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A High-Throughput, Homogeneous, Bioluminescent Assay for *Pseudomonas aeruginosa* Gyrase Inhibitors and Other DNA Damaging Agents

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Abstract

A homogeneous, sensitive, cellular bioluminescent high throughput screen was developed for inhibitors of gyrase and other DNA damaging agents in *Pseudomonas aeruginosa*. The screen is based on a *Photorhabdus luminescens* luciferase operon transcriptional fusion to a promoter that responds to DNA damage caused by reduced gyrase levels and fluoroquinolone inhibition. This reporter strain is sensitive to levels of ciprofloxacin as low as ¼-MIC with Z' scores above 0.5, indicating the assay is suitable for high-throughput screening. This screen combines the benefits of a whole cell assay with a sensitivity and target specificity superior to those of traditional cell-based screens for inhibitors of viability or growth. In duplicate pilot screens of 2,000 known bioactive compounds, 13 compounds generated reproducible signals ≥50% of that of the control (ciprofloxacin at ¼-MIC) using bioluminescence readings after 7h of incubation. Ten are fluoroquinolones known to cause accumulation of cleaved DNA-enzyme complexes in bacterial cells; the other three are known to create DNA adducts. Therefore, all 13 hits inhibit DNA synthesis, but by a variety of different DNA damaging mechanisms. This convenient, inexpensive screen will be useful for rapidly identifying DNA gyrase inhibitors and other DNA damaging agents, which may lead to potent new anti-bacterials.

Keywords

P. aeruginosa; gyrase; high throughput screen; luciferase

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen in animals and humans. It is a common and extremely virulent cause of serious infections in immune-compromised/ suppressed patients (e.g., HIV and cancer), cystic fibrosis patients, and those on mechanical ventilation or with burn wounds.1 Frequent antibiotic resistance and the highly virulent nature of *P. aeruginosa* make it deadlier than many other bacterial pathogens. *P. aeruginosa* exhibits intrinsic drug resistance due to the combined effects of a poorly permeable outer membrane, several multi-drug efflux pumps, and a chromosomally encoded cephalosporinase.1 New chemical classes of antibiotics are critical for continued effective therapy against *P. aeruginosa*, because such drugs are less likely to be subject to existing

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resistance mechanisms. In order to overcome these permeability and efflux obstacles and permit screening of a broad array of targets, we have developed *P. aeruginosa* cell-based assays for identifying inhibitors by using transcriptional reporters.

Transcriptional fusion of reporters to gene promoters, which are up-regulated in response to target depletion, whether by antibiotic inhibition or conditional under-expression, serves to transduce that depletion into a reporter signal. Several reporter assays of this type have been described in *B. subtilis* based on the response of cells to antibiotic treatments and using firefly luciferase as a reporter.2 We have extended this approach to a less permeable species, *P. aeruginosa*, and eliminated the need for luciferase substrate addition while achieving excellent sensitivity and highly positive Z'-scores. The resulting screen is simple, inexpensive, and homogeneous. *In vivo* reporter screens of this type offer substantial benefits, including (a) selection of permeable compounds, (b) ability to monitor multiple metabolic steps simultaneously (e.g., pathway screens), (c) sensitivity (e.g., superior to assays that simply detect growth inhibition), and (d) applicability to biochemically intractable targets (e.g., those with no known function or functions that are difficult to assay).

As a proof of concept, we focused on the known fluoroquinolone antibacterial target, DNA gyrase. This well-validated, druggable target is the essential A_2B_2 protein product of the *gyrA* and *gyrB* genes, which maintains the negative supercoiling of DNA during replication by removing positive supercoils in advance of the replication fork.3 The fluoroquinolone family of antibiotics has been developed through optimization of nalidixic acid, a gyrase inhibitor that was discovered serendipitously.3 A high throughput screen for inhibitors of gyrase has not been feasible because of the difficulty in measuring the substrates and products of the reaction, supercoiled and relaxed DNA, respectively. Previous attempts to screen for inhibitors have relied upon detecting inhibitors of the ATPase activity of the N-terminal fragment of GyrB. This approach has limited utility and runs the risk of identifying toxic compounds with broad anti-ATPase activity. In order to develop a reporter screen for gyrase inhibitors, we identified transcriptional units in *P. aeruginosa* that respond by up-regulation to both reduced expression of the *gyrA* gene and to inhibition of gyrase by the fluoroquinolone ciprofloxacin. Next, we fused responsive promoter regions to the *Photorhabdus luminescens luxCDABE* operon and integrated them into the *P. aeruginosa* chromosome. The most responsive promoter element is known to be up-regulated through the *recA* pathway by a variety of DNA damaging agents, including fluoroquinolones and compounds which create DNA adducts *in vivo*. We used this promoter to build and characterize a simple, sensitive, inexpensive, high throughput screen for compounds with anti-*P. aeruginosa* activity based on DNA damage.

MATERIALS AND METHODS

Strains, plasmids, and growth media

P. aeruginosa PAO-LAC, a version of strain PAO1 in which *lacI*^{q} has been integrated into the ΦCTX site in the chromosome,4 *E. coli* TOP10 (Invitrogen®), *E. coli* DB3.1 (Gateway® host, Invitrogen®), and *E. coli* S17-1 (ATCC 47055), were used as hosts for molecular cloning. Luria-Bertani (LB) medium (liquid and agar) was purchased from Difco. LBIG is LB containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 10 μg/ml gentamicin. Opaque, white, flat-bottom, 96-well microplates (Nunc Cat No. 236108; VWR International) were covered with gas permeable sealant (AeraSeal BS-25; Phenix Research Products, Candler, NC) for reporter screens.

