μ M), or FeSO₄ (100 μ M). Analyses are the average of two independent

experiments. Error bars indicate the standard deviation. Inset: A cartoon depicting the classical view of Fur regulation in *E. coli* (summarized in the text). We performed one-sample Student's *t* tests to test the null hypothesis that the GFP fluorescence intensity mean is equal to 100 (the normalized signal for the DMSO control). ns, nonsignificant; **P* < 0.05; ***P* < 0.01 compared to the DMSO control treatment. (d) Representative microscopy images of *E. coli* BW25113 *tolC* or *E. coli* BW25113 *tolC fur* harboring the plasmid pUA66-*P_{fepA}-gfpmut2* and treated with **1** (6.25 μ M) or FeSO₄ (100 μ M) for 20 h.

Figure 3.

Iron deprivation affects biosynthesis of macromolecules and metabolic activity in bacteria.

(a) Incorporation of ^3H -glucosamine (peptidoglycan), ^3H -thymidine (DNA), ^3H -uridine (RNA), or ^3H -leucine (protein) in *E. coli* BW25113 *tolC* treated with DMSO, 2× MIC of **1** (25 μM), **2** (400 μM), or control drugs. Analyses are the average of three independent experiments. Error bars indicate the standard deviation. Results are expressed as percentage of labeled precursor incorporation compared to the DMSO control (100% incorporation). (b) Measurement of ATP level in *E. coli* BW25113 *tolC* treated with 2× MIC of **1** or **2**. CCCP (2× MIC, 25 μM) was used as a control drug. Analyses are the average of three independent experiments. Error bars indicate the standard deviation. Results are expressed as percentage compared to the DMSO control (100% incorporation). We performed one-sample Student's *t* tests to test the null hypothesis that the tritium-labeled metabolic precursor incorporation or the ATP level intensity mean is equal to 100 (the normalized incorporation or ATP level for the DMSO control). ns, nonsignificant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001 compared to the DMSO control.

Figure 4.

Iron deprivation affects localization of the divisome. Representative fluorescence microscopy images depicting the localization pattern of various cell division proteins in *C. crescentus* CB15N grown for 6 h at 30 °C in PYE broth containing DMSO and 2× MIC of **1** (25 μ M) or **2** (400 μ M). Right: Comparative fluorescence intensity profile of the subcellular distribution of cell division proteins for each treatment tested. a.u., arbitrary units. For this analysis, we only compared cells undergoing division. Analyses are the average of two independent experiments.

Figure 5.

Iron deprivation reduces peptidoglycan incorporation in bacteria. (a) Left panel: Demographs representing normalized signal profiles of HADA incorporation in *C. crescentus* CB15N arranged by increasing cell length. Cells were grown for 6 h at 30 °C in PYE broth containing DMSO and 2× MIC of 1 (25 μM) or 2 (400 μM). Right panel: Representative fluorescence microscopy images depicting the localization pattern of HADA labeling. Bottom panel: Fluorescence intensity profile of the subcellular distribution of HADA. a.u., arbitrary units. For this analysis, we only compared cells undergoing division. (b) Same as part (a) for *C. crescentus* CB15N grown for 16 h. Analyses are the average of two independent experiments.

Figure 6.

Iron deprivation downregulates the expression of cell division genes. (a) Heat map depicting *E. coli* growth in a multicopy suppressor screen. Growth was calculated as the percentage of the absorbance of the **1**-treated culture compared to the same culture treated with DMSO. The measurements are the average of five independent experiments. Squares colored blue and yellow represent full cell growth and complete growth inhibition, respectively. Right: Enhanced view of growth after 48 h of strains overexpressing selected genes. Overexpression of these genes resulted in >10% increase in growth compared to the empty

vector control. (b) Relative gene expression in *E. coli* BW25113 *tolC* treated with 2× MIC of **1** (25 μ M) or **2** (400 μ M). Analyses are the average of four independent experiments. Error bars indicate the standard deviation. We performed one-sample Student's *t* tests to test the null hypothesis that the log₁₀- fold-change mean is equal to 0 (the normalized gene expression for cells treated with FeSO₄). **P* < 0.05; ***P* < 0.01; ****P* < 0.001 compared to the cells treated with FeSO₄.

Figure 7.

Iron deprivation inhibits bacterial cell division. Iron homeostasis in bacteria results from a balance between iron acquisition, storage, and incorporation into proteins. Iron-containing proteins play a major role in a variety of metabolic and developmental processes. Transcription factors (*e.g.*, Fur in *E. coli* and other Gram-negative bacteria) regulate gene expression to maintain precise control of the cytoplasmic levels of iron and prevent iron-induced toxicity. Compound **1** and other iron chelators disrupt this intricate network by depleting the iron pool, interrupting biosynthesis of macromolecules, and preventing assembly of a functional divisome during cell division.