Development and Validation of a High-Throughput Cell-Based Screen To Identify Activators of a Bacterial Two-Component Signal Transduction System

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CpxRA is a two-component signal transduction system (2CSTS) found in many drug-resistant Gram-negative bacteria. In response to periplasmic stress, CpxA autophosphorylates and donates a phosphoryl group to its cognate response regulator, CpxR. Phosphorylated CpxR (CpxR-P) upregulates genes involved in membrane repair and downregulates multiple genes that encode virulence factors, which are trafficked across the cell membrane. Mutants that constitutively activate CpxRA in Salmonella enterica serovar Typhimurium and Haemophilus ducreyi are avirulent in mice and humans, respectively. Thus, the activation of CpxRA has high potential as a novel antimicrobial/antivirulence strategy. Using a series of Escherichia coli strains containing a CpxR-P-responsive lacZ reporter and deletions in genes encoding CpxRA system components, we developed and validated a novel cell-based high-throughput screen (HTS) for CpxRA activators. A screen of 36,000 compounds yielded one hit compound that increased reporter activity in wild-type cells. This is the first report of a compound that activates, rather than inhibits, a 2CSTS. The activity profile of the compound against CpxRA pathway mutants in the presence of glucose suggested that the compound inhibits CpxA phosphatase activity. We confirmed that the compound induced the accumulation of CpxR-P in treated cells. Although the hit compound contained a nitro group, a derivative lacking this group retained activity in serum and had lower cytotoxicity than that of the initial hit. This HTS is amenable for the screening of larger libraries to find compounds that activate CpxRA by other mechanisms, and it could be adapted to find activators of other two-component systems.

The increasing prevalence of multidrug-resistant Gram-negative bacteria has prompted urgent calls for new antibiotics (1). Escherichia coli sequence type 131, a clonal group that expresses extended-spectrum β-lactamases (ESBLs) and quinolone resistance, has emerged as a major cause of community- and health care-associated urinary tract infections in the United States (2–4). The Klebsiella pneumoniae carbapenemase (KPC) has rendered some strains of K. pneumoniae resistant to all β-lactams, while the New Delhi metallo- (NDM-1) β-lactamase-containing plasmid has rendered some strains of E. coli and K. pneumoniae panresistant (5–9). These developments raise the specter that several common infections, such as urinary tract infections due to E. coli or K. pneumoniae, may soon be caused by organisms that are virtually untreatable (5, 8, 9).

The traditional approach to discover antibiotics has been to screen libraries of natural or synthetic products for bacterial killing activity in culture. Unfortunately, this strategy has yielded no new targets or classes of drugs for Gram-negative bacteria over the past 50 years (10–12). More contemporary approaches are aimed at identifying inhibitors of novel targets essential for growth or virulence. Attractive targets include bacterial two-component signal transduction systems (2CSTS), which typically consist of a sensor kinase (SK) and a response regulator (RR), have no mammalian homologs, and involve the phosphorylation of amino acids that differ from the targets of mammalian phosphatases and kinases. Although several inhibitors of 2CSTS have antibacterial activity in vitro (13–16), none have achieved clinical utility in humans. The failure to develop inhibitors may be due to the redundancy of 2CSTS or to the poor selectivity and bioavailability of these compounds, which target the hydrophobic active site of the SK (15, 16). Another approach has been to find nontraditional therapeutics that target 2CSTS and do not cause cell death but downregulate the expression of virulence factors (13, 16, 17). For example, inhibition of the 2CSTS QseBC by the small molecule LED209 increased survival in animals infected with Salmonella enterica serovar Typhimurium or Francisella tularensis (18, 19). Thus, there is a rationale to expand the repertoire of nontraditional therapeutics that target 2CSTS.

