Novel Inhibitors of Bacterial Cytokinesis Identified by a Cell-based Antibiotic Screening Assay*

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The continuous emergence of antibiotic resistance demands that novel classes of antibiotics continue to be developed. The division machinery of bacteria is an attractive target because it comprises seven or more essential proteins that are conserved almost throughout the bacteria but are absent from humans. We describe the development of a cell-based assay for inhibitors of cell division and its use to isolate a new inhibitor of FtsZ protein, a key player in the division machinery. Biochemical, cytological, and genetic data are presented that demonstrate that FtsZ is the specific target for the compound. We also describe the effects of more potent analogues of the original hit compound that act on important pathogens, again at the level of cell division. The assay and the compounds have the potential to provide novel antibiotics with no pool of pre-existing resistance. They have provided new insight into cytokinesis in bacteria and offer important reagents for further studies of the cell division machinery.

Cell division has been of considerable interest as an antibacterial target because it comprises a group of well conserved proteins that are all essential for the viability of a wide range of bacteria, and their activities are completely different from those of the proteins involved in the division of mammalian cells. A number of compounds that act on components of the cell division machinery have been described (1–7). So far, most of the effort has been directed at the FtsZ protein because it has several biochemical activities that can be assayed in vitro. Here we describe a novel approach to the discovery of inhibitors of bacterial cell division using a cell-based reporter assay. We have used the assay to identify a novel class of antibacterial compounds with potential broad-spectrum activity. We show that the compounds act on the highly conserved, essential cell division protein FtsZ, in vitro and in vivo. These compounds represent a potential class of new antibiotics that act by a different mechanism than any of the antibiotics currently in clinical use.

EXPERIMENTAL PROCEDURES

Bacterial Strains—The bacterial strains used in this work are: Bacillus subtilis 168 (trpC2); B. subtilis 1801 (trpC2 chr:pISIZΩble(pBssc-ftsZ b/e)); B. subtilis 2020 (trpC2 Ω[amyE::spe Ppar–gfp–ftsZ]); B. subtilis PL16 (trpC2 Ω[amyE::spolIQ-gus neo] Ω110549 [spolA-lacZ cat]; Enterococcus faecalis ATCC 29212; Escherichia coli ATCC 25922; Haemophilus influenzae ATCC 49247; Moraxella catarrhalis ATCC 25240; Pseudomonas aeruginosa 101021; Staphylococcus aureus ATCC 601055; Streptococcus pneumoniae ATCC 49619.

Molecular Cloning—B. subtilis was transformed by the method described by Anagnostopoulos and Spizizen (8) as modified by Jenkinson (9) or the method described by Kunst and Rapoport (10), except that 20 min after the addition of DNA the transformed cultures were supplemented with 0.66% casamino acids solution. Transformants were selected on Oxoid nutrient agar containing chloramphenicol (5 μg/ml). Sporulation was induced by growth in a hydrolyzed casein medium followed by resuspension in a starvation medium. Starvation medium was as described by Karamata and Gross (11). DNA manipulations and E. coli transformations were carried out as described by Sambrook et al. (12). All cloning was done in E. coli DH5α (Invitrogen).

Cell Division Dual Reporter Assay—B. subtilis PL16 was grown in hydrolyzed casein medium to exponential phase and centrifuged, and cell pellets were frozen at ~80 °C. Frozen cell aliquots of strain PL16 were resuspended in warm starvation medium, incubated with shaking at 37 °C for 50 min (t50), and then added to Greiner 96-well microtiter plates containing compounds and controls (novobiocin, cephalaxin, and 2% Me2SO). Plates were incubated at 37 °C shaking for another 100 min (t150). Assay buffer, containing lysozyme, 4-methyl-umbelliferyl β-D-galactoside, resorufin β-D-glucuronide, and Triton X-100, was added, and after a 60-min incubation at room temperature the fluorescence was measured on a BMG Fluostar Galaxy. Fluorescence was expressed in arbitrary fluorescence units (AFU). Percentage ratio was calculated using the following formula.

