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A Pathway-specific Cell Based Screening System to Detect Bacterial Cell Wall Inhibitors

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A pathway-specific cell-based screen is described to detect compounds that inhibit the biosynthesis of the cell wall of bacteria. The basis for detection is the discovery that the β-lactamase gene from *Citrobacter freundii*, cloned into *Escherichia coli*, is induced when cells are exposed to known cell wall inhibitors, and not just β-lactam-based antibiotics. In a wild type host, cell wall inhibitors such as moenomycin, vancomycin, and ramoplanin, which are excluded by the outer membrane, only induce at high concentrations. However, these compounds, as well as fosfomycin, cycloserine, and cefoxitin, induce at concentrations at or below the MIC of a host carrying the *envA*-mutation, which causes a defect in the outer membrane. As additional proof that induction of β-lactamase is the direct result of cell wall inhibition, a host strain carrying a temperature-sensitive mutation in the *murG* gene, whose product converts the cell wall intermediate Lipid I, to Lipid II, also induced β-lactamase at the restrictive temperature. A protocol is described for screening samples in high-throughput mode.

The emergence of multiply-resistant bacteria has engendered a sense of urgency to the discovery of new antibiotics. In response to this challenge, pharmaceutical and biotechnology companies have adopted strategies to discover novel antibacterial targets, taking advantage of recent advances in genomics research, and technical advances in high-throughput screening[1~4]. Other efforts have concentrated on improving screening methodology for proven targets, with a heavy emphasis on diversity of new chemical sources[5]. At Millennium we have taken on both approaches, searching for novel targets and revisiting proven targets in our search for new antibacterial agents. This paper describes a cell-based screening strategy to detect compounds that inhibit any of the steps in the cell wall biosynthetic pathway, based on induction of β-lactamase in Gram-negative bacteria. The screening system is rapid, robust, sensitive, and suitable for high-throughput.

Cell wall biosynthesis has unique substrates and enzymatic reactions found broadly in bacteria, but not in mammalian cells[5~8]. The first committed step, carried out by MurA, the transfer of a pyruvyl group to *N*-acetyl glucosamine, is a very unusual reaction not found in mammalian cells. MurB then carries out a reduction to form UDP-muramic acid. The amino acids of the pentapeptide are added in stages, including the D-amino acids glutamate and D-alanine, which are absent from mammalian cells. The synthesis of UDP-muramyl-pentapeptide completes the formation of the soluble precursor, UDP muramylpentapeptide. The final steps in the synthesis of cell wall precursor tether the muramyl-pentapeptide to the membrane by covalent bonding to the C55 isoprenoid moiety (Lipid I) through the action of MraY. The subsequent addition of the second saccharide, *N*-acetyl glucosamine. by MurG to form Lipid II, completes the formation of the cell wall precursor, *N*-acetyl-glucosaminyl-muramyl-pentapeptide. The precursor is transported by an unknown mechanism across the inner membrane, which is then accessible to the large penicillin binding proteins, which carry out the transglycosylation and transpeptidation reactions.

Inhibitors have been isolated that block cell wall biosynthesis at various stages. For example, fosfomycin inactivates MurA, D-cycloserine prevents synthesis of D-alanine-D-alanine, bacitracin prevents the formation of

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Lipid I by preventing recycling of the C55 isoprenoid carrier, ramoplanin inhibits formation of Lipid II by MurG, and vancomycin and moenomycin arrest transglycosylation of precursor into the cell wall polymer. The β-lactam-containing antibiotics, which inhibit the transpeptidation reaction, are the best known and most frequently utilized cell wall antibiotics. Clearly cell wall biosynthesis affords multiple opportunities for the preferential inhibition of bacterial cells, as indicated by more than 40 cell wall-active agents that have been approved for clinical use[5].

Previously it was known that in some Gram-negative bacteria such as *Citrobacter freundi*, β-lactamase is induced when cells are grown in the presence of β-lactam antibiotics, such as cefoxitin and imipenem. Curiously, many β-lactams fail to induce, even though cells would be protected from these β-lactams, if β-lactamase were expressed[9]. The prospects of utilizing the regulation of β-lactamase as a broad-based screen for cell wall activities might appear to be remote, given that not even all β-lactams are good inducers. However, regulation of β-lactamase is not based on the recognition of β-lactam structures, but rather on a sensory system that takes inventory of cell wall degradation products and probably the soluble cell wall precursor as well, as described below.