CpxRA is a 2CSTS that allows Gram-negative bacteria to sense...
and respond to envelope stress (20–23). CpxA is an SK that spans the cytoplasmic membrane, and CpxR is its cognate RR. Upon sensing membrane stress, CpxA autophosphorylates on a conserved histidine residue and subsequently donates a phosphate group to a conserved aspartic acid residue on CpxR (20) (Fig. 1). In E. coli, to alleviate membrane stress, phosphorylated CpxR (CpxR-P) regulates the transcription of approximately 100 genes; genes that maintain envelope integrity are upregulated, whereas genes that encode secreted factors are downregulated (24–26). In E. coli, CpxR activity is regulated by two upstream components, CpxP and NlpE (27) (Fig. 1). The periplasmic chaperone CpxP inhibits CpxA kinase activity. Misfolded proteins bind to CpxP and cause it to dissociate from CpxA, activating the system. By an unknown mechanism, surface adhesion induces the lipoprotein NlpE to activate CpxA.

In addition to being an SK, CpxA also has phosphatase activity for CpxR-P (20). In the absence of envelope stress, CpxA acts as a net phosphatase, and CpxR remains inactive. When wild-type cells are grown in minimal medium containing excess carbon, such as 0.4% glucose, CpxR is activated by accepting phosphoryl groups from small-molecule donors, such as acetylphosphate (AcP) (28) (Fig. 1). Glucose-induced activation requires the lysine acetyltransferase YfiQ (also known as Pka and PatZ) and the acetylation of RNA polymerase (RNAP) and YfiQ (pathway 2, right) (29). Compounds that activate CpxRA might target CpxP, NlpE, CpxA, CpxR, or YfiQ (29).

When inoculated into the skin of human volunteers, the H. ducreyi ΔcpxA mutant is avirulent (32). In contrast, an H. ducreyi ΔcpxR mutant, which maintains wild-type levels of virulence determinant expression, is fully virulent in humans (33, 35). Similarly, constitutive activation of CpxRA abolishes S. enterica serovar Typhimurium virulence in mice (36). Mice fed a lethal dose of the wild type and ΔcpxR mutant become infected, while those fed similar doses of the ΔcpxA and cpxA* mutants do not (36). Furthermore, ΔcpxA mutants of uropathogenic E. coli (UPEC) and N. gonorrhoeae are outcompeted by the wild type by three orders of magnitude in their respective murine infection models (A. Jerse and S. Spinola, unpublished data, and H. Mobley and S. Spinola, unpublished data). Taken together, these data led us to hypothesize that activating CpxRA may be a broadly applicable antivirulence strategy and that compounds that pharmacologically activate CpxRA will downregulate virulence determinants and allow the host immune response to clear the infection.

To begin testing this hypothesis, we developed a high-throughput screen (HTS) to detect compounds that activate CpxRA using an E. coli strain containing a CpxR-responsive lacZ reporter (27). To identify the components required for compound-induced activation, we used an isogenic set of cpxRA mutants. Finally, we present validation of the screen and the strategy used to characterize the targets of hit compounds. The screen should be amenable for the discovery of compound leads with the potential to cripple the virulence of multidrug-resistant Gram-negative pathogens.

**MATERIALS AND METHODS**

**Bacterial and mammalian cell growth conditions.** The bacterial strains used in this study are listed in Table 1. The transcriptional fusion reporter strains (27, 29, 37) and the anti-maltose binding protein (MBP)-CpxR antibody (38) were generous gifts from Thomas Silhavy (Princeton University, Princeton, NJ, USA). Bacteria were grown at 37°C in TB7, a medium containing 1% (wt/vol) tryptone and buffered to pH 7.0 with potassium phosphate (100 mM). HepG2 hepatocellular carcinoma cells were a generous gift from Andy Yu (Indiana University, Indianapolis, IN, USA) and were grown in RPMI 1600 medium (Gibco) containing 10% fetal calf serum (Sigma) and 1 mM sodium pyruvate (Sigma) at 37°C with 5% CO₂.
TABLE 1 E. coli strains used in this study