\[
\%\text{Ratio} = \frac{\text{AFU}_{(\text{LacZ/Gus Me2SO})}}{\text{AFU}_{(\text{LacZ/Gus compound})}} \times 100
\]

(Eq. 1)

Cell Division Phenotype Assays—Overnight cultures were grown in starvation medium supplemented with 1% hydrolyzed casein and then diluted in starvation medium supplemented with 3% hydrolyzed casein (B. subtilis) or in Mueller-Hinton medium (E. faecalis, S. aureus) and grown at 37 °C. The culture was diluted to A600 of ~0.06, and 10-μl aliquots were added to transparent 96-well microtiter plates (BD Biosciences Falcon) containing dilutions of compounds in 100-μl volumes of medium. Concentrations of Me2SO, cephalaxin, and 3-methoxybenzamidine were included as controls. To examine the effects of ftsZ overexpression, B. subtilis 1801 was grown in the same medium, supplemented with 0.05–2 mM isopropyl β-D-thiogalactopyranoside (IPTG).

The abbreviations used are: AFU, arbitrary fluorescence units; MIC, minimum inhibitory concentration; MOPS, 4-morpholinopropanesulfonic acid; GFP, green fluorescent protein; IPTG, isopropyl β-D-thiogalactopyranoside.

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After incubation for approximately 5 h (4–5 generations) at 37 °C, 20-μl culture samples were transferred to poly-l-lysine-coated slides for microscopy. Cell morphology was assessed by phase-contrast light and fluorescence microscopy on a Zeiss Axiovert 200 M inverted microscope equipped with a Sony CoolSnap HQ cooled charge-coupled device camera (Roper Scientific) and using Metamorph v6.1 software. Cell length and diameter measurements were determined using Metamorph software.

Minimum Inhibitory Concentration (MIC) Testing—MICs for compounds against each strain were determined by a broth microdilution method according to the National Committee for Clinical Laboratory Standards (now the Clinical Laboratory Standards Institute) guidelines, for which the MIC was defined as the lowest concentration inhibiting visible growth. Absorbance (A₄₀₀) readings were also used to calculate a percentage value for each compound using average A₄₀₀ values for growth and no growth controls. Inhibition was characterized as a reduction in absorbance of ≥85% relative to the controls for all bacterial species, with the exception of S. pneumoniae (≥65%).

GFP-FtsZ Localization Experiments—A colony of B. subtilis strain 2020 was resuspended in Oxoid antibiotic medium no. 3 (Pennisaybroth) supplemented with 0.05% xylose and grown at 37 °C to A₄₀₀ ~ 0.6. Samples of the culture were mixed with an equal volume of medium containing twice the final desired concentration of compound. Ten-microliter volumes were harvested at appropriate time intervals, combined with an equal volume of prewarmed Pennassay broth containing 1% w/v agarose, and 10-μl samples of these were pipetted onto microscope slides, covered, and visualized as soon as possible. Fluorescein isothiocyanate or enhanced GFP settings were used to capture fluorescent images, with a 1500-ms exposure time and binning set to 1.

Resistance Frequencies—To determine the resistance frequency B. subtilis 168 cells were spread on Mueller-Hinton agar (10⁻⁵-10⁷ colony-forming units/plate) containing compound at 1x, 1.5x, 2x, and 4x the MIC. To determine the number of viable cells in the inoculum, dilutions of the culture were plated on compound-free Mueller-Hinton agar. Compound plates were incubated for up to 1 week to allow resistant mutants to grow. By dividing the number of resistant colonies on these compound plates by the number of colony-forming units originally plated the spontaneous resistance frequency was calculated.

Characterization of PC58338-resistant Mutants—The fisZ gene was PCR-amplified from chromosomal DNA of B. subtilis 168 and resistant mutants using oligo ftsZ.fw1 (5’-AAACCTCGAGCGCAACATAATAAACCAGGC-3’) and ftsZ.rv1 (5’-CTGAATTTCTGGTGTACATC-TAACCGAAGGAGG-3’). Two separately amplified PCR products were sequenced using the PerkinElmer Life Sciences AIB PRISM BigDye terminator cycle sequencing kit. ftsZ.fw1 and ftsZ.rv1 were used as primers, and gels were run on an ABI PRISM 377 DNA sequencer (Sir William Dunn School of Pathology Sequencing Service, Oxford, UK). PCR products of wild-type and mutant ftsZ alleles were digested and ligated into pJP1 and pSG1301. Ligated plasmids were transformed into B. subtilis 1801 or B. subtilis 168 with selection for chloramphenicol resistance in the presence of 0.2% xylose as necessary.