PCR and Primers

Synthetic oligonucleotide primers were designed using web-based PRIMER3 (Whitehead Institute) and purchased from Operon, Inc. (see Table 1). Primers were used at 10 μM in PCR amplifications with Failsafe polymerase (Epicentre®), Buffer G (Epicentre®), and 4% DMSO for *P. aeruginosa* chromosomal DNA templates.

Complemented deletion of *gyrA*

A deletion of codons 15-918 of *gyrA* marked with a tetracycline resistance (Tc^R) marker was constructed in PAO-LAC by allelic exchange.5-7 Briefly, N-terminal (1,020 bp) and C-terminal (951 bp) regions flanking *gyrA* were amplified using the primer pairs 1+2 and 3+4, respectively (Table 1, Fig. 1A). The amplified regions were joined to the Tc^R element gene from plasmid pALTER-1 (Promega) (amplified with primers 9+10, Table 1) in a three-fragment splicing by overlap extension (SOE) PCR,8 using the outside primers 1+4 (Table 1). This was followed by several amplification cycles with GW-Universal-attB1/GW-Universal-attB2 to complete the Gateway® sites for cloning. Products were gel-purified and cloned using the Gateway system into pEX18GWAp, a Gateway®-adapted pEX18Ap (GenBank AF004910), provided by Drs. X. Liang and S. Lory (Harvard Medical School). The resulting construct was conjugated from *E. coli* S17-1 into *P. aeruginosa* PAO-LAC (Fig. 1B). A merodiploid resulting from a single cross-over integration was transformed to Gm^R by electroporation with mini-Tn7-GW-*lux* carrying the promoter region from *P. aeruginosa* gene PA0614 (see below for construction) and helper plasmid pTNS29 (Fig.1C). The Gm^R element was removed by electroporation to Sp^R with pFLP2-Sp^R (AF048702, modifed by replacement of the *Xba*I Gm^R fragment with Sp^R) followed by screening for Gm^S colonies (Fig. 1D). Cells lost pFLP2-Sp^R rapidly upon growth in the absence of spectinomycin selection. Finally, a wild-type copy of the *P. aeruginosa gyrA* gene was amplified with PCR primers 7+8 (Table 1) and inserted into pUCP24GW, a Gateway® (Invitrogen) adapted version of pUCP24 (GenBank U07167) which was constructed as follows. The multiple cloning site and a portion of the *lacZ*-alpha gene were deleted using an outward PCR reaction with primers pUCP24-UP and pUCP24-DN. A Gateway® Vector Conversion System (Cat. No. 11828-029, Invitrogen, Inc.) cassette was ligated with blunt ended PCR product and used to transform *E. coli* DB3.1 cells to Gm^R. Complemented *gyrA* deletions were isolated by selection for growth on sucrose in the presence of 1 mM IPTG and screened for a Tc^R Ap^S Gm^R phenotype (Fig. 1E). Deletions were confirmed by PCR with primers outside the region carried on pEX18GWAp (PaeGyrA-outF/PaeGyrA-outR). Deletions could only be isolated in the presence of the complementing *gyrA* copy on pUCP24GW-*gyrA*, and deletions failed to grow in the absence of IPTG, consistent with the essentiality of the *gyrA* gene.

Construction and use of mini-Tn*7*-GW-*luxCDABE*

We modified the *P. aeruginosa* site-specific integrating vector, mini-Tn*7*-GW-Gm (GenBank AY737004)9 to create a vector for directionally cloning luciferase transcriptional fusions to promoter fragments generated by PCR. We generated a 2-way SOE PCR product8 consisting of the *lac* promoter and operator region bounded on both sides by *Dra*III restriction endonuclease sites and fused to the *P. luminescens luxCDABE* operon. Primers 15+16 and 17+18 (Table 1) were used to amplify and join fragments from the plasmid pUC-*lux*.10, 11 The 3-nt ambiguity in the *Dra*III recognition site was used for directional cloning of promoter fragments in place of the *lacOP* stuffer fragment. The SOE-PCR product was cloned into mini-Tn*7* -GW-Gm by Gateway®, and a suitably luminescent clone was selected by its strong emission of light on medium containing 1 mM IPTG. Promoter regions to be inserted into the vector were amplified by PCR from genomic DNA by using primers containing two different *Dra*III sites as tails (e.g., PA0612-F+Dra/PA0612-R+Dra; PA0614-F+Dra/PA0614-R+Dra; and PA0617-F+Dra/ PA0617-R+Dra; see Table 1). *Dra*III digestion

of the gel-purified PCR products and ligation into *Dra*III-cut mini-Tn*7*-GW-GM-LUX resulted in directionally cloned promoter regions upstream of *luxCDABE*. Resulting constructs were integrated into PAO1 or into the complemented *gyrA* deletion strain by co-electroporation with helper plasmid pTNS2.9 The Gm^R marker could be eliminated readily by electroporation with pFLP2-Sp^R, which was lost after a few generations of growth without spectinomycin selection. This maneuver allowed the re-use of the Gm^R marker (see above).