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β-galactosidase assay. The E. coli PcpP− lacZ reporter strains were cultured overnight at 37°C in TB7 supplemented with 0.4% glucose. The following day, the cultures were diluted to an optical density at 600 nm (OD600) of 0.1 in 1.7X TB7 with glucose, and 30 µl of the diluted culture were distributed to wells of a 384-well plate containing 20 µl of vehicle or compound and grown at 37°C without shaking. After 5 h, 50 µl of All-in-One β-galactosidase reagent (Pierce), which was diluted 2.5-fold with TB7 medium, was added. The OD420, OD500, and OD600 of the wells were measured using a Molecular Devices SpectraMax 384 spectrophotometer, and the Miller units were calculated as described previously (39). When appropriate, 10% (vol/vol) human AB serum (HyClone) was supplemented. The effect of 10% serum on the activity of the compound was determined by dividing the Miller units obtained in the presence of DMSO or DMSO alone (192 wells for each strain/treatment). After 5 h of incubation at 37°C, the wells were pooled and harvested by centrifugation. All processing was carried out at 4°C. The cells were washed once in phosphate-buffered saline (pH 7.4) and suspended in 2× Laemmli lysis buffer, according to the bacterial pellet weight. As controls, 10 µM His6-CpxR, expressed and purified as previously described (30), was incubated with 0 or 20 mM AcP at 30°C for 15 min (30). Twenty-microliter and 5-µl aliquots of each cell lysate and AcP treatment, respectively, were separated on a Phos-tag gel, which was prepared according to the procedure described by Lima and colleagues (30) and the manufacturer’s protocol, with some modifications. Phos-tag acrylamide was purchased from Wako. The stacking gel contained 4% acrylamide/bis-acrylamide prepared in 350 mM bis-Tris (pH 6.8). The separating gel contained 10% acrylamide/bis-acrylamide, 25 µM Phos-tag acrylamide, and 50 µM Zn(NO3)2 prepared in 350 mM bis-Tris (pH 6.8), and was degassed with stirring for 2 min prior to pouring. The gel was run at 4°C in morpholinepropanesulfonic acid (MOPS) buffer (0.1 M MOPS, 0.1 M Tris, 5 mM sodium bisulfite, and 0.1% SDS) for 2 to 3 h at 40 milliamps, and the buffer was refreshed each hour. The gel was washed for 15 min in Towbin transfer buffer containing 1 mM EDTA and then for 30 min in standard Towbin transfer buffer. The proteins were transferred to a polyvinylidene difluoride membrane using a wet transfer method. Tris-buffered saline containing 0.1% Tween 20 (TBST) was used for washing, and TBST supplemented with 5% skim milk was used for blocking and antibody incubations. The membrane was blocked for 1 h and probed overnight at 4°C with 1:10,000 anti-MBP-CpxR antibody. The secondary antibody goat anti-rabbit IgG-horseradish peroxidase conjugate was used at a 1:5,000 dilution for 1 h at room temperature. Densitometry values were determined using Photoshop, and the ratio of CpxR-P to CpxR was analyzed by an one-tailed paired Student’s t test.

Cytotoxicity assay. To measure cytotoxicity, 20,000 HepG2 (50 µl for the 5-h assay and 100 µl for the 24-h assay) cells were plated per well of tissue culture-treated 96-well plates and allowed to adhere for 3 h. The cells were treated with an equal volume of medium containing either 1% DMSO (vehicle control) or 2X compound, such that the final concentration of DMSO was 0.5%. After 5 and 24 h, lactate dehydrogenase (LDH) release was measured using the CytoTox 96 nonradioactive cytotoxicity assay (Promega), according to the manufacturer’s instructions. We determined the percent cell death by adjusting the vehicle-treated and Triton X-100-treated cells to 0 and 100% death, respectively. The 50% inhibitory concentration (IC50) after 24 h of incubation was determined using the GraphPad Prism 6.0 software. The relative cytotoxicities of compounds 1 and 1a were analyzed using a mixed-effects ANOVA model.
that antibiotics indirectly activate CpxRA (42, 43). For example, untreated wild type.