FtsZ Sedimentation Assay—B. subtilis FtsZ protein and sedimentation assays were prepared generally as described elsewhere (13). Samples of protein were prespun at 25 p.s.i. (127,000 × g) for 20 min in an Airfuge (Beckman) to remove pre-existing polymers and aggregates. FtsZ was diluted to 5 μM final concentration in polymerization buffer containing M₆SO or compound at the indicated concentration. GTP was added to a final concentration of 1 mM followed by 0.1 mg/ml DEAE-dextran hydrochloride. Total reaction volumes were 100 μl. Reactions were incubated at 37 °C for 10 min, and 90-μl samples were centrifuged at 22 p.s.i. (119 000 × g) for 10 min to collect polymers. Supernatants were carefully removed and combined with sample loading buffer. Pellets were resuspended in 100-μl volumes of sample loading buffer. Samples were boiled for 5 min and resolved by 12% PAGE. Gels were stained with Coomassie Brilliant Blue, dried, and scanned.

FtsZ GTPase Assay—Conversion of GTP to GDP by FtsZ was measured by monitoring the release of phosphate using malachite green dye in an end point enzyme assay. Reactions contained 5 μM FtsZ and compound at the concentrations indicated in reaction buffer (50 mM MOPS, pH 7.2, 5 mM MgCl₂, 200 mM KCl). Reactions were started by the addition of 2 mM GTP and incubated at 37 °C for 10 min, and multiple samples (10 μl) were added to 100-μl volume of malachite green dye. After 6 min the A₄₀₀ was measured using a Tecan Spectrofluor. E. coli FtsZ protein was purchased from Bioquote Limited.

RESULTS

A Cell-based Screening Assay Utilizing the Dependence of σF Activation during Sporulation on Cell Division—A cell-based assay would require the use of one or more elements that are responsive to specific inhibition of cell division. Surprisingly, E. coli (14) and B. subtilis, at least, do not appear to have any checkpoint system that alters gene expression in response to inhibition of division. However, we and others (reviewed in Ref. 15) have shown previously that during sporulation of B. subtilis, activation of the σF transcription factor that initiates gene expression in the prespore compartment is dependent on formation of the asymmetric sporulation septum (which generates the prespore and separates it from the mother cell). The formation of the sporulation division septum is dependent on all of the proteins that are known to be required for vegetative division (16). The nature of the mechanism that regulates σF in response to sporulation division is not known, but we reasoned that this dependence could be exploited in a screening assay for inhibitors of division. The basis of the assay is illustrated in Fig. 1. The assay relies on a strain of B. subtilis (PL16) bearing two reporter genes. The first measures activity of σF by a fusion of the strong, σF-dependent spolIQ promoter to a gus reporter (encoding β-glucuronidase). The second acts as a control for nonspecific inhibitors and is a fusion of the promoter of the locus encoding σF (spolIA) to lacZ (encoding β-galactosidase). The spolI promoter becomes active just before septation but is insensitive to septum formation. Expression of spolI is normally shut down soon after σF becomes active, so β-galactosidase accumulation is actually enhanced by inhibition of division (17). In the presence of a specific inhibitor of cell division, the activity of the spolI reporter gene should be reduced, whereas that of the spolIA reporter should be increased (Fig. 1B). Nonspecific inhibitors would eliminate the activity of both reporters (Fig. 1C). Measurement of the ratio of the two reporter genes should provide a robust means of identifying specific inhibitors of division.