When the *ampC* and *ampR* genes, encoding β-lactamase and its regulator from *Citrobacter freundii*, were introduced into *Escherichia coli*, the regulation of β-lactamase expression was retained. The cell wall is degraded by lytic transglycosylases that produce anhydromuramyl peptides as degradation products[10,11]. *In vitro* transcription studies show that the AmpR protein responds to cell wall degradation products *in vitro* by inducing the transcription of the β-lactamase gene. Additional *in vitro* experiments suggest that UDP-muramyl-pentapeptide, the soluble precursor of cell wall biosynthesis, may be a negative effector of AmpR-mediated transcription[12]. Thus β-lactamase expression may reflect the titration of cell wall precursor and cell wall breakdown products. In support of this regulatory model, *in vivo* studies have shown in that reduction of cell wall degradation products due to genetic lesions results in diminished expression of β-lactamase[10~13], whereas mutations leading to increased accumulation of cell wall degradation products[12,14], leads to an increase in β-lactamase.

Because the regulation of β-lactamase is apparently governed by an integrative signal that takes into account both cell wall degradation and synthesis, it was possible that inhibition at stages of cell wall biosynthesis other than transpeptidation might also cause induction of β-lactamase. It was therefore of great interest to determine if other cell wall activities induced the expression of *ampC* derived from *C. freundii* cloned into *E. coli*.

Material and Methods

Strains

All strains used were derivatives of *E. coli* K-12. The wild type strains were D21 and TP71, each carrying plasmid pNU305[15], which contains the *ampC* and *ampR* genes from *C. freundii* encoding β-lactamase and its regulator, respectively, a colEI origin of replication, and a tetracycline resistance determinant. The *E. coli* strain D22, which contains the *envA*-mutation resulting in a hyperpermeable outer membrane due to a defect in lipid A synthesis[16,17], was transformed with plasmid pNU305 selecting for tetracycline resistance. Strain D22/pNU305 is referred to as the screening strain.

Growth Media

Cells were grown in L Broth (LB, 5 g yeast extract (Difco), 10 g tryptone (Difco), and 10 g NaCl per liter). The screening strain was supplemented with E Salts (400 ml E Salts per 100 ml LB). E Salts contain, per liter, 10 g $MgSO_4 \cdot 7H_2O$, 100 g citric acid$\cdot H_2O$, 500 g K_2HPO_4 (anhydrous), and 175 g $Na(NH_4)HPO_4 \cdot 4H_2O$.

Agar plates contained LB with 10 μg/ml tetracycline to assure maintenance of plasmid pNU305, and E salts to supplement the *envA*- mutation of the screening strain.

Preparation of Cells and Screening Protocol

For testing compounds or for screening samples, cells were streaked on agar plates and incubated overnight at 30°C or 37°C, or incubated for up to 3 days at room temperature. A second passage on agar plates was accomplished by streaking a single colony and similarly incubating. Multiple colonies from this second passage were used to inoculate the starter culture in LB without tetracycline, at a cell density of ≤0.05 OD (650 nm, Beckman Spectropotometer). The culture was well aerated (*e.g.* 1/10 volume of an Erlenmeyer Flask, shaking at ≥200 rpm in a New Brunswick Series 25 Incubator Shaker) at 37°C. When the cell density reached 0.25~.8 OD, the culture was diluted to 0.083 OD, and 90 μl were immediately dispensed into 96-well microtiter plates which contained 10 μl of compound or sample. Cells were incubated for 1 hour at 37°C, with no greater than two plates in a stack, to allow induction to occur at an even temperature. Four control wells per plate contained fosfomycin at 25 μg/ml as positive controls.

Immediately following the one hour incubation, 30 μl of cells were added to 90 μl of reaction buffer to assay β-lactamase. The OD at 490 nm was measured immediately and after 2 hours incubation at room temperature (Biorad Model 3550 UV plate reader).

The reaction buffer was identical to Z buffer for assaying β-galactosidase, with detergents which arrest cell growth and make cells permeable to substrates[18], with the important exception that β-mercaptoethanol was excluded. The composition of Z buffer is 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 246 mg $MgSO_4 \cdot 7H_2O$, 200 mg CTAB (hexadecyl trimethylammonium bromide), and 100 mg Na deoxycholate per liter, pH 7.0. A 1/50 volume of nitrocefin solution (25 mg/ml DMSO, stored in aliquots at $-80°C$) was added to the reaction buffer, and after vigorous mixing, the mixture was filtered using 0.45 micron filter, to remove particulates. The reaction buffer was prepared and used for the day.