Development and validation of a HTS assay for CpxR activators.

In E. coli, cpxP is the promoter most highly upregulated by phosphorylated CpxR (25). To detect CpxR activation, we used an isogenic set of E. coli reporter strains that contain a chromosomal cpxP promoter-lacZ fusion (27) (Fig. 1). We developed a 384-well plate assay to detect compounds that activate the reporter in the wild type, using either the ΔcpxA or the cpxA* mutants as standards for cpxP transcription and growth. The assay was performed in TB7 broth containing 0.4% glucose, which fosters the formation of AcP. We reasoned that this growth condition would allow the detection of compounds that activate CpxR by augmenting CpxA kinase activity or inhibiting CpxA phosphatase activity.

After 5 h of growth in the glucose-enriched medium, the β-galactosidase activity of the ΔcpxA and cpxA* mutants was approximately 10- and 20-fold greater, respectively, than that of their wild-type parent (Fig. 2A and B). The quality of our assay was assessed by calculating the Z’ factor using the Miller units of the WT strain; each compound was tested in a single well. This screen resulted in 340 putative hits. Of 324 compounds available for re-screening, 10 activated the reporter and did not inhibit growth by ≥3 standard deviations from the mean; the 10 hit compounds be-

RESULTS
Development and validation of a HTS assay for CpxR activators.

To exclude indirect activators, such as antibiotics, we established growth parameters for the screen. After 5 h, the average optical density at 600 nm of the wild type and the ΔcpxA and cpxA* mutants was 0.5 ± 0.03 (mean ± standard deviation), 0.24 ± 0.01, and 0.21 ± 0.01, respectively (Fig. 2C and D). The growth impairment in the activating mutants suggested that compounds that activate CpxR in the wild type would likely reduce growth to the extent seen in the ΔcpxA or cpxA* mutants. Thus, a second criterion for a compound to be considered a hit is that it may not inhibit the growth of the wild type more than the mean minus three times the standard deviation of the growth of the cpxA* mutant.

We performed a mock screen in which the wild type was inoculated in 372 wells of the plate, and the ΔcpxA and cpxA* mutants were inoculated in 5 wells each. After 5 h of growth, the reporter activity of the mutants was clearly distinguishable from that of the wild type (Fig. 3). Thus, compounds that activate CpxRA in the wild type should be distinguishable from background.

Whole-cell screen for CpxRA activators. A 36,000-compound small-molecule library was screened at approximately 15 μM for the induction of β-galactosidase activity in the wild-type reporter strain; each compound was tested in a single well. This screen resulted in 340 putative hits. Of 324 compounds available for re-screening, 10 activated the reporter and did not inhibit growth by optical density at 600 nm criterion (Table 2) and satisfied both hit criteria. As controls, we included the bactericidal antibiotic ciprofloxacin and the bacteriostatic antibiotic spectinomycin; both antibiotics activated the reporter in the wild type and caused complete growth inhibition (Table 2). The 10 hit compounds be-
shown and Fig. 4A), validating the specificity of the screening procedure. At their optimal activating concentrations, the candidate quinolone and one candidate nitroindole caused a 102-fold reduction in viable CFU (data not shown), suggesting that compound 1 either generates a CpxA-activating signal or inhibits CpxA phosphatase activity. Since compound 1 activated the reporter primarily in concentrations ranging from 10 to 100 μM (data not shown). Importantly, no compounds increased reporter activity in the ΔcpxR mutant (data not shown and Fig. 4A), validating the specificity of the screening assay.