Measurement of bioluminescence of reporters

Strains were grown overnight from frozen stocks at 37°C on LB agar containing 1 mM IPTG and 10 μg/ml gentamicin. Cells from the agar plate were used to inoculate liquid LB medium containing IPTG and gentamicin at an OD_{600} ~0.05. Cultures were grown for about two hours to an OD_{600} = 0.4, and then 200 μl of culture was added to each well of a 96-well microtiter dish containing compound (2 μl in DMSO) to initiate an assay or screen. Luminescence was measured at various times in kinetic mode or at a single time in endpoint mode in a Wallac Microbeta Trilux Luminometer or a Wallac Victor^{2}V 1420 Multilabel HTS counter. In some cases, relative luminescence units (RLU) were normalized to cell number by using OD_{600}. Inhibition was measured as luminescence relative to that of the positive control containing 0.5-fold MIC of ciprofloxacin (0.03 μg/ml) in the pilot screen and was calculated as follows: % of positive control = [RLU of sample – Average RLU of negative control) / (Average RLU of positive control – Average RLU of negative control)] × 100, with the negative control consisting of incubation with 1% DMSO but without compound addition. Z and Z' scores were calculated as previously described12 based on positive and negative controls as described above. The MIC of ciprofloxacin for *P. aeruginosa* strain PAO1 was determined according to NCCLS recommendations13 and is consistent with that of previous reports.14, 15

RESULTS

Construction of the reporter strain

The principle of the type of whole-cell reporter assay described here is the coupling of the transcriptional regulatory response produced by the depletion of an antibacterial target to a suitable reporter. A detectable signal, in this case bioluminescence, is produced when the quantity of active target is reduced by inhibition. First, it is necessary to identify promoters that respond to depletion of the potential target or to treatment with a known antibacterial agent which affects that target. We chose gyrase as the target for establishing the proof of principle for this type of reporter screen because it is a well-validated anti-bacterial target with several known inhibitors, many of which are marketed as antibiotics.3 Recently, Brazas and Hancock compared the transcription profile of *P. aeruginosa* cells grown in the presence and absence of the fluoroquinolone ciprofloxacin.16 They demonstrated that several genes, including a cluster of genes encoding the R and F type pyocins17 and spanning a contiguous region of over 28 kb in the genome (genes *PA0612 – PA0648*), were up-regulated significantly in cells treated with 0.3× and 1.0× MIC of the antibiotic. We have confirmed this result by expression profiling with microarrays and with luciferase fusions, and we have also demonstrated that the pyocin-encoding region is among the loci up-regulated when *gyrA* gene expression is reduced in a deletion strain complemented by a regulated copy of *gyrA*. We tested the entire upstream region from each of three of the earliest genes in the cluster for the ability to drive luciferase production in a ciprofloxacin-regulated manner as follows. We fused 451nt, 353 nt, and 150 nt of predicted non-coding sequence upstream from *PA0612*, *PA0614*, and *PA0617*,18,19 respectively, to the *P. luminescens luxCDABE* operon in the site-specific integrating vector mini-Tn*7*-Gm-GW-LUX and integrated them into the chromosome of *P. aeruginosa* PAO-LAC (Fig. 1C). The

resulting strains were grown without ciprofloxacin and with a range of three different concentrations of ciprofloxacin, and the ratio of luminescence +/- ciprofloxacin was measured. In this experiment an initial inoculum of 0.2 ml of cell suspension grown in LB to an OD_{600} of 0.4 was incubated at 37°C +/- ciprofloxacin for 330 min. As shown in Figure 2, the response of the *PA0614* promoter region was at least 10-fold stronger than those of the other two regions. For example, at 0.03 μg/ml ciprofloxacin (0.5 × MIC) the increase in bioluminescence as compared to untreated cells was 1.9-fold, 11.2-fold, and 0.8-fold for *PA0612*, *PA0614*, and *PA0617* promoter regions, respectively.

To measure the effect of GyrA depletion on bioluminescence, we inserted mini-Tn*7*-Gm-GW-LUX carrying the PA0614 promoter region into PAO-LAC and generated a deletion of the *gyrA* gene complemented by a *lac* repressor-regulated copy on the replicating extrachromosomal vector pUCP24GW (Fig. 1). The conditionally-complemented Δ*gyrA* strain failed to grow in LB + 0.001 mM IPTG, and growth was impaired at 0.0125 mM IPTG, confirming dependence of growth on expression of the complementing gene copy. Growth of cells in low IPTG (0.0125 mM) vs. high IPTG (1 mM) concentrations resulted in a decrease in *gyrA* mRNA levels by about 50% as judged by microarray analysis (data not shown) and an increase in bioluminescence of almost 2-fold (average of 10,764 +/-916 vs. 5,797 +/-713 RLU/OD_{600} in low vs. high IPTG-grown cells in two independent experiments).

In summary, as predicted by transcriptional profiling, the *PA0614* upstream region behaved as a promoter that is up-regulated by ciprofloxacin and decreased GyrA levels. Since the ciprofloxacin-induced up-regulation of the *PA0612* and *PA0617* upstream regions was less significant than that of the *PA0614* region, we used the *PA0614* construct as the basis of a reporter screen. The resulting reporter assay strain, MBX-623, consists of the promoter region from *PA0614* fused to *P. luminescens luxCDABE* operon and integrated at the Tn*7* attachment site in the PAO-LAC chromosome. The strain carries a Tc^R-marked deletion of *gyrA* in the chromosome and is complemented by a *lac* repressor-regulated copy of *gyrA* on pUCP24GW carrying a Gm^R marker.