Scoring Hits

In order to standardize values, and take into account day-to-day variations in temperature, plate-to-plate variations, and other factors that could adulterate the results, the data from each plate was normalized as follows. The background reading in every well was taken into account by subtracting the initial OD reading from the OD measured after the 2 hour incubation. This corrected measurement was used for all subsequent calculations. When few positive compounds were tested on a microtiter plate, the median OD change was determined for each plate, which served as the enzyme activity of uninduced cells. Alternatively, for a plate containing a high proportion of active samples, the average of four or more samples of untreated cells was the activity of untreated cells. Then the Induction Value, or I value, was determined as the ratio of the difference between a sample and activity of untreated cells, compared to the difference in the OD of the four positive control wells relative to activity of untreated cells. In algebraic terms, the induction value (I) can be calculated as follows:

$$I = (\text{OD of sample} - \text{OD of untreated cells}) / (\text{OD of induced cells} - \text{OD of untreated cells})$$

An Induction Value of 0.2 was found to be consistently above the noise level of the screen, which can be considered the cutoff for a hit. A higher number can be chosen if a more discriminating cutoff is desired.

This is an endpoint screen designed to discriminate weak positive samples from background noise. The concentration and time parameters of the screen do not necessarily detect the differences among stronger inducing activities. For positive controls of fully induced cells, a reaction time of approximately 30 minutes was sufficient to reach the full OD value. Therefore a sample showing full induction only after 2 hours, rather than 30 minutes, would have an Induction Value of 1, even though enzyme activity was actually 25% of full induction. A sample having an Induction Value of 0.2 would suggest just 5% of the induction of positive control cells, because the positive controls reached the maximal OD in just 30′.

MIC Testing

MIC testing was carried out by inoculating cells from a fresh LB agar plate into LB to a concentration of 5×10^5 cells per ml, in 100 μl cultures of a 96-well microtiter tray. The MIC was defined as the minimum concentration resulting in a cell density less than 0.01 OD (Biorad Model 3550 UV plate reader), which corresponded to no visible growth, after incubating for 17 hours at 37°C.