The three most potent compounds in terms of cpxP transcription that also passed the growth inhibition criterion included two nitroindoles and one quinolone. At concentrations ranging from 5 to 80 μM compound, we measured the viable CFU of the treated wild type and that of the untreated wild type and the ΔcpxA and ΔcpxR mutants. By quantitative culture, after 5 h, the untreated wild type increased CFU counts by 42-fold, the ΔcpxA mutant by 13-fold (data not shown). In contrast, the CFU of the ciprofloxacin-treated wild type decreased almost 10^2-fold (data not shown). At their optimal activating concentrations, the candidate quinolone and one candidate nitroindole caused a 10^2-fold reduction in viable CFU (data not shown), suggesting that they activated the system by killing the bacteria and were not valid candidates. However, in the presence of 40 μM the other candidate nitroindole (6-nitro-2,3,4,9-tetrahydro-1H-carbazol-1-amine; catalog no. 5302860; ChemBridge), here referred to as “compound 1,” the wild-type CFU increased 4.5-fold (data not shown), suggesting that compound 1 was not activating the system through an antibiotic effect.

Characterization of compound 1. Since compound 1 activated cpxP transcription and permitted bacterial growth, we performed dose-response assays in the wild type and ΔcpxR mutant; the untreated ΔcpxR mutant was included for comparison. A dose-dependent increase in reporter activity was observed in wild-type bacteria but not in the ΔcpxR mutant (Fig. 4A). In the presence of the highest concentrations of the compound, reporter activity in the wild type was induced to levels higher than those observed in the untreated ΔcpxA mutant (Fig. 4A, dashed lines). In contrast, the antibiotic ciprofloxacin induced reporter activity similar to the lower level of activity observed in the ΔcpxA mutant. No additional reporter activity was observed at concentrations of >5 μM ciprofloxacin, likely due to its bactericidal activity (Fig. 4B).

Wild-type bacteria treated with compound 1 mimicked the β-galactosidase activity and growth observed with the ΔcpxA mutant; thus, we hypothesized that the compound inhibited CpxA phosphatase activity. If this were true, compound 1 should activate the reporter only in the presence of glucose, CpxA, CpxR, and YfiQ (29). Therefore, we treated the wild-type strain and its isogenic ΔcpxA, ΔcpxR, and ΔyfiQ mutants grown in the presence or absence of glucose with 80 μM compound 1. We calculated the fold change in reporter activity in compound-treated versus untreated wells. Compound 1 maximally activated the reporter in the presence of glucose in the wild type (P = 0.001) (Fig. 5A); it was not active in the ΔcpxA (P < 0.0001), ΔcpxR, or ΔyfiQ mutants, suggesting that compound 1 likely targeted CpxA. To investigate potential upstream targets, 80 μM compound 1 was tested with the ΔnlpE and ΔcpxP reporter strains (27). The fold change increase in β-galactosidase activity in the ΔnlpE and ΔcpxP mutants was not significantly different than the activity in their wild-type parent (data not shown). Taken together, the data suggest that compound 1 either generates a CpxA-activating signal or inhibits CpxA phosphatase activity.

To test the hypothesis that compound 1 inhibits CpxA phosphatase activity, we used the cpxA*I allele cpxA101 (20). In vitro

FIG 3 Validation of HTS assay. β-Galactosidase activity of 372 wells of the wild type (○) and 5 wells each of the cpxA* (◆) and cpxA (■) mutants.

FIG 4 Compound 1 activates CpxRA. Shown are the dose responses of compound 1 (A) and ciprofloxacin (B) with wild-type (WT) and ΔcpxR reporter strains. The reporter activity was determined after 5 h of incubation with increasing concentrations of compound 1 or ciprofloxacin. Data are means ± standard deviations from three independent experiments. The dashed lines represent the mean plus and minus three standard deviations of the untreated ΔcpxA mutant.

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* Ciprofloxacin
  | 9.05 | 0.20 |
  | Spectinomycin | 10.61 | 0.21 |

\(^a\) A, B, and C are the 3 classes of hits identified in the high-throughput screen: nitroaromatics, quinolones, and the furoxan-pyridazine compound, respectively.