Optimization of the assay

We examined the sensitivity of the assay by testing two-fold dilutions of ciprofloxacin over a 128-fold range from 0.24 μg/ml (equivalent to 4-fold above the MIC) to 0.002 μg/ml (equivalent to 32-fold below the MIC). Strain MBX-623 produced sufficient luminescence to yield Z' scores at certain time points above 0.5 for all ciprofloxacin concentrations from 0.24 ug/ml through 0.015 μg/ml (4-fold below the MIC), but not for ciprofloxacin concentrations below that. We have not examined ciprofloxacin concentrations greater than 4-fold above the MIC, but it is interesting that the luminescence signal increased for 7 h even when cells were incubated with ciprofloxacin up to 4-fold MIC (data not shown). The kinetics of luminescence from reporter strain MBX-623 incubated with three concentrations of ciprofloxacin (0.015, 0.03, or 0.06 μg/ml, equivalent to 0.25, 0.5, and 1-fold MIC, respectively), and in the absence of ciprofloxacin are shown in Figure 3A. Cells were grown in LBIG to a cell density of OD_{600} ~0.4, added to microtiter wells containing ciprofloxacin, and incubated for the time indicated. Increased luminescence in response to ciprofloxacin treatment was apparent after about 3 h and peaked after about 7 h. Luminescence was directly proportional to the concentration of ciprofloxacin added and was significantly stimulated by as little as 0.25-fold MIC. Luminescence declined after 7 h, but remained elevated above the zero ciprofloxacin control after 23 h. We calculated the Z' score, which is a measure of the variability and signal to background ratio, at several time points (Fig. 3B). A Z' score > 0.5 is indicative of the suitability of a screen for HTS.12 Cells treated with 0.03 μg/ml and 0.06 μg/ml ciprofloxacin exhibited Z' scores >0.5 from 350 min through the last measurement at 1,385 min (23 hr), indicative of an adequate window for detection of

inhibitors. Cells treated with the lowest ciprofloxacin concentration (0.015 μg/ml) exhibited positive Z' scores from 350 min, but the Z' score failed to reach 0.5 until the 23 hr timepoint. Concentrations of DMSO up to 2% had no significant effect on luminescence signal intensity (data not shown). The assay was tested with initial cell densities ranging from OD_{600} = 0.25 to 0.50. All produced robust signals at 6-7 h, but lower initial cell densities yielded slightly higher luminescence readings.

In principle, the assay strain should be genetically stable in un-supplemented growth medium (LB) since all modules are integrated stably into the chromosome (promoter-*luxCDABE* at the Tn*7* attachment site; stable deletion of *P. aeruginosa gyrA* gene) or on pUCP24GW, which is maintained by expression of the essential *gyrA* gene. IPTG is the only necessary supplement; however, gentamicin was also added in the pilot screen to ensure that the pUCP24GW-*gyrA* plasmid levels were maintained at a high level. Steady-glow bioluminescence from the *P. luminescens luxCDABE* operon products requires no reagent addition. Growth medium and minimal aeration (plates were covered with a gas permeable sealant but not shaken) are required for cells to produce ATP and light.

Application of the assay to known compounds -- a pilot screen

We screened 2,000 compounds from a biologically active and structurally diverse set of known drugs, experimental bioactives, and pure natural products ("Spectrum" library; Microsource Discovery, Inc.). To initiate the screen, 200 μl of a fresh culture of strain MBX-623 (OD_{600} ~ 0.40) in LBIG was added directly to opaque white flat bottom 96-well microtiter dishes containing 2 μl of library compounds (25 μM final concentration in 1% DMSO). Positive controls consisted of ciprofloxacin at 0.06 μg/ml (~1× MIC) and at 0.03 μg/ml (~0.5× MIC, the concentration used to calculate % of positive control values) final, and negative controls consisted of wells with DMSO but no compound addition. Plates were incubated at 37°C, and bioluminescence was read at 7 h in a Wallac Microbeta Trilux Luminometer plate reader. Percent bioluminescence of the positive control was calculated as described in Materials and Methods.

The pilot screen was performed in duplicate. The first screen was accomplished over a period of three days, testing 8-10 plates from the 25-plate library each day; the duplicate screen was done in a single day. Z' scores, signal-to-background (S/B), and coefficient of variation (CV; standard deviation of positive control as a percentage of positive control) values were calculated from the positive (0.5× MIC ciprofloxacin, 0.03 μg/ml) and negative controls (no ciprofloxacin) for each day of the screen. These values, Z' (S/B, %CV) for the three-day screen were 0.48 (6.8, 12%), 0.45 (5.2, 13%), and 0.30 (5.8, 16%), and for the single day screen were 0.45 (6.8, 12%). The luminescence for one of the replicates was read after overnight incubation for 23 h, yielding a Z' score of 0.56 (8.7, 11%). Bulk reagent dispensing was done by hand with multi-channel pipets, suggesting that better Z' scores could be obtained with more automated dispensing of cells.