We developed a microtiter plate assay for inhibition of cell division. The assay was tested for sensitivity and specificity using a panel of antibiotics, nonspecific inhibitors, and M₆SO (examples shown in Fig. 2). The optimized assay was evaluated by determining the Z-factor, and values for this parameter were typically in the range of 0.78 to 0.95 for both signals. This categorizes the quality of the assay as excellent (18). The specificity of active compounds was assessed by plotting the ratio of % inhibition. As positive controls we used cephalaxin, which is a β-lactam antibiotic that in E. coli, at least, has a high affinity for the septum-specific penicillin-binding protein PBP3 and at certain concentrations causes division inhibition (19), and 3-methoxybenzamide, which

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A hit compound, designated PC58538, which specifically inhibited cell division, was identified as a result of this process (Fig. 3A). Fig. 3B shows the original screening hit response to compound PC58538. The reporter ratio was clearly altered by this compound, compared with the low level fluctuations seen across the plate with other compounds. Fig. 3, C and D, demonstrates that compound PC58538 also inhibits division in vegetative cells of wild-type B. subtilis. Untreated cells (Fig. 3C) had a typical short rod morphology. Cells that had been treated with compound (Fig. 3D) had the form of extremely long aseptate filaments. This effect was quantified (Fig. 3E). The average length of untreated cells was about 3.6–4 μm at different time points. After 2 h (about three generations) in the presence of 128 μg/ml PC58538 the average cell length had increased about 5-fold, commensurate with a near complete block in cell division.

FtsZ Is the Target for PC58538—At least seven proteins are known to be required for efficient cell division in B. subtilis (16). Any one or more of these could be the target for PC58538. We used several approaches to identify the specific target. The division proteins are known to assemble into a “cytokinetic ring” at the site of impending division. Genetic experiments have established a hierarchy for this assembly in which FtsZ comes first, as all other proteins are dependent on it for assembly. Experiments with various GFP fusions to division proteins (FtsZ, FtsA, and FtsL) revealed that all of these proteins were prevented from assembling by treatment with the compound (data not shown). This would be consistent with the compound acting at the top of the hierarchy to prevent assembly of the FtsZ ring. Fig. 4, A and B, shows a typical result nated by secondary assays that we developed to look directly at cell division by microscopic methods (see below).

Identification of a Novel Drug-like Inhibitor of Cell Division—The microtiter plate assay was used to screen a library of about 105,000 synthetic compounds at a single concentration (32 or 40 μg/ml). Possible hit compounds were retested in triplicate and then at multiple concentrations. Compounds that showed robust and reproducible activity against the assay were subjected to a secondary test for inhibition of cell division in vegetatively growing B. subtilis, based on phase-contrast microscopy.

appears to be an inhibitor of FtsZ, although it is active only at quite high concentrations (1). Cephalaxin and 3-methoxybenzamide showed high values for the ratio across a range of concentrations (Fig. 2). Some other β-lactam antibiotics also had a specific effect as judged by the assay readout (e.g. carbenicillin, Fig. 2), even though they ought not to be specific for cell wall synthesis in the septum but should have a more general effect on cell wall synthesis. We believe that this is so because during the starvation conditions of sporulation, general cell wall synthesis is greatly reduced, and synthesis in the sporulation septum is the major site of synthesis. A wide range of antibiotics and growth inhibitors that affects other cellular processes, including vancomycin, erythromycin, kanamycin, spectinomycin, monensin, ofloxacin, phosphomycin, and bacitracin (several of which are shown in Fig. 2), affected both reporter genes similarly and did not generate a significant effect on the reporter activity ratio, except for weak effects at a very narrow concentration range. False positives were likely to be rare and could be elimi-
for cells bearing an FtsZ-GFP fusion. In the absence of compound the normal pattern of localization was observed, with bands of FtsZ-GFP fluorescence at the midpoints of each of the cells (Fig. 4 A). After 10 min of treatment with PC58538 (128 µg/ml) the bands were completely dissipated (Fig. 4 B).

If FtsZ were the target for PC58538, it is possible that overproduction of the protein might partially overcome the inhibitory effects of the compound. To test this we used a strain of B. subtilis in which the ftsZ gene has been placed under the control of the IPTG-inducible promoter Pspac. In the presence of relatively low levels of IPTG (0.075 mM), strain 1801 grows well with normal sized cells, indicating that sufficient FtsZ is being made for normal division (not shown). At this level of IPTG, addition of 32 µg/ml PC58538 resulted in an increased cell length (Fig. 4 C). With the same amount of compound but increased levels of FtsZ (0.5 mM IPTG) the cell length was reduced to near normal (Fig. 4 D).