\(^b\) ID, identification; CB, ChemBridge; CD, ChemDiv.

\(^c\) MU, Miller units; WT, wild type.
phosphorelay assays showed that CpxA101 retains autokinase and CpxR kinase activity but lacks CpxR-P phosphatase activity (20). We treated the wild-type strain and the cpxA101 mutant in the presence or absence of glucose with 80 μM compound 1 and calculated the fold change in reporter activity in compound-treated versus untreated wells. As observed previously, the compound maximally activated the reporter in wild-type bacteria in the presence of glucose, whereas significantly less activity was observed in the absence of glucose (P < 0.0001) (Fig. 5B). In contrast, the compound-induced reporter activity did not differ in the presence or absence of glucose in the cpxA101 mutant (Fig. 5B). The compound-induced reporter activity of the wild type was significantly higher than that in the cpxA101 mutant (P < 0.0001) (Fig. 5B). These results also suggested that compound 1 predominantly acts by inhibiting CpxA phosphatase activity.

**Compound 1 causes accumulation of phosphorylated CpxR.**

We reasoned that if compound 1 inhibits CpxA phosphatase activity, it should induce an increase in CpxR-P levels. Lima and colleagues (30) recently used Phos-tag SDS-PAGE to detect CpxR-P in E. coli grown in the presence of 0.4% glucose. To test if compound 1 induces CpxR-P accumulation, we cultured the wild type, untreated or treated with 20 mM AcP, along with the untreated ΔcpxA mutant grown in the presence of glucose. We harvested the cells after 5 h, prepared cell lysates, separated proteins by Phos-tag SDS-PAGE, and detected endogenous CpxR and CpxR-P by Western immunoblot with anti-MBP-CpxR antiserum. As shown in Fig. 6A, the treatment of CpxR with AcP increased the fraction of CpxR-P. Treatment with compound 1 induced an accumulation of CpxR-P levels in wild-type cells equivalent to that in the activated ΔcpxA mutant. Total CpxR levels were higher in the ΔcpxA mutant and treated wild type, consistent with the fact that CpxR-P positively autoregulates its transcription. To accurately compare the samples, the two CpxR fractions were quantified by densitometry. Compared to the untreated wild type, compound 1 treatment trended toward increasing the ratio of CpxR-P to CpxR in the wild type by 3-fold (P = 0.057) (Fig. 6B).

**Activity of compound 1 derivatives.** To determine whether the nitro and amine moieties of compound 1 were necessary for activity, we obtained two derivatives: compound 1a (2,3,4,9-tetrahydro-1'H-carbazol-1-amine; catalog no. 8019-9961; Chem-Div), which lacks the nitro group, and compound 1b (3-nitro-5,6,7,8,9-pentahydro-4aH-carbazole; catalog no. ST024298; TimTec), which lacks the amine group (Fig. 7A). The activity of the three compounds was evaluated in the wild-type reporter strain in the presence of 0.4% glucose. The activity of compound 1a was not significantly different than that of compound 1, whereas compound 1b elicited little reporter activity (P < 0.005 versus compound 1 and P = 0.007 versus compound 1a) (Fig. 7B). Thus, activation of the reporter depended on the presence of the amine group and not the nitro group. Using the maximum re-

![FIG 5](https://example.com/fig5)

**FIG 5** Compound 1 activity requires glucose, CpxA, CpxR, YfiQ, and CpxA phosphatase activity. The fold changes in reporter activity after treatment with 80 μM compound 1 in the presence or absence of glucose in wild type (WT) and the ΔcpxA, ΔcpxR, and ΔyfiQ mutants (A) or in WT and the cpxA101 mutant (B) are shown. For each strain, the fold change was calculated by dividing the β-galactosidase activity (in Miller units) of the compound-treated wells by the Miller units of the untreated wells. The data are the average and standard deviation from three independent experiments.