Thirteen of the 2,000 tested compounds produced luminescence ≥50% of that of the positive control in both replicates at 7 h, and were defined as hits in the screen (Table 2). Results of the two duplicate screens were in agreement with regard to the compounds identified as hits. Variation between the two values averaged 18% for the hits, but was higher (57%) for those compounds that caused luminescence below the level defined as a hit. Ten of the hits were identified as fluoroquinolones and are thus "true" positives in the same drug class as ciprofloxacin. Four early generation quinolones, cinoxacin, pipemidic acid, piromidic acid, and oxolinic acid, and a 1st generation fluoroquinolone, flumequine, are present in the library but are not very potent vs. *P. aeruginosa*, exhibiting MICs ~25 μg/ml (~80 μM).[20-22] These were not detected as hits, but all except cinoxacin and piromidic acid (the least potent with MIC's >64 and 200 μg/ml, respectively) produced modest luminescence

increases of 12-47% of the positive control in the cell-based reporter screen, consistent with potencies at the limit of detection of the screen. These compounds represent all of the quinolones and fluoroquinolones in the screening library. The three non-quinolone hits are known to affect DNA synthesis by forming DNA adducts. These include a nitrofuran, furazolidone,23 a bifunctional alkylating agent, mechlorethamine,24 and an anti-neoplastic agent trichlormethine.25 Clearly, all thirteen hits share a common mechanism of damaging DNA by forming adducts, either directly or catalyzed by gyrase/topoisomerase action. Thus, the false positive rate in this pilot screen was <0.05% (<1/2,000).

Several of the 132 compounds in the library annotated as having antibacterial activity are non-quinolone, anti-pseudomonal compounds, including carbenicillin, piperacillin, cefotaxime, ceftriaxone, tobramycin, amikacin, gentamicin, spectinomycin, and minocycline.1 The screening strain carries a gentamicin-resistance marker on pUCP24GW, and no effect was observed on RLU values by gentamicin. However, tobramycin, piperacillin, cefotaxime, and minocycline reduced luminescence slightly (-14%, -3%, -13%, and -9%, respectively, of the positive control at 7 h) consistent with their inhibition of the growth or viability of the screening strain without affecting DNA or DNA synthesis. Carbenicillin, ceftriaxone, amikacin, and minocycline had no detectable effects on the luminescence values at 7 h. Spectinomycin produced a slight increase in luminescence, 7% of the positive control at 7 h. None of these anti-pseudomonal compounds had a significant effect on the screen.

Interestingly, nine of the ten fluoroquinolones, but none of the three DNA adduct forming compounds identified as hits at 7h exhibited luminescence ≥50% of the positive control at 23 h. Thus, while the 23 h data exhibit adequate Z' scores (see Fig. 3), they fail to identify one fluoroquinolone and three known DNA damaging agents, trichlormethine, furazolidone, and mechorethamine, suggesting a false negative rate of 0.2% (4/2,000). Furthermore, the 23 h data identify as inhibitors two compounds that do not act by damaging DNA, polymyxin B and sildenafil, suggesting a false positive rate of ≥0.1% (2/2,000). Finally, the DNA alkylating agent carboplatin26 only qualifies as a hit at 23 h, and thus, could be considered a false negative at 7 h.

DISCUSSION

The whole cell reporter screen described here is a successful transduction of the effects of depletion or inhibition of GyrA in *P. aeruginosa* to a bioluminescent readout. The approach of determining the cellular transcriptional response to depletion of a target and fusing appropriate promoter regions to a luciferase operon is a general one, which may be applied to many antibacterial targets. A similar approach has been used to build *B. subtilis* reporter screening strains, but use of those strains for HTS required an additional pipeting step for the addition of luciferase substrate prior to measuring luminescence.2 We used the entire luciferase operon from *P. luminescens* to build strain MBX-623. The resulting reporter strain requires no reagent addition for production of bioluminescence, is genetically stable, and produces high levels of luminescence in 4-7 hours of incubation. Despite the inherent variability of cell-based screens, strain MBX-623 produced acceptable Z' scores and no known false positives in the pilot screen reported here. The number of false negatives is more difficult to assess since many compounds fail to gain entry into *P. aeruginosa*, or are effluxed rapidly, and thus would not be expected to generate luminescence even if their mechanism involved DNA damage. For example, five of the fifteen quinolones in the library, cinoxacin, pipemidic acid, piromidic acid, oxolinic acid, and flumequine, are weakly potent against *P. aeruginosa* and were not detected as hits, but this result is not surprising considering the screening concentration was significantly less than the MIC values for these

compounds. If these compounds are considered true negatives, then the false negative rate for this pilot screen is < 0.05% (1/2000).

While this reporter screen was developed using ciprofloxacin and gyrase as the model inhibitor-target pair, the promoter chosen to drive the luciferase reporter is known to respond to DNA damage in general. All three genes, PA0612, PA0614, and PA0617, for which promoters were examined in this study, are part of a bactericin locus (pyocin) that responds to DNA damage through the RecA pathway.27 This property of regulation by DNA damage is shared with the *B. subtilis* genes used by Hutter et al.2 to generate *B. subtilis* reporter strains responding to fluoroquinolones, but the *P. aeruginosa* and *B. subtilis* genes and their predicted proteins are unrelated by sequence homology. The three *P. aeruginosa* genes encode products (Zn^{++} finger protein, holin, and assembly protein W, respectively) related to the tails of contractile (myoviridae) bacteriophages, but produce an R-type pyocin with the capability of killing certain strains of *P. aeruginosa*.17 The pronounced response of the promoter region upstream of PA0614 to DNA damage is fortuitous and extremely useful for reporter strain construction. This regulation by DNA damage is consistent with the results of the pilot screen, since the three non-quinolones among the 13 replicated hits are all known to damage DNA. It may be possible to construct a more gyrase-specific reporter screen by using other promoter regions to drive luciferase. In fact, several additional genes and apparent operons are up-regulated in response to ciprofloxacin treatment.16,28 However, this general DNA damage pathway reporter screen is useful for both identifying potential antibacterial candidates and also for eliminating DNA-damaging agents from further consideration.