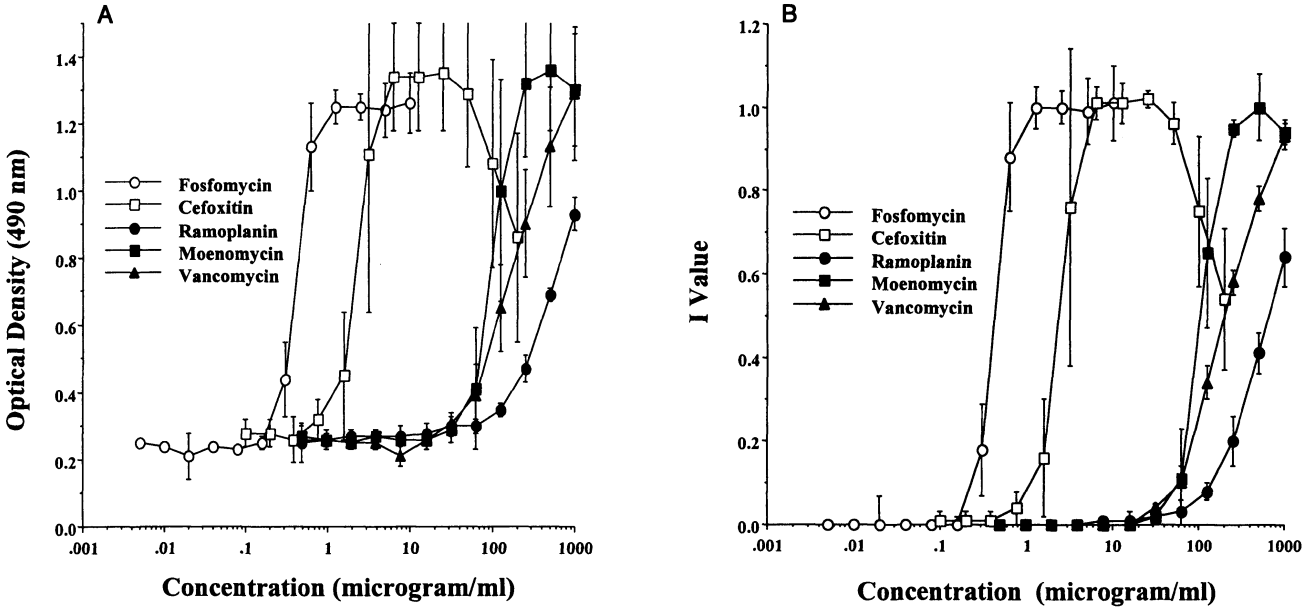
Results

Cells Respond to Cell Wall Inhibitors by Inducing β-Lactamase

Strain TP71, which contains the *ampC* and *ampR* genes from *C. freundii*, encoding β-lactamase and its regulator, were exposed to cell wall inhibitors for one hour in order to test if β-lactamase would be induced, as described in Materials and Methods. Figure 1 shows that the baseline level of expression was very low. However, when cells were exposed to the cell wall inhibitors, cefoxitin, fosfomycin, or cycloserine, then significant induction was observed. The hypothesis that compounds affecting cell wall biosynthesis would trigger the induction of β-lactamase, proved to be correct. This inducing effect was particularly important because fosfomycin, an inhibitor of the first committed step of cell wall biosynthesis, induced as effectively as a late stage inhibitor, the β-lactam cefoxitin. Ciprofloxacin, which inhibits growth of cells by affecting DNA gyrase, exerted no such induction.

Ramoplanin, which is known to inhibit Lipid II formation, as well as moenomycin and vancomycin, which inhibit the transglycosylation reaction, failed to induce β-lactamase except at very high concentrations (≥ 100 μg/ml, Figure 1). It was possible that these late stage inhibitors of cell wall biosynthesis were ineffective inducers. However, ramoplanin, moenomycin, and vancomycin are all larger antibiotics that cannot traverse the outer membrane of Gram-negative bacteria with facility. Therefore the

Fig. 1. β-Lactamase induction by cell wall antibiotics in *envA*+ cells.



Strain TP71 containing plasmid TP55 were exposed to antibiotics for 1 hour at 37°C and assayed for β-lactamase as described in Materials and Methods. Experiments were conducted at least 3 times and the standard deviations are indicated. A. Optical density at 490 nm. B. Calculated induction value (I value) using positive and negative controls on the microtiter plate.

possibility remained that these compounds failed to induce at lower concentrations simply because they were excluded from their sites of action. To address this possibility, we transformed plasmid pNU304 into strains that were defective in outer membrane permeability.

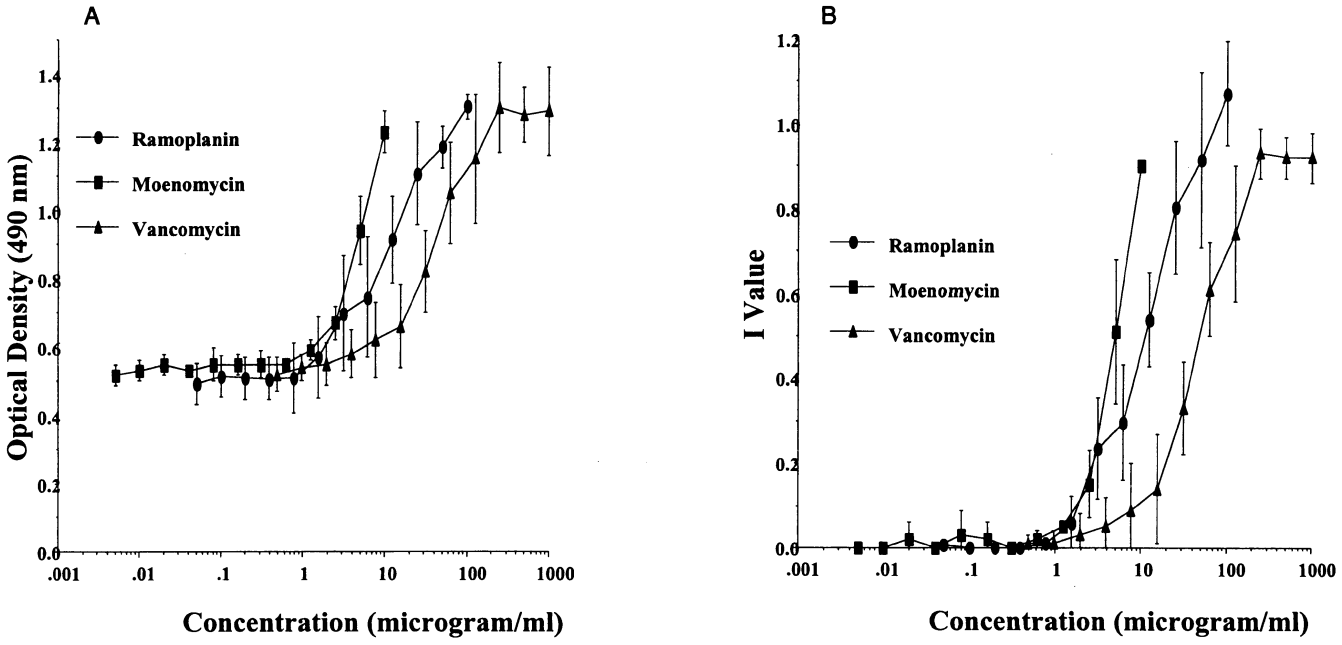
An *envA*-mutant strain also Induces β-Lactamase in Response to Cell Wall Inhibitors