![FIG 6](https://example.com/fig6)

**FIG 6** Compound 1 induces phoso-CpxR accumulation. (A) Composite representative Western blot of His6-CpxR incubated with 0 or 20 mM AcP, and the ΔcpxA mutant and WT grown in medium with 0.4% glucose and 0 or 80 μM compound. Note that His6-CpxR migrates slower than native CpxR. (B) The ratio of CpxR-P to CpxR was determined using densitometry; the data are the mean and standard deviation results from 4 independent experiments.
DISCUSSION

Because bacterial 2CSTS frequently have essential functions and lack mammalian homologs, there has been considerable interest in targeting 2CSTS for antimicrobials (13–15, 18, 47). The mode of action of reported 2CSTS-targeted compounds is the inhibition of SK activity or interference with the binding of the RR to its promoter (16, 18, 48). To the best of our knowledge, this is the first report describing a compound that activates, rather than inhibits, a 2CSTS.

In this study, we developed and validated a novel HTS based on genetic activation of the cpxRA system. We established growth and reporter activity cutoffs using the untreated wild type and ΔcpxA and cpxA* mutants grown in the presence of glucose. As shown in Fig. 2 and 3, reporter activity in the ΔcpxA and cpxA* mutants was readily distinguishable from the wild-type reporter activity, providing a robust HTS assay. The facile nature of the β-galactosidase assay makes this a broadly applicable screening strategy.

Most biochemical screens performed in our core facility have typical hit rates of 0.1 to 0.2%. Our limited screen of 36,000 compounds yielded 340 hits, 10 of which were confirmed, giving a hit rate of 0.027%. Performing the screen in wild-type E. coli may account for the low hit rate, due to limited uptake and/or efflux of the small molecules. The archetype of multidrug efflux pumps in Enterobacteriaceae is the AcrAB-ToIC system; inactivation of these transporters increases susceptibility to multiple antibacterial agents (reviewed in references 49 and 50). By allowing more compounds to reach their intracellular target, E. coli mutants lacking AcrAB or ToIC are frequently used in HTS assays to increase the hit rate. However, the deletion of tolC activates the CpxRA system in E. coli (51). Similarly, CpxRA is activated by the deletion of the efflux pump genes mtrC in H. ducreyi (52) and vexAB or vexGH in Vibrio cholerae (53). Thus, wild-type bacteria must be used to identify CpxRA activators.

A major advantage of our cell- and reporter-based screening assay is its ability to identify compounds that specifically activate the CpxRA system. In theory, hit compounds might target different members of the Cpx pathway (i.e., CpxP, NlpE, CpxA, and CpxR), the acetyltransferase YfiQ, or enzymes involved in central metabolism. None of the hit compounds increased reporter activity or interference with the binding of the RR to its promoter (16, 18, 48). To the best of our knowledge, this is the first report describing a compound that activates, rather than inhibits, a 2CSTS.

Because bacterial 2CSTS frequently have essential functions and lack mammalian homologs, there has been considerable interest in targeting 2CSTS for antimicrobials (13–15, 18, 47). The mode of action of reported 2CSTS-targeted compounds is the inhibition of SK activity or interference with the binding of the RR to its promoter (16, 18, 48). To the best of our knowledge, this is the first report describing a compound that activates, rather than inhibits, a 2CSTS.

In this study, we developed and validated a novel HTS based on genetic activation of the cpxRA system. We established growth and reporter activity cutoffs using the untreated wild type and ΔcpxA and cpxA* mutants grown in the presence of glucose. As shown in Fig. 2 and 3, reporter activity in the ΔcpxA and cpxA* mutants was readily distinguishable from the wild-type reporter activity, providing a robust HTS assay. The facile nature of the β-galactosidase assay makes this a broadly applicable screening strategy.