As another approach to confirm FtsZ as the target for PC58538 we attempted to isolate resistant mutants. This might also flag possible future problems with drug resistance. Cultures of B. subtilis 168 were plated in the presence of various concentrations of PC58538 and incubated to allow the growth of resistant mutants. Candidate resistant mutants were isolated by restreaking on the same concentration of compound. The MIC of compound under the conditions used was 128 µg/ml. No bona fide mutants were isolated on agar containing 256 and 512 µg/ml PC58538 (256 and 512 µg/ml, respectively) indicating a spontaneous resistance frequency of 10⁻⁹. Small colonies were obtained after prolonged culture on agar containing 192 and 256 µg/ml PC58538 (and in the absence of compound) were further analyzed. Chromosomal DNA was isolated from the compound-resistant mutants, and the ftsZ gene of each mutant was PCR-amplified and sequenced. All of the mutants have a single point mutation in the ftsZ gene, consistent with FtsZ being the target for the inhibitor. Four different mutations were identified, which resulted in the following amino acid changes: A26V, A47T, A49V, and R184H. Fig. 4, F–I, shows the responses of the different mutants to treatment with PC58538 (128 µg/ml) compared with the wild-type strain (Fig. 4 E). All of the mutants showed a decreased effect of the
compounds on cell length. For three of them cell length was almost normal under these conditions, whereas for one mutant the length was not quite restored to normal (Fig. 4G). The amplified ftsZ alleles from wild-type B. subtilis 168 and each of the mutants were cloned and transformed back into B. subtilis, and these constructs were tested in the cell division phenotype assay. The same phenotypic effects were observed confirming that the mutant alleles were sufficient to confer increased resistance to PC58538.

Interestingly, the amino acid substitutions conferring partial resistance all clustered on one surface of the FtsZ protein, based on modeling with the crystal structure of the Methanococcus jannaschii FtsZ protein (20) (Fig. 4f). These sites are located, in general, around the edge of the GTP-binding pocket on the protein.

As final confirmation that the compound worked on FtsZ we turned to in vitro experiments with purified FtsZ protein. In Fig. 5A, a pelleting assay was used to measure GTP-dependent polymerization of B. subtilis FtsZ protein. Sedimentation of the protein did not occur significantly in the absence of GTP (Fig. 5A, lane 1; control) but did in its presence (Fig. 5A, lane 2). In the presence of GTP and PC58538, pelleting was essentially eliminated (Fig. 5A, lane 3). We also tested the effects of PC58538 on the GTPase activity of FtsZ. As shown in Fig. 5B, GTPase activity was inhibited in a dose-dependent manner by PC58538, with an IC50 value of 136 μg/ml (362 μM; Kd = 82 μM).

As part of an ongoing medicinal chemistry program an extensive series of analogues of PC58538 has been generated. One of the more potent of these is PC170942 (Fig. 3B). This was used in GTPase assays against B. subtilis and E. coli FtsZ proteins. As shown in Fig. 5B, this compound was active against both forms of FtsZ, and it exhibited IC50 values of 24 μg/ml (44 μM; Kd = 10 μM) and 594 μg/ml (1100 μM) against the B. subtilis and E. coli enzymes, respectively. Kinetic analyses indicated that the mechanism of inhibition of B. subtilis FtsZ by PC170942 was competitive (data not shown).