The sensitivity of the screen might be improved by modifiying the assay strain or screening protocol. First, an efflux deficient assay strain could increase sensitivity of the assay. The efflux pump encoded by the *mexAB-oprM* locus is known to play a major role in fluoroquinolone resistance in *P. aeruginosa*.29 Screening with a strain carrying markerless deletions of *mexAB-oprM* and other efflux pumps would likely permit identification of a wider range of hits, some of which might be optimised to avoid efflux from wild-type *P. aeruginosa* cells. In addition, we reasoned that screening at lower IPTG levels might sensitize the assay to inhibitors by reducing the amount of gyrase in the cell, thereby lowering the inhibitor concentration threshold required for luciferase induction. However, such experiments failed to increase the sensitivity to ciprofloxacin, possibly because energy and light production as well as growth were limited due to insufficient *gyrA* expression. Therefore, the assay strain could be simplified to include the *PA0614* promoter region driving *luxCDABE* without the complemented *gyrA* deletion. In fact, we have examined such a strain and found it to be as responsive to DNA damaging agents as MBX-623. However, the complemented deletion assay strain configuration offers a significant advantage because it allows the substitution of heterologous *gyrA* genes for inhibitor screening and profiling of hits. For example, we have substituted the *Burkholderia pseudomallei gyrA* gene for the *P. aeruginosa gyrA* gene to permit screening for inhibitors of this Select Agent bacterium in a surrogate BSL2 strain (DTM and MD, unpublished).

In summary, the results of the pilot screen support the utility of this whole cell reporter screen for DNA damaging agents. Analysis of the results indicate the following: (a) a reasonable inhibition cut-off value (e.g., 50% of the RLU produced by 0.5xMIC ciprofloxacin) may be used to eliminate all false positives, at least in this set of 2,000 compounds at a 7 h endpoint; (b) hits in the assay share a common mechanism of damaging DNA by forming adducts, either directly or catalyzed by gyrase/topoisomerase action; and (c) the screen may used as a simple rapid method for identifying DNA damaging agents in compound libraries. Note that some hits may provide sufficient selectivity to be suitable for optimization studies as potential anti-bacterials.

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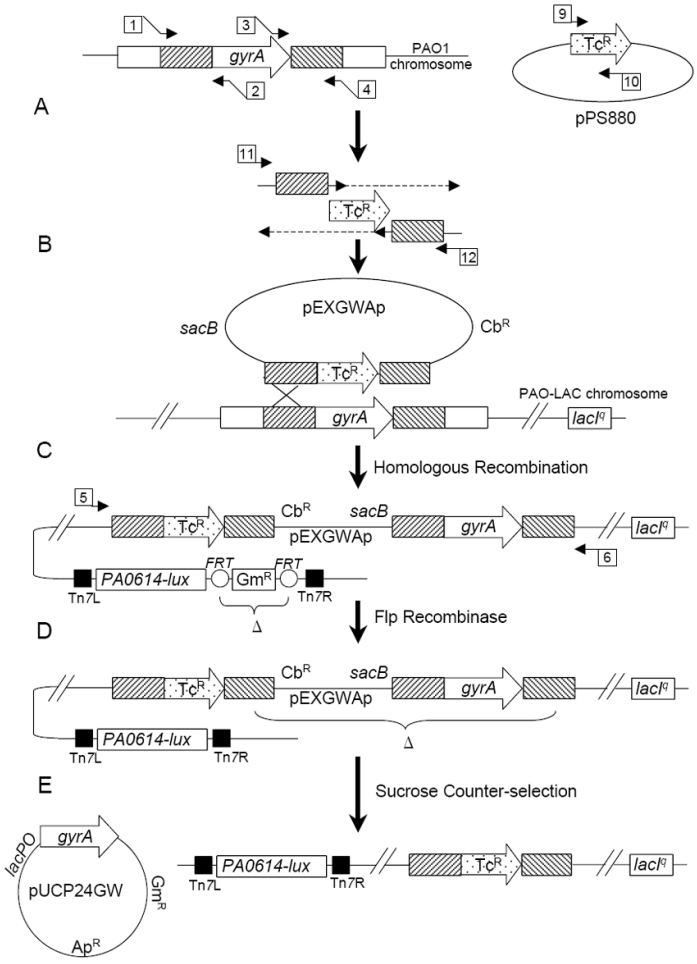

Figure 1.