Plasmid pNU305 was transformed into strain D22, which carries the *envA*-mutation resulting in a defect in lipid A synthesis[17]. The *envA*-transformant will be referred to as the screening strain, and it showed the advantage that it was induced effectively not only by fosfomycin, cycloserine, and cefoxitin, but also by the larger cell wall inhibitors. Figure 2A shows that the screening strain induced β-lactamase when exposed to 100 fold less of ramoplanin, moenomycin, or vancomycin, when compounds were tested as described in Materials and Methods. The disadvantage of the screening strain was that it showed a high background of activity, so that the signal to noise ratio is not as favorable as the *envA*+ strain (compare Figure 1A with Figure 2A). However, this disadvantage was minimized by calculating I values, utilizing positive and negative control samples on each microtiter plate, as shown in Figure 2B.

Reproducibility of the Screening System

In order to determine if this system of detecting cell wall-active compounds was sufficiently reproducible using the screening strain, we did a series of comparisons using a battery of 1840 compounds from the Wyeth-Ayerst compound collection, distributed on 20 microtiter trays, each tray containing 92 test compounds and 4 positive controls. Compounds were tested multiple times, and induction values were determined, as described in Materials and Methods. Reproducibility was assessed in two different ways. Figure 3 shows the results of two different runs of all 1840 compounds, using a scatter plot. If there is a perfect agreement in the two runs, then all values should lie on a diagonal line. The figure shows good reproducibility, especially with regard to the positive samples having induction values of >0.2. There were 32 positives in run 1 and 30 positives in run 2; 21 samples tested positive for both runs. This is very good performance considering that several of the samples in question were only weakly positive. The scoring criteria that we have adopted is to retest samples that had induction values ≥ 0.2, setting the

Fig. 2. β-Lactamase induction by cell wall antibiotics in *envA*- cells.



D22 cells carrying the *envA*-mutation and containing plasmid TP55 (the screening strain) were exposed to antibiotics for 1 hour at 37°C and assayed for β-lactamase as described in Materials and Methods. Experiments were conducted at least 3 times and the standard deviations are indicated. A. Optical density at 490 nm. B. Calculated induction value (I value) using positive and negative controls on the microtiter plate.

threshold at 0.15 for the retest, a value that is still well above background levels for this screen. This method improves the retest frequency.

A second method of testing reproducibility was to compare the results of positive samples for several different runs. Among 10 positive samples that were tracked in 10 different runs, 8 of the 10 samples tested positive every time, or every time but one run, whereas two samples tested positive in all runs but two (data not shown). From both of these methods, the screening system appears to have good reproducible results when run in high-throughput mode.

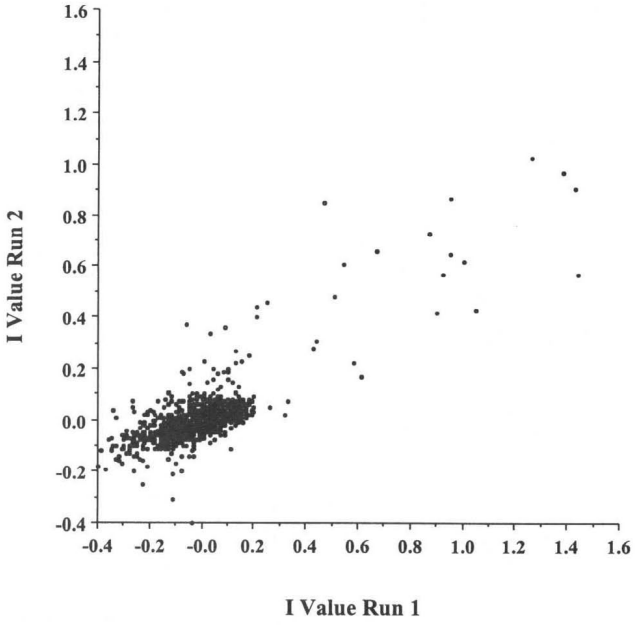
Genetic Challenge to the Screening System