Most biochemical screens performed in our core facility have typical hit rates of 0.1 to 0.2%. Our limited screen of 36,000 compounds yielded 340 hits, 10 of which were confirmed, giving a hit rate of 0.027%. Performing the screen in wild-type E. coli may account for the low hit rate, due to limited uptake and/or efflux of the small molecules. The archetype of multidrug efflux pumps in Enterobacteriaceae is the AcrAB-ToIC system; inactivation of these transporters increases susceptibility to multiple antibacterial agents (reviewed in references 49 and 50). By allowing more compounds to reach their intracellular target, E. coli mutants lacking AcrAB or ToIC are frequently used in HTS assays to increase the hit rate. However, the deletion of tolC activates the CpxRA system in E. coli (51). Similarly, CpxRA is activated by the deletion of the efflux pump genes mtrC in H. ducreyi (52) and vexAB or vexGH in Vibrio cholerae (53). Thus, wild-type bacteria must be used to identify CpxRA activators.

A major advantage of our cell- and reporter-based screening assay is its ability to identify compounds that specifically activate the CpxRA system. In theory, hit compounds might target different members of the Cpx pathway (i.e., CpxP, NlpE, CpxA, and CpxR), the acetyltransferase YfiQ, or enzymes involved in central metabolism. None of the hit compounds increased reporter activity or interference with the binding of the RR to its promoter (16, 18, 48). To the best of our knowledge, this is the first report describing a compound that activates, rather than inhibits, a 2CSTS.
phosphorelay assays are under way to elucidate the effect of compound treatment on CpxA enzymatic activity.

The activity of a compound in the presence of serum is important when considering potential efficacy in vivo. The presence of 10% human serum reduced compound 1 activity by approximately 50%. In contrast, compound 1a retained full activity in the presence of serum (Fig. 8A and B). Thus, compound 1a does not appear to nonspecifically bind to or react with proteins found in serum. Since the compounds retained activity in serum, it was appropriate to examine cytotoxicity. HepG2 hepatocellular carcinoma cells were chosen as the model because the liver metabolizes most drugs. As nitro groups are known to be cytotoxic (46), it is not surprising that removal of the nitro group relieved the cytotoxicity of compound 1 by 2-fold (Fig. 8C and D). Thus, compound 1a is an optimized first-generation derivative: it activates CpxRA, retains complete activity in serum, and is less cytotoxic than compound 1. In a 5-h assay, 80 μM compound 1a maximally activated CpxRA and caused negligible cell death (2.2%). Thus, medicinal optimization is under way to develop compound 1a into a highly potent lead compound.

A consideration for the clinical utility of cpxRA activators is the recent implication of the requirement of cpxRA activation in the mode of action of bactericidal antibiotics. Some studies suggest that bactericidal antibiotics kill E. coli through a “final common death pathway,” which is mediated in part by cpxRA activation and is inhibited by the deletion of cpxA or cpxR (42, 43, 55). However, Mahoney and Silhavy (44) showed that cpxR is not required for killing by bactericidal antibiotics and that an E. coli cpxA* mutant is as susceptible to ampicillin and norfloxacin as the wild type (44). However, a cpxA* mutant is less susceptible to gentamicin than the wild type, perhaps because the preactivation of CpxR by CpxA* prevents membrane damage induced by the misfolded proteins (44). Although an E. coli ΔcpxA mutant is less susceptible than the wild type to 5 μg/ml gentamicin, the cpxA* mutant is as susceptible as the wild type to 15 μg/ml gentamicin (43), a level that is exceeded clinically with once-daily dosing regimens. Thus, depending on the level of activation and the antibiotic concentration, activating compounds may interfere with aminoglycosides but not with other antibiotics.

In conclusion, we developed a robust screening strategy to identify and characterize CpxRA activators. We plan to extend our HTS and confirmatory screening assay to identify more potent...
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REFERENCES


Novel High-Throughput Screen for CpxRA Activators