Construction of reporter strain consisting of a *P. aeruginosa gyrA* deletion complemented by a *lac*-regulated copy of *gyrA* on plasmid pUCP24GW and carrying a chromosomally inserted mini-Tn*7* element expressing *P. luminescens luxCDABE* (*lux*) from the *PA0614* gene promoter. **A**. SOE PCR was used to replace *gyrA* with a Tc^R element between ~1 kb of flanking homology (primers numbered as in Table 1); **B**. the PCR product was cloned into pEXGWAp using Gateway® technology and conjugated into *P. aeruginosa* PAO-LAC, resulting in integration of the pEXGWAp deletion construct into the *P. aeruginosa* chromosome (confirmed by PCR with primers outside of the region cloned); **C**. A mini-Tn*7*-GW-Gm construct containing the *PA0614* promoter fused to *luxCDABE* was co-electroporated into the merodiploid together with the helper transposase plasmid pTNS2 resulting in integration of the mini-Tn*7* element at the *att*Tn*7* locus; **D**. Plasmid pFLP2-Sp^R was introduced by electroporation to eliminate the Gm^R marker, then pFLP2-Sp^R was lost by growth without selection; **E**. A pUCP24GW construct carrying a *lac*-regulated complementing copy of *gyrA* was introduced by electroporation, and a deletion of the chromosomal copy of *gyrA* was selected on sucrose-containing medium and confirmed by PCR.

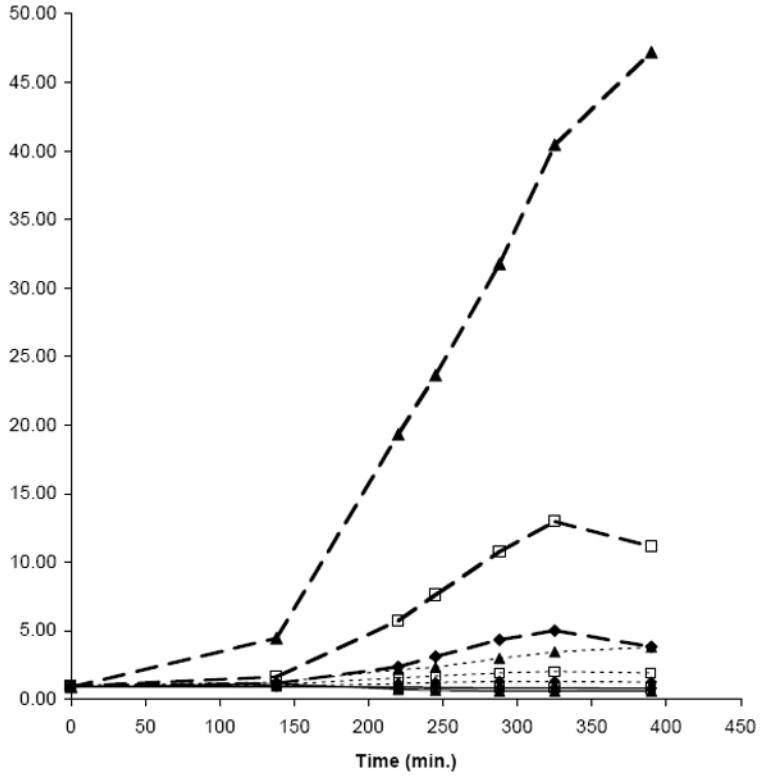

Figure 2.

Ratio of luminescence during growth in the presence and absence of ciprofloxacin by *P. aeruginosa* strains carrying three different pyocin promoter regions fused to *luxCDABE*. Cells with appropriate mini-Tn*7* luciferase fusions integrated into the chromosome were grown to OD_{600} = 0.2 in LB, and 0.2 ml were added to each well of a 96-well opaque white microplate. The ratios of relative luminescence units (RLU) from cells grown in the presence of ciprofloxacin at the following concentrations to that of cells grown in the absence of ciprofloxacin (24 wells each) is plotted, ♦, 0.015 μg/ml, □, 0.03 μg/ml; and ▲, 0.06 μg/ml (1× MIC). Promoter regions were the entire predicted untranslated sequence upstream from *PA0612* (451 nt), dotted line; *PA0614* (353 nt), dashed line; *PA0617* (150 nt), solid line.

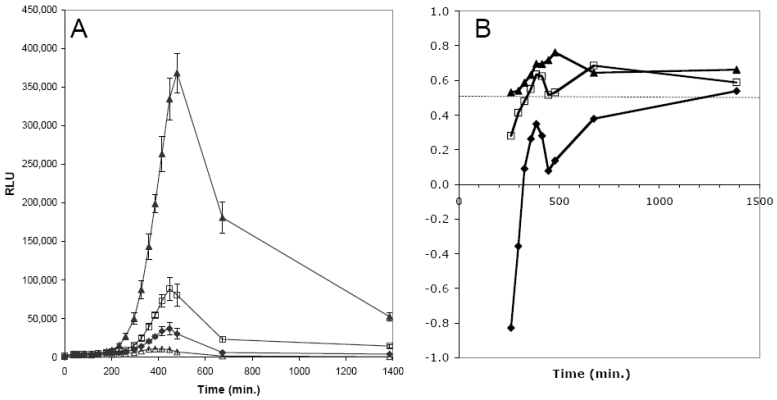

Figure 3.