Exposure of the screening strain to ramoplanin, moenomycin, and vancomycin resulted in strong induction of β-lactamase. The most plausible explanation for the reduced induction in *envA*+ strains exposed to these drugs was that cells were not sufficiently permeable to these inhibitors. If this hypothesis is correct, then the inhibition of a late step in cell wall biosynthesis in an *envA*+ cells, due to a conditional lethal mutation, should result in induction. The plasmid pNU305 was transformed into *E. coli* wild type strain K802 as well as strain MNts10, both from the Millennium strain collection by selecting for tetracycline resistance. Strain MNts10 carried a temperature-sensitive lesion in the *murG* gene, which results in the loss of the ability to form Lipid II, the final cell wall precursor, at the restrictive temperature[19]. Figure 4 shows that wild type cells carrying pNU305 synthesized a low, baseline level of β-lactamase when grown at 30°C, or when shifted to 37°C or 42°C. However, MNts10 cells carrying plasmid pNU305 showed a marked increase in β-lactamase when cells were shifted to 37°C, and an even greater induction of β-lactamase when cells were shifted to 42°C. Thus when the *envA*+ cells (TP71) was blocked in cell wall biosynthesis, due to the temperature-sensitive genetic lesion, then induction of β-lactamase resulted.

Sensitivity of the System

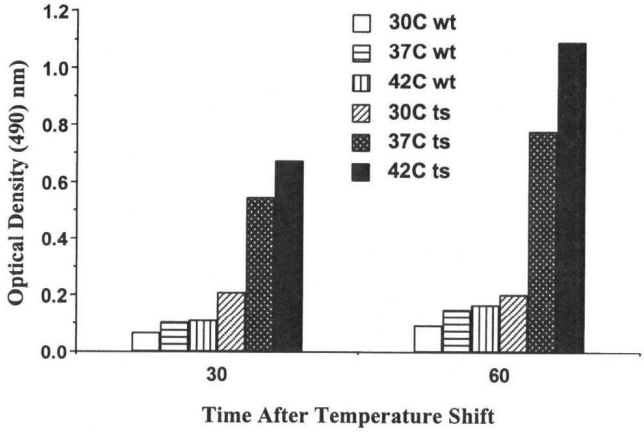
Table 1 shows that the antibiotics ciprofloxacin (DNA gyrase inhibitor), rifampicin (which inhibits RNA polymerase), and chloramphenicol (inhibiting translation) failed to induce β-lactamase. However, every non-β-lactam cell wall inhibitor was effectively detected at a low

Fig. 3. Comparison of the I values for two runs.



The screening strain was tested for induction using 20 microtiter plates containing 1840 compounds, as described in the Materials and Methods. The 80 positive control samples (4 per plate) are not included.

Fig. 4. Induction by temperature shift of a murGts mutant.



Production of β-lactamase was monitored in either the wild type host strain, K802, or the isogenic strain MNts10, carrying a temperature-sensitive mutation in *murG*, affecting the formation of Lipid II at the restrictive temperature. Both strains contained the pNU305 plasmid. Cells were pre-grown at 30°C for several generations and then diluted into fresh LB medium and grown at 30°C (the permissive temperature) or shifted to 37°C (partially restrictive temperature) or 42°C (the restrictive temperature). β-Lactamase was measured after 30′ and 60′, as described for the Induction Assay in Materials and Methods.

concentration in the screening strain, carrying the *envA*-mutation. Detection of fosfomycin (MurA inhibitor), D-cycloserine (inhibiting D-alanine dipeptide formation), ramoplanin (inhibitor of Lipid II formation), vancomycin (tranglycosylation inhibitor), and moenomycin (transglycosylation inhibitor), and cefoxitin (inhibiting transpeptidation) were detected at a concentration below the MIC of biological activity. Bacitracin (inhibiting the regeneration of the isoprenoid carrier) was detected in the screening organism at a concentration near the MIC. Strains that carried plasmid pNU305 in an envA+ background (either strain TP71 or strain D21) were also capable of detecting cell wall inhibitors at concentrations below the MIC (data not shown), but were insufficiently sensitive to large molecules such as vancomycin, moenomycin, or ramoplanin to be as useful in detecting as broad a range of cell wall-active molecules.