PC58538 Analogs That Have Increased Potency and Broad Spectrum of Activity—The range of PC58538 analogues made so far has been systematically tested for potency and spectrum of activity against different pathogenic bacteria. PC58538 had relatively weak antibacterial activity, with MICs detectable only against three of the organisms tested and these all at ≥128 μg/ml (TABLE ONE). Results obtained for some of the more potent PC58538 analogues are shown in TABLE ONE. Although generally the compounds tested so far were more active against Gram-positive bacteria, many, including all of the compounds shown in TABLE ONE, were active against at least one Gram-negative bacterium, M. catarrhalis, including one compound with a single-digit MIC against this organism. PC170942 showed an 8- or 16-fold improvement in potency over PC58538 in tests on B. subtilis and M. catarrhalis, respectively, and it was also active against S. aureus and E. faecalis. The increased potency of PC170942 against B. subtilis reflected its higher activity against purified FtsZ protein (Fig. 5B).

We showed recently (21) that depletion of FtsZ in S. aureus has a different effect than in rod-shaped cells, in that the cells enlarge and become multinucleate but remain essentially spherical. Such enlarged and depleted cells become inviable, presumably because they cannot generate a functional division machine once they have exceeded a critical cell diameter. This is different from rod-shaped cells in which elongated, multinucleated filaments are formed because of delocalization of FtsZ (22). The more potent derivatives of PC58538 appeared to retain specificity for the division machinery in both rods and pathogenic cocci, as judged by morphological phenotype. Fig. 6B shows the action of compound PC170942 producing filamentation and lysis of B. subtilis at 8 μg/ml. Another derivative, PC170945, which also caused filamentation in B. subtilis and inhibited FtsZ GTPase activity in vitro (data not shown), generated enlarged, multinucleate, rounded cells of E. faecalis at 256 μg/ml as shown in Fig. 6D. The increase in cell diameter in the presence of compound was statistically significant (control = 0.80 μm, treated = 1.36 μm; p < 0.01). This represents a novel description of a phenotype in E. faecalis consistent with inhibition of cell division.

**DISCUSSION**

The problem of increasing resistance to existing antibiotics is well documented. One solution to this problem would be to identify novel classes of compounds that act on new targets. Cell division has been identified as a potentially important target by pharmaceutical companies and other researchers. Most previous attempts to obtain inhibitors of cell division have focused on the FtsZ protein and have been based on the use of in vitro assays against either the intrinsic GTPase activity or specific protein-protein interaction (with ZipA). We chose to develop a cell-based assay with the idea that compounds identified should be active in vivo. We hoped that by making the assay discriminate between specific and nonspecific inhibitors, we could avoid compounds with off-target activity.

We exploited the observation that in sporulating cells of B. subtilis, completion of the sporulation septum is a morphological checkpoint to which activation of the transcription factor, σ^F, is coupled. We showed that the assay could pick up inhibitors of cell division. It seems also to detect certain general inhibitors of cell wall synthesis, including a range of β-lactams, which could be useful in terms of drug development, as wall synthesis remains an important target for antibiotics. This is likely due to wall synthesis being mainly required for division in sporulating...
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TABLE ONE

| Strain                  | MIC PC58538 (μg/ml) | MIC PC170942 (μg/ml) | MIC PC175515 (μg/ml) | MIC PC175568 (μg/ml) |
|-------------------------|---------------------|----------------------|----------------------|----------------------|
| B. subtilis 168         | 128                 | 16                   | 32                   | 64                   |
| E. faecalis ATCC 29212  | >256                | 128                  | >256                 | >256                 |
| E. coli ATCC 25922      | >256                | >256                 | >256                 | >256                 |
| H. influenzae ATCC 49247| >256                | >256                 | >256                 | >256                 |
| M. catarrhalis ATCC 25240| 128               | 8                    | 128                  | 128                  |
| P. aeruginosa 101021    | >256                | >256                 | >256                 | >256                 |
| S. aureus ATCC 601055   | >256                | 64                   | 32                   | 128                  |
| S. pneumoniae ATCC 49619| 256                 | 256                  | 256                  | 128                  |

FIGURE 6. Effects of PC58538 derivatives on cell division and cell morphology. Cells of B. subtilis 168 (A and B) or E. faecalis ATCC 29212 (C and D) were cultured in the absence (A and C) or presence of 8 μg/ml PC170942 (B) or 256 μg/ml PC170945 (D), and samples were taken, stained with 4‘,6-diamidino-2-phenylindole, and studied by phase-contrast (A and B) or fluorescence (C and D) microscopy.

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