Luminescence and Z' scores of strain MBX-623 *vs*. time and ciprofloxacin concentrations. Cells of MBX-623 were grown to OD_{600} = 0.4 in LBIG, and 0.2 ml were added to each well of a 96-well opaque white microplate. Ciprofloxacin was present at the following concentrations (24 wells each): Δ, 0 μg/ml; ♦, 0.015 μg/ml, □, 0.03 μg/ml; and ▲, 0.06 μg/ml (1× MIC). Relative luminescence units (RLU) were measured in each well in a Wallac Victor^{2}V 1420 Multilabel HTS Counter at the times indicated. Error bars represent the standard deviation over 24 wells per time point. **A**. Kinetics of luminescence of reporter assay strain MBX-623. **B**. Z' scores throughout the time course of the MBX-623 reporter assay of **3A**. Z' scores were calculated for the luminescence at each ciprofloxacin concentration *vs*. the no ciprofloxacin

TABLE 1

Oligonucleotide Primers Used in this Study

No.	Primer Name	Primer Sequence
1	PaeGyrAupF+GWL	TACAAAAAAGCAGGCTtctggagcgaatgaaagagg
2	gyrA UpR+Tc	TCCTGCGTTATCCCCTGATTCTGTGGATAActtcgatattgaccgggaga
3	gyrA DwnF+Tc	GCTAACGGATTCACCACTCCAAGAATTGGAcggaaggcaacgaagagtaa
4	PaeGyrAdwnR+GWR	TACAAGAAAGCTGGGTctggcatcgatggtcttgta
5	PaeGyrA-outF	aggaggtctggctcgaacac
6	PaeGyrA-outR	ggatcgggttggtgtagaag
7	PaeGyrA-F+SD+GWL	TACAAAAAAGCAGGCTaggaaacagctatgggcgaactggccaaagaaat
8	PaeGyrA-R+GWR	TACAAGAAAGCTGGGTccgagccttactcttcgttg
9	pPS880Tet-FRT-F	ttatccacagaatcaggggataacgcagga
10	pPS880Tet-FRT-R	tccaattcttggagtggtgaatccgttagc
11	GW-Universal-attB1	ggggacaagtttgtacaaaaaagcaggct
12	GW-Universal-attB2	ggggaccactttgtacaagaaagctgggt
13	pUCP24-UP	TCCCCCGGGGTGTGAAATTGTTATCCGCTCACAATTCCACAC
14	pUCP24-DN	TCCCCCGGGAACGTCGTGACTGGGAAAACC
15	PlacO-F+GWL	TACAAAAAAGCAGGCTTTAATTAAtcgaGGCGCGCCatcgCACtatGTGattaatgcagctggcacgac
16	PlacO-R+Dra4CTX	tatttgccatccatttaatgCACcgcGTGtgtgaaattgttatccgctca
17	Lux-F5	cattaaatggatggcaaatATGa
18	Lux-R2+GWR	TACAAGAAAGCTGGGTcgcaagcattccacttacaa
19	PA0612-F+Dra	catcgctccCACtatGTGggcaatctacagaccgatgg
20	PA0612-R+Dra	catcgctccCACcgcGTGgaaagcctccctggcgt
21	PA0614-F+Dra	catcgctccCACtatGTGcTGAgttcctggaccggata
22	PA0614-R+Dra	catcgctccCACcgcGTGcctggggacgcaccttta
23	PA0617-F+Dra	catcgctccCACtatGTGccacacccatTGAacatacg
24	PA0617-R+Dra	catcgctccCACcgcGTGgctccggcagagacagg

TABLE 2

Compounds Producing Reproducible Significant Inhibition in the Pilot Screen

Compound	Therapeutic Use	Class/Activity	% of Control, 7h (1st replicate)	% of Control, 7h (2nd replicate)	% of Control, 23h
Enoxacin	antibacterial	Fluoroquinolone	361	215	429
Pefloxacine mesylate	antibacterial	Fluoroquinolone	314	336	429
Lomefloxacin HCl	antibacterial	Fluoroquinolone	331	246	153
Gatifloxacin	antibacterial	Fluoroquinolone	277	259	153
Moxifloxacin HCl	antibacterial	Fluoroquinolone	252	164	138
Ofloxacin	antibacterial	Fluoroquinolone	332	246	133
Ciprofloxacin	antibacterial	Fluoroquinolone	182	101	24
Levofloxacin	antibacterial	Fluoroquinolone	167	223	145
Norfloxacin	antibacterial	Fluoroquinolone	132	72	107
Sarafloxacin HCl	antibacterial	Fluoroquinolone	127	299	127
Trichlormethine	antineoplastic	Alkylating agent	141	214	6
Furazolidone	antibacterial	Forms DNA adducts	125	85	15
Mechlorethamine	antineoplastic	Alkylating agent	50	135	7
Flumequine	antibacterial	Fluoroquinolone	30	20	-2
Cinoxacin	antibacterial	Quinolone	1	-8	0
Pipemidic acid	antibacterial	Quinolone	15	47	-1
Oxolinic acid	antibacterial	Quinolone	12	21	-2
Piromidic acid	antibacterial	Quinolone	2	-12	2
Sildenafil	impotency therapy	Phosphodiesterase inhibitor	22	3	60
Carboplatin	antineoplastic	Alkylating agent	2	19	97
Polymyxin B sulfate	antibacterial	Membrane active cyclic lipopeptide	-19	-33	80

The “Spectrum” library of known bioactives (2,000 compounds) was screened at 25 μM in duplicate. The whole cell reporter screen was incubated at 37°C, and readings were taken in a Wallac Microbeta Trilux Luminometer. The first duplicate was read after both 7 h and at 23 h; the second duplicate was only read after 7 h. A screening hit is defined as a compound causing ≥50% of the luminescence observed from the 0.5× MIC ciprofloxacin control, calculated as follows: % of control = $[(LUX_{cpd} - LUX_{NegCtl})/(LUX_{PosCtl} - LUX_{NegCtl})] \times 100$, where PosCtl = 0.03 μg/ml ciprofloxacin, and NegCtl = no addition