Discussion

It was known that some β-lactam antibiotics regulate expression of β-lactamase cloned from *C. freundii* into *E. coli*, and that regulation appeared to involve a general monitoring of the balance of cell wall synthesis and degradation[10~12]. Our finding that inhibitors of other steps in cell wall biosynthesis, lacking β-lactam structures, also induced, showed that β-lactamase could be used as a reporter in a screening system to detect cell wall-active compounds. The optimal screening strain carried the *envA*-mutation, a strain which was far more sensitive in detecting larger compounds that do not significantly penetrate the outer membrane of wild type cells.

The fact that not all β-lactam antibiotics are effective inducers of β-lactamase[9], may have obscured the utility of Gram-negative induction systems as a pathway specific screening system. There have been reports of induction of β-lactamase by components of growth medium[20], but to our knowledge, no previous report of induction by inhibitors other than the transpeptidase step. One report that vancomycin failed to induce might be explained by the lack of permeability to the drug in the strain that was used; we have no easy explanation for the observation that D-

Table 1. Minimal induction concentration and minimal inhibitory concentration (MIC) of the screening strain.

Compound	[Induction][a] (μg/ml)	MIC (μg/ml)	MIC/[Induction]
Fosfomycin	1.3	0.6	0.5
Cycloserine	25	12.5	0.5
Bacitracin	3.3	12.5	3.8
Ramoplanin	1.6	20	12.5
Moenomycin	1.3	2	1.5
Vancomycin	3.9	10	2.6
Cefoxitin	2.5	7	2.8
CTAB	4	0.5	0.1
Monensin	None	None	0
Nystatin	None	None	0
Polymyxin B	0.2	0.4	2
SDS	4	2	0.5
Triton X 100	100	500	5
Ciprofloxacin	None	0.01	0
Erythromycin	None	0.8	0
Rifampicin	None	0.06	0

[a][Induction]; minimal concentration required to induce β-lactamase in the screening strain to an I value of 0.1. Values are the geometric mean of at least 3 trials.

[b]MIC; minimal inhibitory concentration. Values are the geometric mean of at least 3 trials.

[c]MIC/[Induction]; ratio of concentrations required to induce β-lactamase and to inhibit growth.

cycloserine also did not induce for the other investigators[21]. It is ironic that these bacteria induce β-lactamase when the bacteria encounter inhibitors that the enzyme cannot degrade, yet often fail to induce in response to several β-lactam antibiotics, when the enzyme could be protective. It has been suggested that the most effective positive effector molecule is the anhydromuramyl-pentapeptide cell wall breakdown product[21]. This possibility, coupled with the different binding capacities to PBPs and inducing attributes of β-lactams[9], could account for the curious fact that many β-lactams fail to induce effectively.

This pathway-specific screening system has the advantage of detecting multiple targets in one measurement. Other screening systems have been described that detect only inhibitors of transglycosylation[22], specific structures inhibiting transglycosylation[23~25], transpeptidation inhibitors[26,27], or inhibitors of D-alanine formation[28]. This pathway-specific screen has advantages compared to other cell wall pathway screens that have been described[29,30], which rely on comparing the sensitivities of two strains, or making microscopic observations that are not as amenable to high-throughput screening. This β-lactamase screening system is very complementary to a biochemical screening system that is designed to detect

inhibitors of cell wall precursor formation[5]. Both the biochemical approach as well as the cell-based screening system will prove valuable in discovering new drugs. It is essential to confirm that positive hits in the β-lactamase screening system are, in fact, specific inhibitors of cell wall biosynthesis, given that detergents can test positive (Table 1).

The purpose of the primary screen is to identify positive hits that may hit the desired target. By filtering out the vast majority of samples, from compound libraries as well as from crude or partially purified natural products, the primary screen allows for the more careful investigation of a relative handful of positive samples. The accompanying paper shows that the β-lactamase screen serves this purpose well in the search for inhibitors of the late stages of cell wall biosynthesis, which cannot be screened in high-throughput with such facility[31].

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